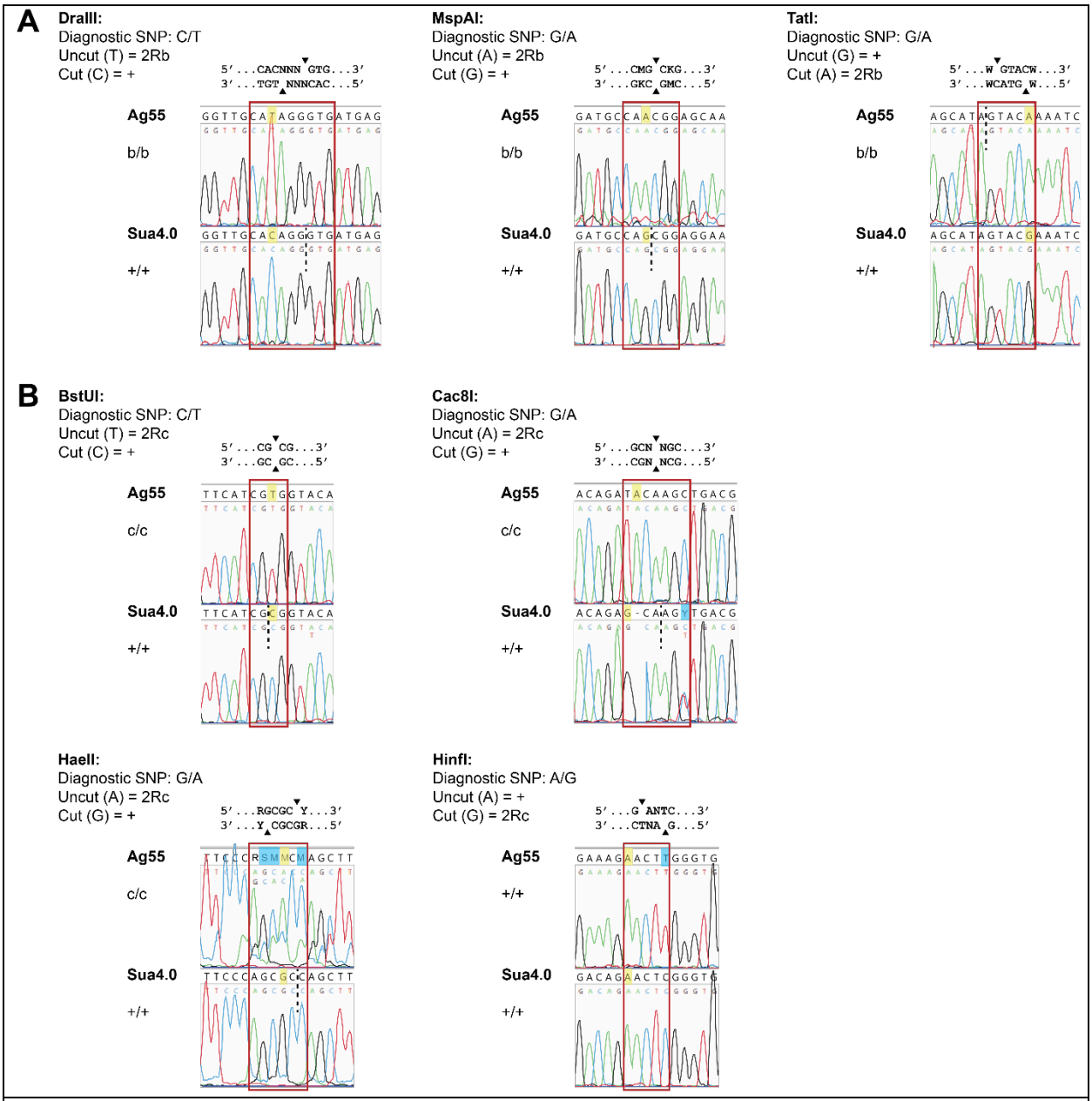

Figure S1. Molecular karyotyping assays of 2Rb and 2Rc on Ag55 and Sua4.0 cell lines. **A-B)** Ag55 and Sua4.0 cells were karyotyped for 2Rb (**A**) and 2Rc (**B**) using primers designed for previously published RFLPs for molecular karyotyping. PCR products were sequenced to identify the diagnostic SNP utilized by the RFLP assays. The diagnostic SNP (also highlighted in yellow on each electropherogram) and the inversion karyotype for cut vs uncut product are listed for each restriction enzyme. Ag55 electropherograms are shown above Sua4.0, and the concluded inversion karyotype for each diagnostic test is shown to the left of the electropherograms. The restriction enzyme recognition sites are shown above the electropherograms, with black triangles indicating cut sites. The red box over the electropherograms highlights the restriction enzyme recognition site, and a dotted black line indicates where diagnostic cutting would occur. Nucleotides highlighted in blue indicate additional SNPs within the restriction enzyme recognition sequence that destroy the site. The single nucleotide peak for each diagnostic SNP indicates homozygosity for the inversion

karyotypes. In the case of HaeII for 2Rb, neither of the two nucleotides in Ag55 at the diagnostic SNP would result in cutting. (**A**) All three tests for 2Rb (DraIII, MspAI, TatI) concur that Ag55 is b/b and Sua4.0 is +/+. (**B**) Three of the four tests for 2Rc (BstUI, Cac8I, HaeII) concur that Ag55 is c/c and Sua4.0 is +/+.

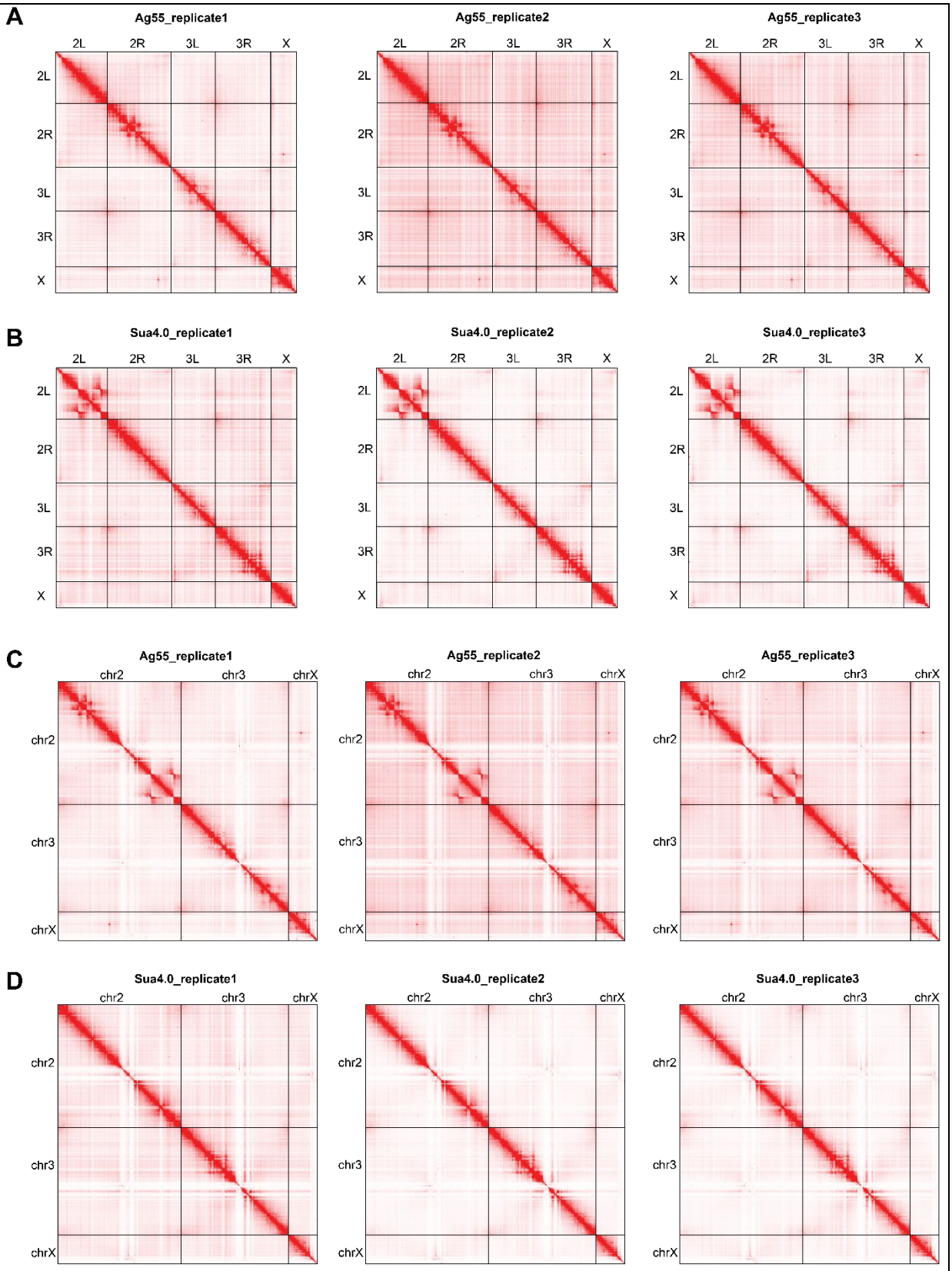

Figure S2. Contact matrices for all replicates mapped to both PEST 2L+[a] and MOPTI 2La reference genomes. Micro-C libraries were made from three biological replicates of

Ag55 cells which are fixed for 2L+^{a} (**A, C**) and three biological replicates of Sua4.0 cells which are fixed for 2La (**B, D**), and reads were mapped to PEST 2L+^{a} (**A, B**) or MOPTI 2La (**C, D**) reference genomes. The PEST reference genome splits chromosomes 2 and 3 into their L and R arms while the MOPTI reference genome includes the L and R arms in chromosomes 2 and 3. All three replicates of each cell line produce highly similar contact matrices.

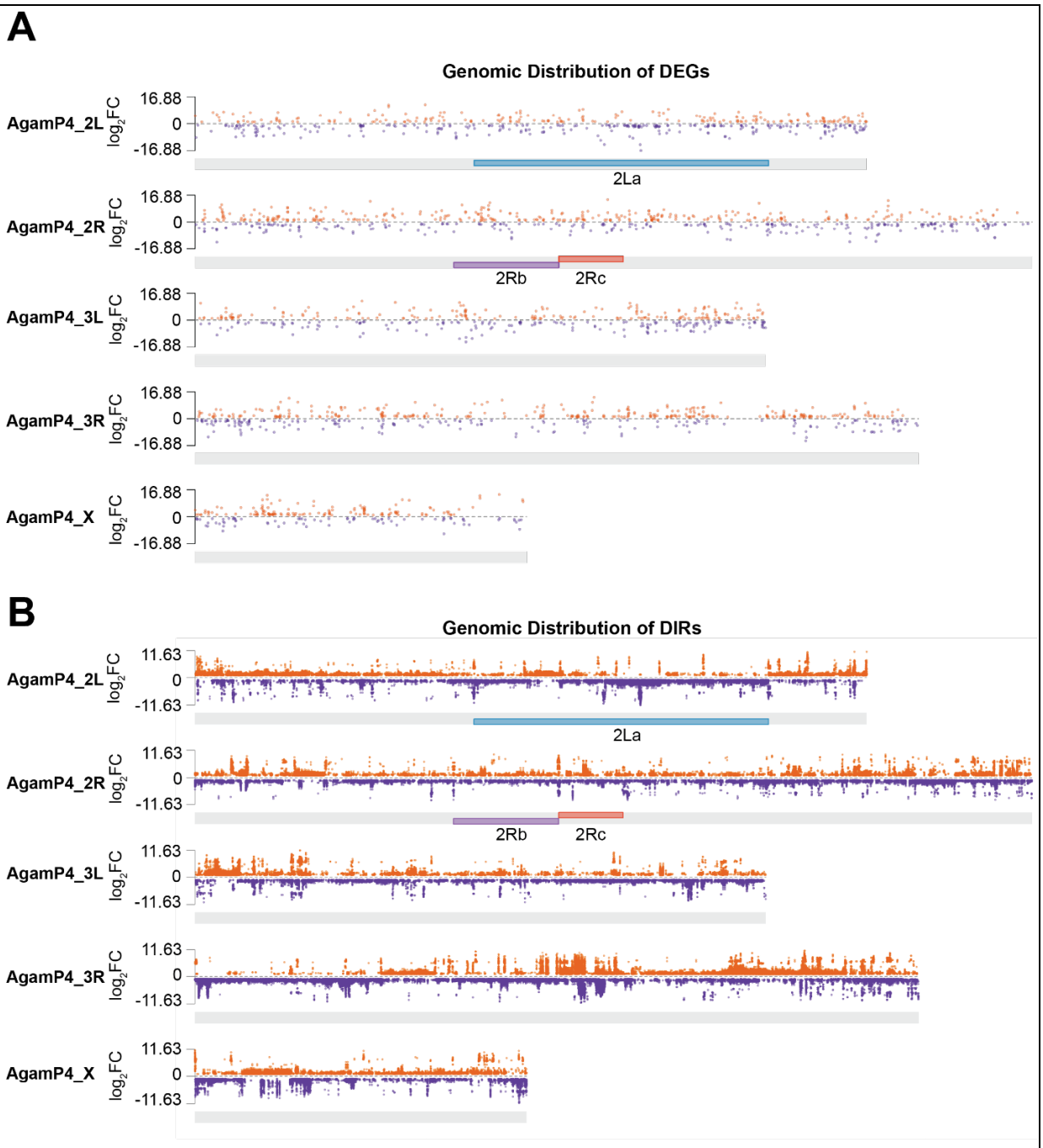

Figure S3. Genomic distribution of differentially expressed genes (DEGs) and differentially interacting regions (DIRs) between Ag55 and Sua4.0 cell line samples.

(A) The genomic positions of significantly (|log2FC|>1, p<0.05) upregulated (orange dots above x=0) and downregulated (purple dots below x=0) genes in Sua4.0 samples as compared to Ag55 samples are plotted for each chromosome arm. **(B)** The genomic positions of both anchors of interacting regions that are significantly (|log2FC|>1, p<0.05) more frequent (orange dots above x=0) and infrequent (purple dots below x=0) in Sua4.0 samples as compared to Ag55 samples are plotted for each chromosome arm. The 2La inversion (blue bar), 2Rb inversion (purple bar), and 2Rbc inversion (red bar), of which Ag55 and Sua4.0 samples have opposite inversion karyotypes, are shown along the x-axis of the appropriate chromosome arms. The brighter colors in the DIR plot compared to the DEG plot are due to the greater number of DIRs (130,662, times two for each anchor) compared to DEGs (2,297).

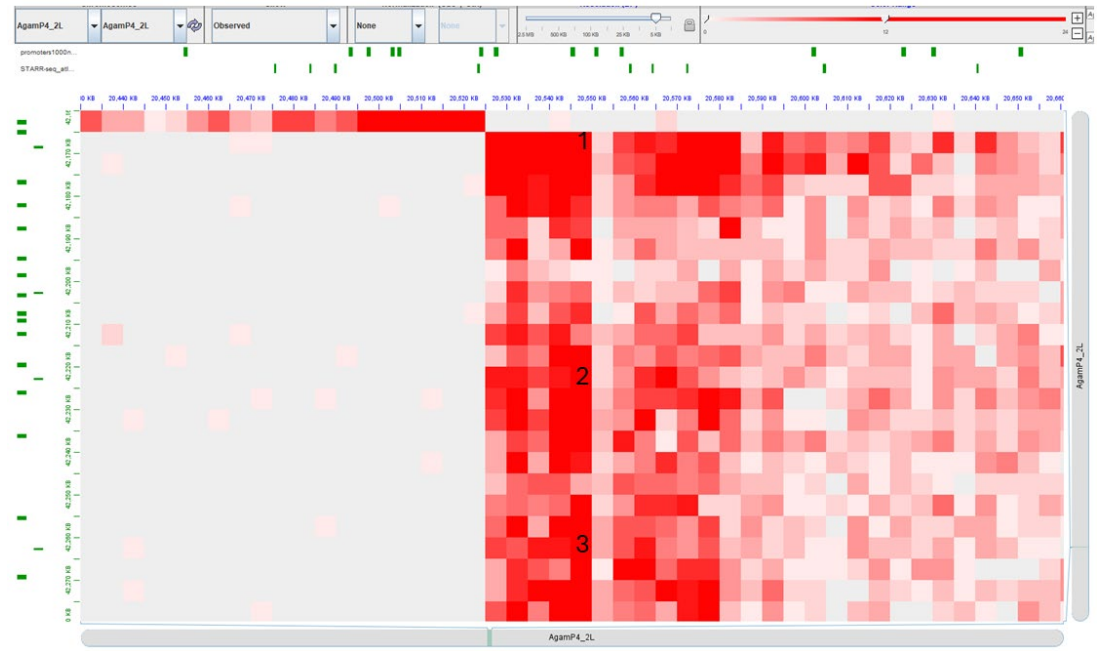

Figure S4. Enlarged image of Sua4.0_replicate1 Micro-C contact matrix mapped to the PEST reference genome showing interaction between ED1/ED2/ED3 and AGAP005781. Screenshot of Sua4.0 replicate 1 Micro-C contact matrix mapped to PEST at 5kb resolution. Window shown is of x-axis range ~20.43MB-20.66MB and y-axis range ~42.27MB-42.16MB. The tracks of green bars along the x- and y-axes are promoter regions (tracks farthest from matrix) and STARR-seq identified enhancers (tracks closest to matrix). The red boxes within the matrix labeled 1, 2, and 3 are the interactions between AGAP005781 and ED1 (1), ED2 (2), and ED3 (3). All other reported enhancer-promoter interactions look similar to this and are therefore shown only as interaction arcs in the manuscript.

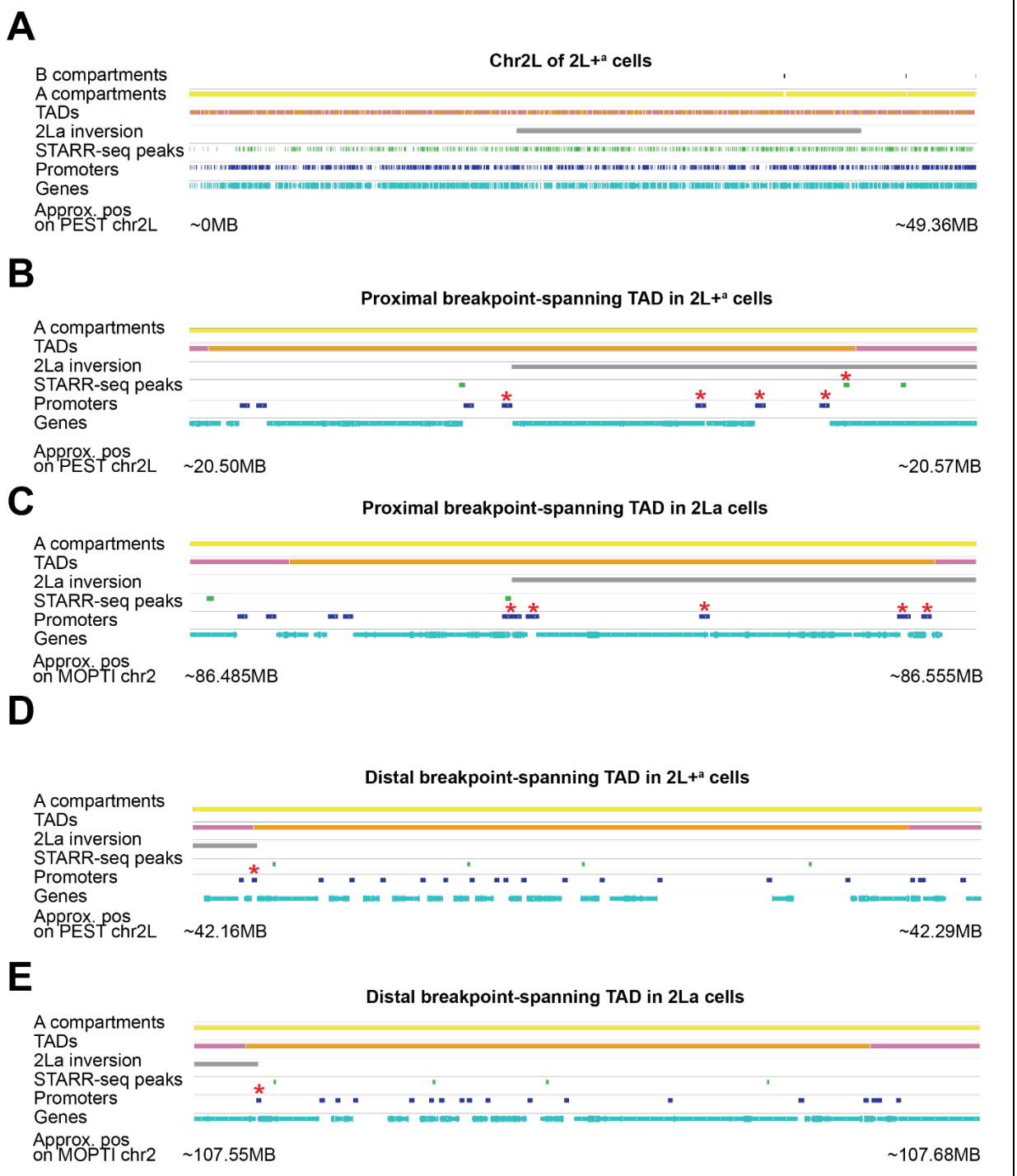

Figure S5. Visualization of 2La inversion breakpoint-containing TADs relative to enhancers and promoters. (A) Window showing the entirety of chromosome 2L of Ag55 (2L+^{a}/2L+^{a}) cells mapped to PEST with tracks from top to bottom showing 50kb resolution B (inactive) compartments (black), 50kb resolution A (active) compartments (yellow), 5kb

resolution TADs (alternating orange and pink), the 2La inversion (gray), STARR-seq identified enhancers (green), promoters (navy), and genes (teal).**(B-C)** Windows 70kb in size showing the proximal breakpoint-containing TAD for Ag55 ($2L+^a/2L+^a$) cells mapped to PEST (B) and Sua4.0 (2La/2La) cells mapped to MOPTI (C). The proximal breakpoint-spanning TAD is 60kb in both $2L+^a$ and 2La cells, 65.6% of which overlaps the 2La inversion in $2L+^a$ cells compared to 53.2% in 2La cells (chi-square goodness-of-fit $p<0.0001$). **(D-E)** Windows 130kb in size showing the distal breakpoint-containing TAD for Ag55 ($2L+^a/2L+^a$) cells mapped to PEST (D) and Sua4.0 (2La/2La) cells mapped to MOPTI (E). The distal breakpoint-spanning TAD is 110kb in $2L+^a$ cells, 2.0% of which overlaps the 2La inversion. In 2La cells, the distal breakpoint-spanning TAD is 115kb, 0.5% of which overlaps the 2La inversion (chi-square goodness-of-fit $p<0.0001$). Tracks shown in (B-E) are the same as (A) but excluding B compartments as none are within the zoomed in windows. The red asterisks mark the genomic elements that are not in the same TAD in the alternative inversion karyotype due to the physical rearrangement of chromatin.

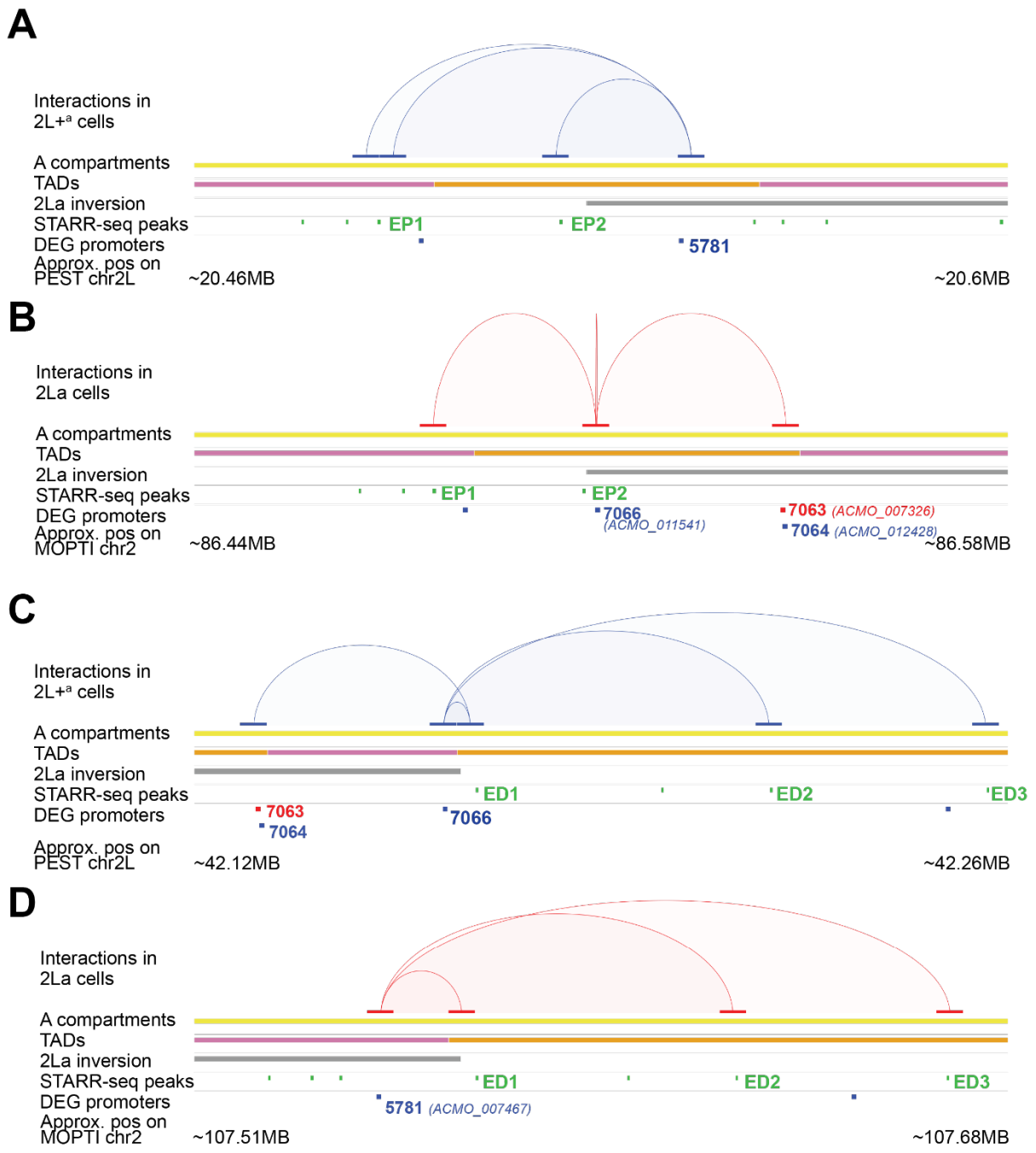

Figure S6. Visualization of the 13 differential interactions involved in enhancer wiring and rewiring over 2La breakpoints relative to TADs and A compartments. (A-B) Differential interactions over the proximal 2La inversion breakpoint involved in enhancer reciprocal rewiring that are more frequent in 2L+^{a} cells, shown mapped to PEST (A) and more

frequent in 2La cells, shown mapped to MOPTI (B). **(C-D)** Differential interactions over the distal 2La inversion breakpoint involved in enhancer wiring and rewiring that are more frequent in 2L+^{a} cells, shown mapped to PEST (C) and more frequent in 2La cells, shown mapped to MOPTI (D). Tracks shown from top to bottom are 50kb resolution A compartments (yellow), 5kb resolution TADs (alternating pink and orange), the 2La inversion (gray), STARR-seq peaks (green), promoters (navy), and genes (teal). The enhancers and DEG promoters involved in the 13 differential interactions involved in enhancer reciprocal rewiring over 2La breakpoints are labeled, as in Figure 2B. Some of the 13 differential interactions occur across TADs. Namely, in 2L+^{a} cells, EP1 and AGAP005781 interact over a TAD boundary, and the interactions between ED1, ED2, ED3, and AGAP007066/AGAP007064/AGAP007063 occur over TAD boundaries. In 2La cells, EP1 interacts with AGAP007066, AGAP007064, and AGAP007063 over a TAD boundary, and ED1, ED2, and ED3 interact with AGAP005781 over a TAD boundary. The proximal breakpoint-spanning TAD in 2L+^{a} cells mapped to PEST has coordinates 20,500,001- 20,560,000bp. The proximal breakpoint-spanning TAD in 2La cells mapped to MOPTI has coordinates 86,490,001- 86,550,000bp. The distal breakpoint-spanning TAD in 2L+^{a} cells mapped to PEST has coordinates 42,165,001- 42,280,000bp. The distal breakpoint-spanning TAD in 2La cells mapped to MOPTI has coordinates 107,555,001-107,665,000bp. All of the 13 differential interactions occur within A compartments, whose coordinates in PEST are 2- 37,300,000bp at the proximal end of the breakpoint and 37,400,002- 44,950,000bp at the distal end and in MOPTI are 85,200,002- 95,800,000bp at the proximal end and 97,100,002- 110,000,000bp on the distal end.

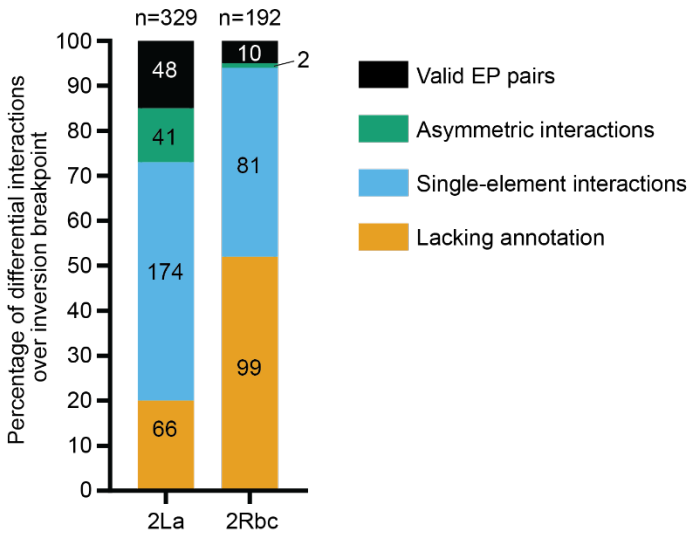

Figure S7. Distribution of chromatin interaction types differentially occurring over inversion breakpoints differ between the 2La and 2Rbc inversions. Valid EP pairs are interactions in which one anchor contains an enhancer while the partner anchor contains a promoter. Asymmetric interactions are interactions in which one anchor contains both an enhancer and a promoter while the partner anchor contains neither an annotated enhancer nor promoter. Single-element interactions are ones in which one anchor contains either an enhancer or a promoter while the partner anchor contains neither an annotated enhancer nor promoter. Interactions lacking annotation are interactions in which both anchors lack an annotated enhancer or promoter. For differential interactions over 2La breakpoints, 48 (14.6%) are valid EP pairs, 41 (12.5%) are asymmetric interactions, 174 (52.9%) are single-element interactions, and 66 (20.1%) are lacking annotation. For differential interactions over 2Rbc breakpoints, 10 (5.2%) are valid EP pairs, 2 (1.0%) are asymmetric interactions, 81 (42.2%) are single-element interactions, and 99 (51.6%) are lacking annotation. The distributions between 2La and 2Rbc differential interactions are statistically different by chi-square goodness-of-fit test ($p<0.0001$).

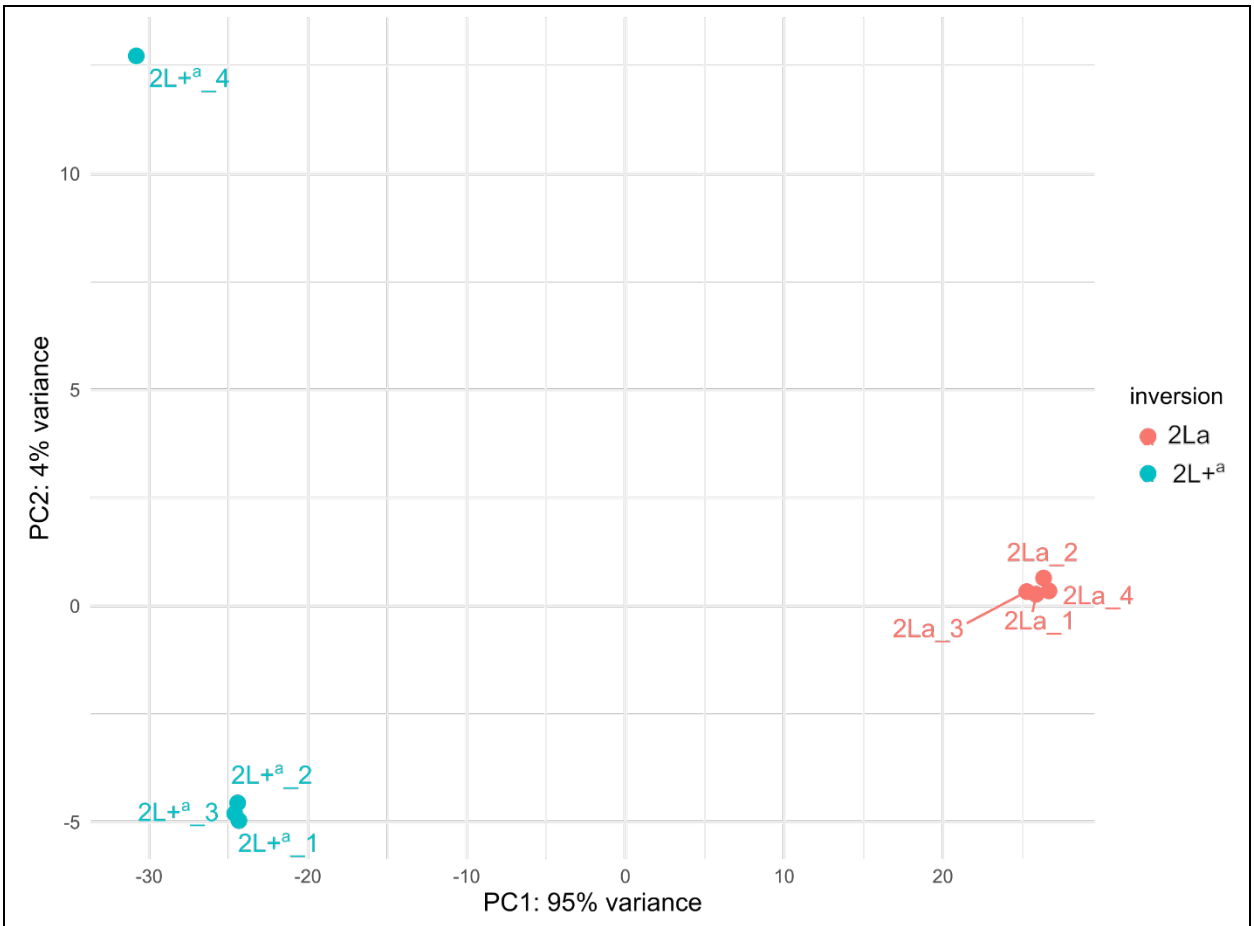

Figure S8. Prioritization of samples for Micro-C by PCA analysis of the 4 bulk RNA-seq replicates. RNA-seq data were rlog-transformed using DESeq2 (blind=TRUE) prior to principal component analysis. PCA analysis was performed using the plotPCA function in DESeq2, with samples grouped by inversion karyotype. The percentage of variance explained by each principal component was extracted and plotted using ggplot2. Replicates 1,2, and 3 were used in Micro-C experiment. Note, for all replicates, samples were simultaneously prepped for bulk RNA-seq and Micro-C.