Supplemental Methods

RNA-seq library preparation and sequencing

100 pairs of testes from < 2 day old male flies were used per replicate. The testis were dissected in PBS on ice. Total RNA was extracted using RNeasy Mini Kit (Qiagen 74104) and ribosomal RNA was depleted using Ribo-Zero rRNA Removal Kit (Illumina MRZH116). Library preparation was then carried out using SMARTer Stranded RNA-Seq Kit (Clonetech 634839).

Sequencing was done at the Stanford Functional Genomics Facility with NextSeq 500 75bp paired-end, with 9 libraries pooled in each run. 34~40 million reads were obtained per replicate and each condition had 2 biological replicates.

RNA-seq data analysis

Adapters and low quality bases were trimmed with trimGalore (0.4.1) (Martin 2011), and mapped to *Drosophila melanogaster* genome build dm6 using STAR (2.5.3b) (Dobin et al. 2013). Reads that fell within gene regions were counted with STAR using Ensembl annotation BDGP6.84. Only genes that are protein-coding and located on the main chromosomes were included in our analysis, which took up 50% of the total reads. Differential expression analyses were carried out using the DESeq function with default settings in DEseq2, which does a Wald test. For plotting in Fig. 1, log transformation and between library normalization was done by the rlog function in DESeq2 (1.18.1) (Love et al. 2014).

CAGE library preparation and sequencing

300 pairs of testes from < 2 day old male flies were used per replicate. Testis were dissected in PBS on ice. RNA was extracted and purified with Trizol (Invitrogen 15596026) followed by Qiagen RNeasy Mini Kit (Qiagen 74104). 20~50ug of total RNA was sent to DNAform (https://www.dnaform.jp/en/products/library/cage/) for CAGE library preparation using published

protocol nAnTi-CAGE (non-amplifying-non-tagging illumina CAGE) (Murata et al. 2014) and sequenced using Illumina HiSeq with 75bp single-end sequencing. Around 25~30 million reads were obtained per replicate, and each condition had 2 biological replicates.

CAGE data analysis

Low quality bases were trimmed using TrimGalore (0.4.4_dev) (Martin 2011) and mapped to *Drosophila melanogaster* genome build dm6 using STAR (2.5.4b) (Dobin et al. 2013). The most 5' mapped nucleotide of the uniquely mapped reads were counted using bedtools coverage (2.27.1) (Quinlan and Hall 2010) and used as input into CAGEr to build CAGE clusters (1.20.0) (Haberle et al. 2015). The Gs occasionally added to the 5' end of reads by reverse transcriptase were not specifically corrected unless they do not map to the genome.

TSS cluster identification and gene classification

For TSS tag clustering with CAGEr, replicates were merged, resulting in ~49 million reads for each condition. Individual TSS with more than 18 CAGE reads were included for building TSS clusters, TSSs within 40bp of each other were clustered together, and only clusters with more than 90 total reads from all TSS positions were considered expressed and kept for further analysis. Using lower read count cutoffs lead to more clusters, and also tended to merge nearby clusters. As a result, varying the cutoffs changed the number of genes in each group but did not change the main conclusions about sequence motifs.

Given our approach to use the same cutoff for TSS at each genomic location across all genes, we noticed an overall correlation between the total width of a TSS cluster and gene expression level. Conceivably, if a gene is more highly expressed, it will tend to have more TSSs that exceeded the cutoff and so were included into the cluster, and as a result, a wider TSS cluster. We therefore discarded 10% of reads on either end of the clusters and defined the RETI, which does not correlate

with the overall expression level and were less likely to be influenced by the level of gene expression and the depth of sequencing (Haberle et al. 2014).

For quantification and differential expression analysis, the CAGE clusters generated from bam-/- and 72hrPHS testes were merged into one set of clusters with CAGEr (1.20.0) (Haberle et al. 2015), and bedtools coverage (Quinlan and Hall 2010) was used to count reads falling within each cluster in each sample. Between sample normalization was carried out using DESeq2 (1.18.1) (Love et al. 2014).

TSS clusters were first classified as either “non-72hrPHS-specific CAGE clusters” or “72hrPHS-specific CAGE clusters”. “72hrPHS-specific CAGE clusters” have CAGE read counts in 72hrPHS upregulated greater than or equal to 16-fold compared to in bam-/- testis. All the other CAGE clusters were classified as “non-72hrPHS-specific CAGE clusters”. TSS clusters were then assigned to expressed protein-coding genes based on falling within 500bp upstream of the annotated TSS or overlapping with any exons of the gene on the same strand based on Ensembl annotation BDGP6.84. The genes expressed were then classified based on how transcript levels changed in the time course based on RNA-seq, and the type of CAGE clusters they contained. Genes that contained only non-72hrPHS-specific CAGE clusters and that were downregualed more than 2 fold based on RNA-seq in 72hrPHS compared to *bam-/-* testes were assigned to the 'downregulated' group. Genes containing only 72hrPHS-specific CAGE clusters and that were upregulated more than 8 fold in 48hrPHS compared to *bam-/-* or 16 fold in 72hrPHS compared to *bam-/-* testes were designated 'off-to-on'. Genes containing both non-72hrPHS-specific CAGE clusters and 72hrPHS-specific CAGE clusters were placed in the 'alternative promoter' group.

Some genes contained multiple CAGE clusters of the same type: for example, some extremely highly expressed genes tended to have small CAGE clusters along the gene body. Also, some genes had CAGE clusters within the 3’UTR, as previously reported (Hoskins et al. 2011). To capture the one CAGE cluster that most likely corresponded to the main promoter being used in the time point of

interest, for each downregulated and off-to-on gene, only the CAGE clusters that were both the most upstream and at the same time most expressed were kept for the following bioinformatics analysis. For genes with alternative promoters, one “non-72hrPHS-specific CAGE clusters” and one “72hrPHS-specific CAGE clusters” were kept. With this filtering step, 1153 downregulated genes, 1640 off-to-on genes and 1036 genes with alternative promoters were carried forward to further analysis.

ATAC-seq library preparation and sequencing

ATAC-seq was carried out with a modified version of published protocols (Buenrostro et al. 2015). For each batch, 10~20 pairs of testes from < 1 day old male flies were dissected in PBS on ice, and immediately digested for 8 minutes at room temperature in 0.5mg/ml of dispase (Worthington LS02109) and 0.5mg/ml of collagenase XI (Sigma C7657) dissolved in PBS with 0.3mM of $CaCl_2$. Testis were washed once in cold PBS and broken open by pipetting 7 ~ 10 times in 50ul of cold PBS. Cells were spun down at 2000rpm for 1min, cold lysis buffer (Buenrostro et al. 2015) were added, and the pellet gently resuspended by flicking the tube. After another 2000rpm 2min spin, 20ul of transposase mix from the Nextera Kit (Illumina, cat. no. FC-121-1030) was added and the pellet gently resuspended by flicking the tube. The reaction was incubated at 30^oC for 25min with the tube flicked once during incubation. The DNA was purified using Qiagen MinElute column (Qiagen 28004) and the entire sample used for PCR with NEBNext (NEB M0541S) for a total of 8~9 amplification cycles.

For samples enriched for spermatocytes, the transposase reaction would fail if there were more than 10 testes in one tube, possibly due to the large size of the nuclear pellet interfering with diffusion of transposase. So 10 pairs of testes were processed per batch and libraries from 1 or 2 such batches done in parallel using testis from the same cross were combined as one biological replicate. At least 2 biological replicates were generated per condition.

Sequencing was done with HiSeq 4000 at the Stanford Functional Genomics Facility, with 75bp pair-end reads. 10~30 million reads were obtained for each biological replicate.

ATAC-seq data analysis

Adapters and low quality bases were trimmed using trimGalore (0.4.1) (Martin 2011), mapped to *Drosophila melanogaster* genome build dm6 using bwa aln (0.7.10) (Li and Durbin 2009). PCR duplicates were removed using Picard tools (1.130) and only reads uniquely mapped to main chromosomes (50~60% of total reads) were used for downstream analysis.

Reproducibility was checked across biological replicates and the replicates were combined to plot heatmaps using DeepTools (3.3.0) (Ramírez et al. 2016). In heatmaps in Fig. 2I and Fig. 3, read counts at each genomic position was divided by library size and then times 10,000,000, which was effectively 10 fold of RPM. The scale were chosen to avoid maximum data range less than 1. For plotting, data point more than 6 were plotted as 6 to show dynamic range. Combined data were also used to calculate nucleosome positions using NucleoATAC (0.3.2) (Schep et al. 2015), and the autocorrelation score (nucleoatac_signal.bedgraph.gz generated by NucleoATAC) were used in Fig. 3D with data point less than 0 plotted as 0.

Figure S1

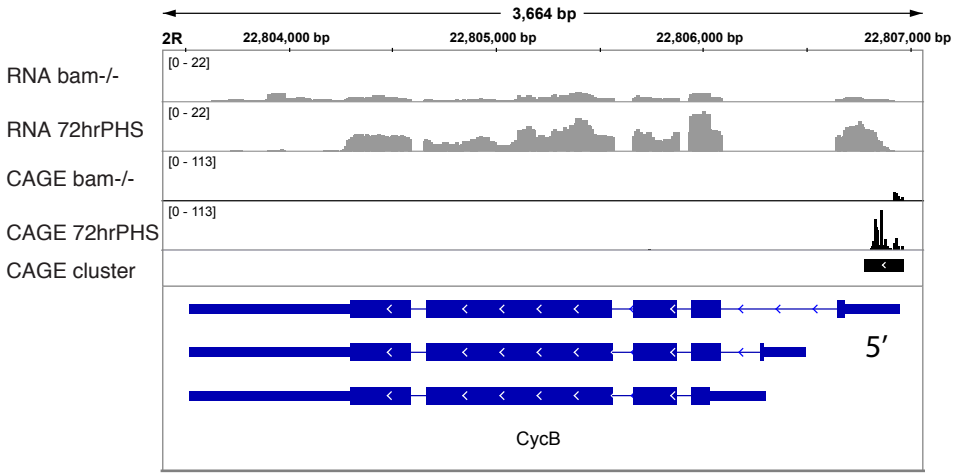

Figure S1. RNA-seq and CAGE profile for Cyclin B1 locus. Cyclin B1 is known to be expressed from an Aly-independent promoter in spermatogonia and an Aly-dependent promoter in spermatocytes (White-Cooper et al. 1998). Consistent with this, CAGE signal appeared in a new position in the 72hrPHS sample. However Cyclin B1 was not included in the group of genes with alternative promoters because the new CAGE cluster at 72hrPHS was too close to the old CAGE cluster in bam-/- to be called as a separate cluster (see Supplemental Methods for CAGE cluster calling. At least 40bp of low or no CAGE signal was required to separate two nearby clusters).

Figure S2

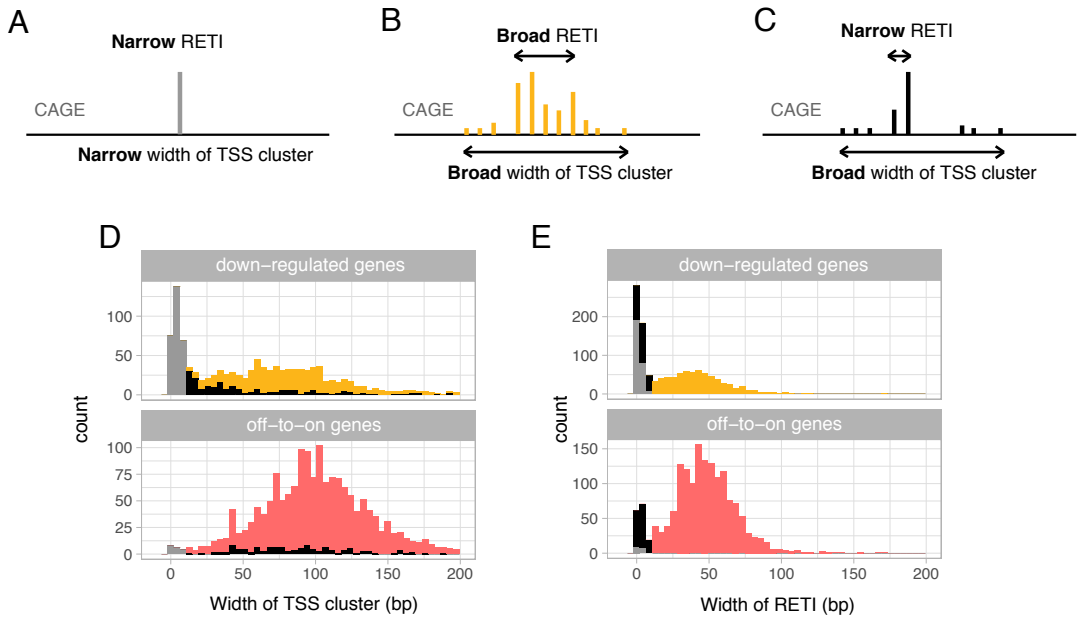

Figure S2. (A-C) Comparing the width of region of efficient transcript initiation (RETI) with the overall width of the CAGE cluster revealed three kinds of promoters. (A) In narrow/narrow promoters, almost all mature transcripts initiate from one or a few adjacent positions and both the RETI and the total width of the TSS cluster spanned less than 11bp. (B) In broad/broad promoters, mature transcripts initiated from many sites, with the RETI often spanning over tens of nucleotides. (C) In narrow/broad promoters, the vast majority of mature transcripts initiated in a focused RETI spanning <11bp but some transcripts did initiate across a considerably broader region. Suggesting that although a wide span across the promoter was available, only a small region of it was preferentially used for productive transcript initiation. The cutoff for both measures of width was 11bp, chosen to separate the two peaks in the distributions in D,E. (D) Distribution of width of total TSS cluster and (E) Distribution of width of RETI from the testis data. Grey: narrow/narrow promoters. Orange/Red: broad/broad promoters. Black: narrow/broad promoters. (D, E top panels) The vast majority of the genes downregulated upon differentiation had narrow/narrow or broad/broad promoters, with a small fraction of narrow/broad promoters. (D, E bottom panels) Most of the off-to-on genes had broad/broad promoters, with a small fraction of genes expressed from narrow/broad (black) promoters, and only very few genes expressed from narrow/narrow (grey) promoters. For all analysis presented in the rest of the paper, only the width of RETI was used and discussed.

Figure S3

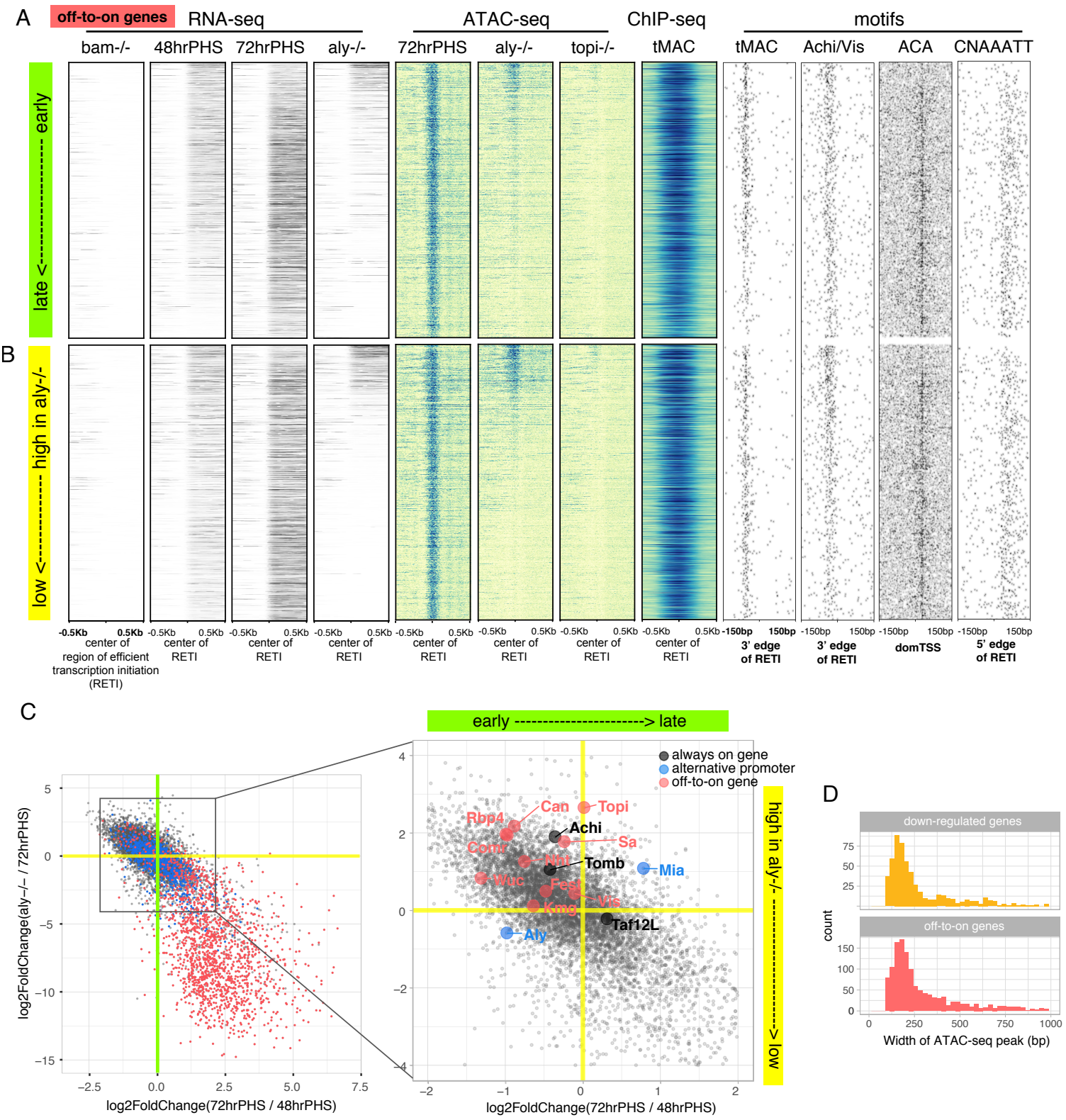

Figure S3. The special group of off-to-on genes that are expressed early after onset of spermatocyte differentiation and do not require Aly for expression. (A-B) RNA-seq, ATAC-seq, tMAC ChIP-seq profiles and motif occurrence for promoters of off-to-on genes. (A) sorted by how early the genes become fully expressed, approximated by ratio of RNA level in 72hrPHS to 48hrPHS, and (B) sorted by how depedent they are on Aly to be expressed, approximated by ratio of RNA level in aly-/- to 72hrPHS testes. These promoters still require Topi for expression, have tMAC ChIP peaks, enrich for tMAC motif and Achi/Vis motif, but lack ACA and CNAAATT motifs. (C) Left panel: scatter plot of off-to-on genes (red), genes with alternative promoters (blue) and other genes (grey) showing the timing of their expression in spermatocyte stage (X-axis) and their dependency on Aly to be expressed (Y-axis). Right panel: important regulators of transcription in spermatocytes (highlighted) are among genes that are expressed early and high in aly-/- testes. (D) Distributions of size of open promoter region for indicated gene groups based on ATAC-seq.

Figure S4

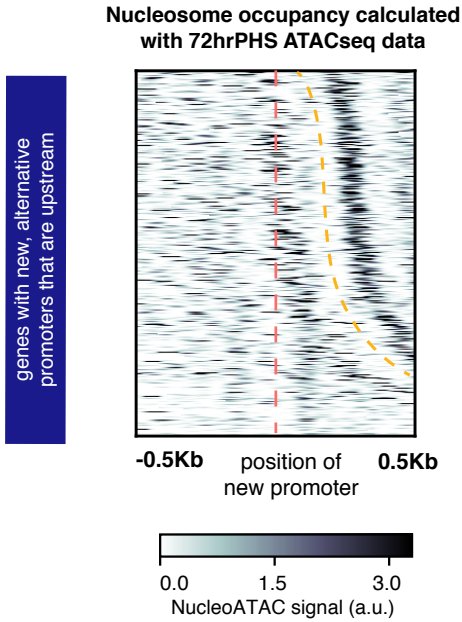

Figure S4. Although chromatin clearly opened at the site of the new upstream promoter based on ATAC-seq (Fig. 3B, 72hrPHS), displacement of the -1 nucleosome of the old promoter was not clearly observed in the 72hrPHS sample (compare Figure S4 with Fig. 3D right panel). This is likely due to the 72hrPHS testes having both proliferating bam-/- spermatogonia and maturing spermatocytes and have a mixture of ATAC-seq signal. Moreover, the transposase ATAC-seq uses also show clear sequence bias (Adey et al. 2010; Buenrostro et al. 2013). The downregulated promoters tended to have stronger ATAC-seq signal compared to spermatocyte-specific promoters (including off-to-on and alternative new promoters in speramotyctes), likely due to transposase preference for nucleotide sequence composition. This can be seen comparing the data range in the legend for ATAC-seq in Fig. 2I (downregulated promoters) and Fig. 3A (spermatocyte-specific promoters).

Figure S5

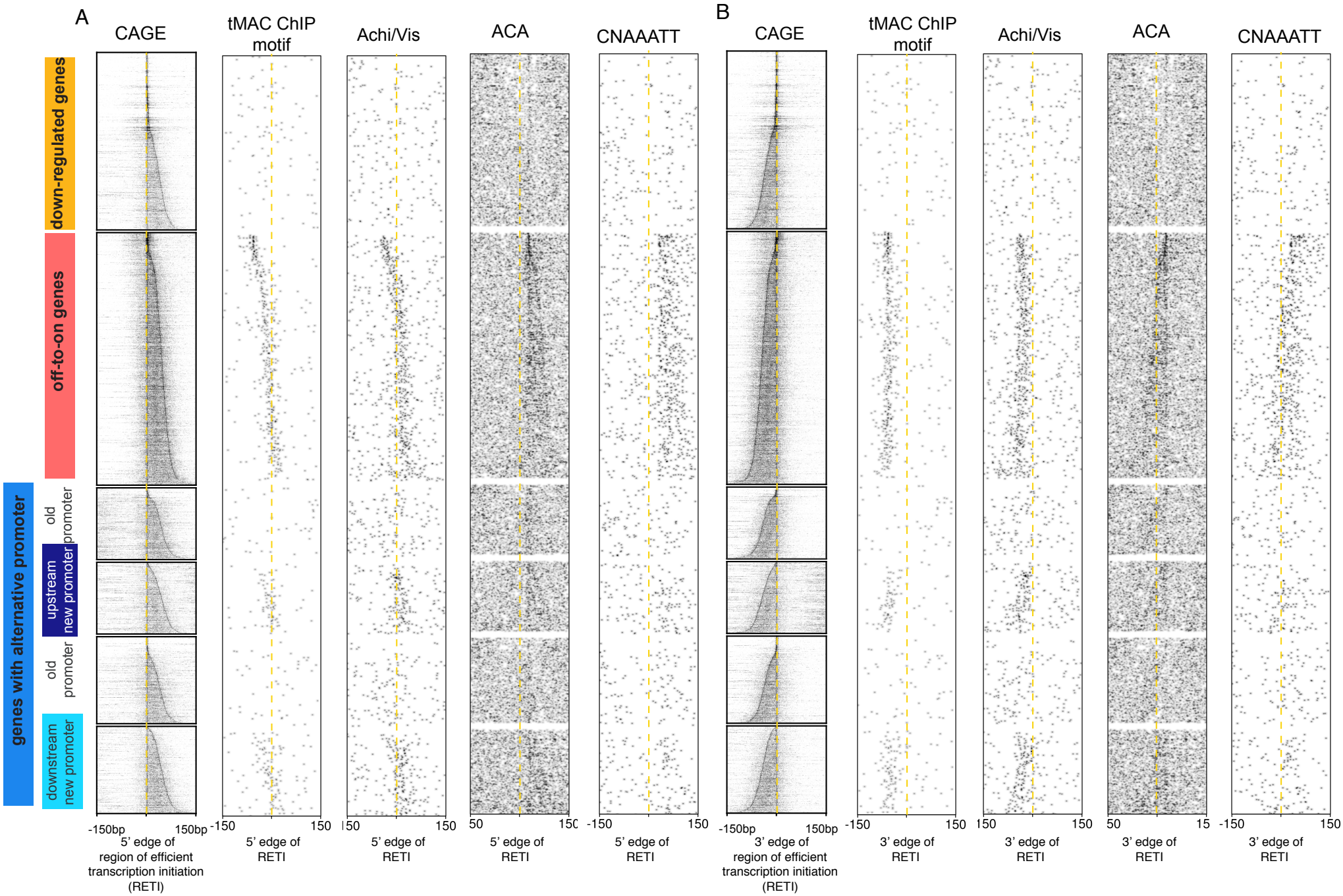

Figure S5. Occurrences and positions of specific motifs in the indicated groups of genes. (A) Genes aligned by the 5' edge of the RETI. (B) Genes aligned by the 3' edge of the RETI. All panels sorted by width of the RETI.

Figure S6

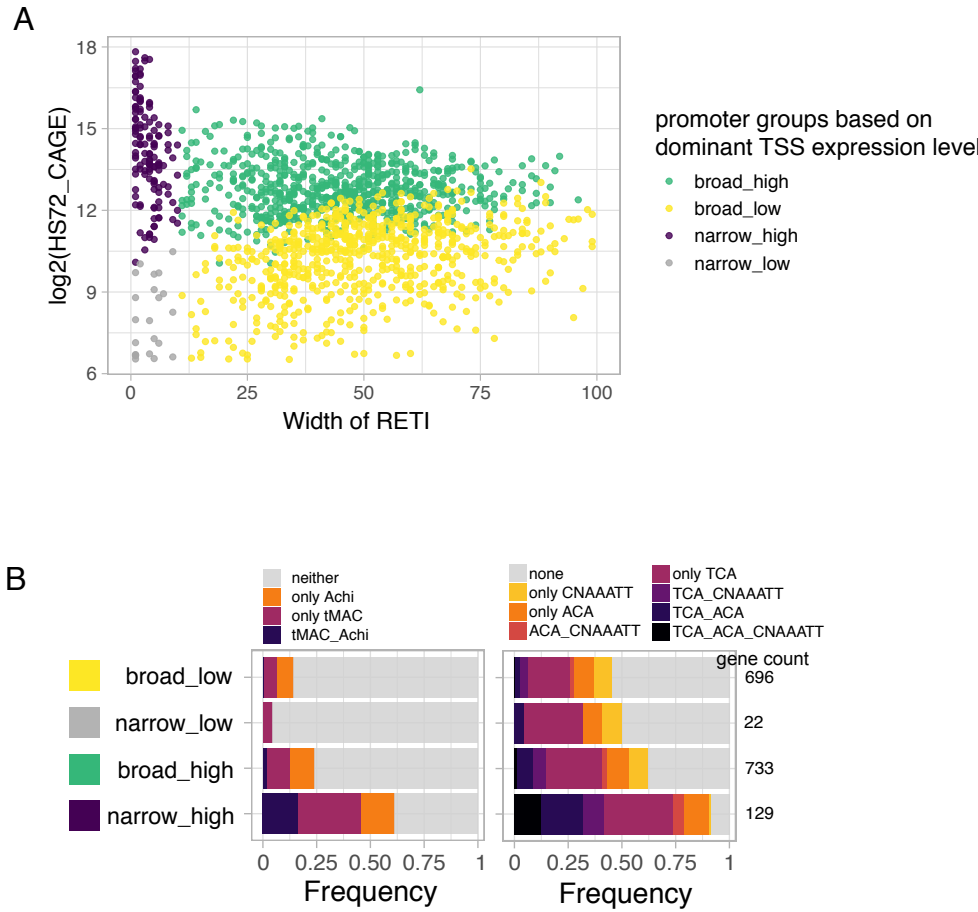

Figure S6. (A) The grouping of off-to-on promoter types based on overall promoter expression level largely agrees with grouping by dominant TSS expression level. The color is based on grouping by dominant TSS level as in Figure 7A, and the Y-axis is overall promoter expression level in 72hrPHS. (B) Motif occurrence in the four groups of off-to-on genes as defined in Figure 7A. Note the motifs were only counted if they were at optimal position relative to the dominant TSS as in Figure 7: tMAC ChIP motif between -70 to -40; Achi/Vis motif between -50 to -20; ACA at +26, +28, or +30; CNAAATT between +29 to +62.