Supplementary Materials and Methods

Immunofluorescence

Larvae were dissected in batches of 5-20 in PBS at room temperature and transferred to ice. Samples were then fixed in PBS plus 4% paraformaldehyde (Electron Microscopy Services) for 25-minutes at room temperature on an orbital shaker. Fixed samples were washed in PBS + 0.15% Triton X100 (PBT). Antibodies were incubated either for 2hrs at room temperature or overnight at 4°C. After secondary antibody incubation, samples were washed once with PBT, once with PBT + 0.2µg / ml DAPI, and once with PBT. The following antibody concentrations were used: 1:750 mouse anti-EcR (DSHB DDA2.7, concentrate), 1:4000 rabbit anti-GFP (Abcam ab290), 1:3500 mouse anti-Dl (DSHB C594.9b, concentrate), 1:200 mouse anti-FLAG M2 (Sigma F1804), 1:1000 anti-Br (DSHB 25E9.D7, concentrate), 1:500 anti-Dcp-1 (Cell Signaling Technology 9578). Secondary antibodies were: 1:1000 goat anti-rabbit, or goat anti-mouse, conjugated with either Alexa-488 or Alexa-594 (ThermoFisher A11037, A11034). Samples were imaged on a Leica Sp5 or Leica Sp8 confocal microscope. Salivary gland DNA content and dimensions were measured using the ROI manager in FIJI.

Sample preparation for RNA-Seq

A minimum of 60 wings or salivary glands were prepared as previously described (58) from either *Oregon R (WT)* or *yw; vg-GAL4, tub>CD2>GAL4, UAS-GFP, UAS-FLP / UAS-EcR-RNAi^{104} (EcR-RNAi)*. For library construction, 50-100ng RNA was used as input to the Tecan Genomics Universal RNA-Seq with NuQuant, Drosophila. Library preparation followed the manufacturer's instructions with the following modifications: 1) after second-strand cDNA synthesis, samples were sonicated 5x20-seconds (30-seconds rest between cycles) on high power in a BioRupter bath sonicator; 2) qPCR was performed to determine the optimal cycle number using manufacturer's recommendations; 3) after library amplification, an additional, 1.2:1 SPRI bead-cleanup was performed. Paired-end, 2x75 sequencing was performed on an Illumina HiSeq X using Novogene Co.

Sample preparation for CUT&RUN

A minimum of 75 wings or 50 salivary glands from *w; EcR^{GFSTF}/Df(2R)BSC313* were dissected in wash buffer (20mM HEPES-NaOH, 150mM NaCl, 2mM EDTA, 0.5mM Spermidine, 10mM PMSF). The rest of the protocol was performed as described in[1]. Fragments that diffused out of the nucleus ("supernatant" sample), as well as size-selected DNA from the nuclei ("pellet" samples) were prepared and sequenced. For library preparation, the Takara ThruPLEX DNA-seq kit with unique dual-indexes was used following the manufacturer's protocol until the amplification step. For amplification, after the addition of indexes, 16-21 cycles of 98C, 20s; 67C, 10s were run. A 1.2x SPRI bead cleanup was performed (Agencourt Ampure XP). Libraries were sequenced on an Illumina HiSeq 4000 with 2x75 reads. The following antibody concentrations were used: 1:300 mouse anti-FLAG M2; 1:200 rabbit anti-Mouse (Abcam ab46450); 1:400 Batch#6 protein A-MNase (gift of Steven Henikoff).

Sample preparation for FAIREseq

Larvae from either *Oregon R (WT)* or *yw; vg-GAL4, tub>CD2>GAL4, UAS-GFP, UAS-FLP / UAS-EcR-$RNAi^{104}$ (EcR-RNAi)* were dissected in 1xPBS in batches of 5-10 then fixed at RT for 10-minutes in 4% paraformaldehyde, 50mM HEPES (pH 8.0), 100mM NaCl, 1mM EDTA (pH 8.0), 0.5mM EGTA (pH 8.0). Fixation was quenched by incubation for 5m in 1xPBS, 125mM Glycine, 0.01% Triton X-100 and then transferred to 10mM HEPES (pH 8.0), 10mM EDTA (pH 8.0), 0.5mM EGTA (pH 8.0), 0.25% Triton X-100, 1mM PMSF. Wings or salivary glands were dissected off cuticles and snap frozen in liquid nitrogen. Samples were lysed in 2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris (pH 8.0), 1mM EDTA. Following lysis, a minimum of 40 wings or salivary glands were pooled together and homogenized using 2.38mm tungsten beads with 6 cycles of 1min on and 2min off and then sonicated using a Branson Sonifier with 5 cycles of 30-seconds (1-second on, 0.5-second off) while letting the samples rest for at least 2-minutes on ice between cycles. An aliquot was removed as an input fraction. The remaining samples were subjected to phenol-chloroform and chloroform extractions and then

precipitated with ethanol. Input and experimental samples were heated overnight at 65C to reverse cross links and then treated with RNase A for 1-hour at 37°C. DNA was purified with a Qiagen QIAquick PCR Purification Kit eluting in nuclease free water. Samples were used as input into the Takara ThruPLEX DNA-seq kit following manufacturer's instructions.

RNA Sequencing Analysis

Reads were trimmed using bbmap (v38.75) with parameters ktrim=r ref=adapters rcomp=t tpe=t tbo=t hdist=1 mink=11. Reads were aligned with STAR (2.7.3a)[2]. Indexes for STAR were generated with parameter --sjdbOverhang 74 using genome files for the dm6 reference genome. The STAR aligner was run with parameters --alignIntronMax 50000 --alignMatesGapMax 50000. Samtools (v1.9) was used to filter reads to those with a q-score greater than 2. RSubread (v2.0.1) was used to count reads mapping to genes using a gtf file from flybase.org (r6.32) using parameters: annot.ext = gtfPath, isGTFAnnotationFile = T, isPairedEnd = T, strandSpecific = 1, nthreads = 4, GTF.featureType = 'exon', allowMultiOverlap = F[3]. DESeq2 (v1.26.0) was used to identify differentially expressed genes using the lfcShrink function to shrink log-fold changes and with each genotype and time-point as a separate contrast[4]. Differentially expressed genes were defined as genes with an adjusted p-value less than 0.05 and an absolute log2 fold change greater than 1. Normalized counts were generated using the counts function in DESeq2. For c-means clustering, normalized counts were first converted into the fraction of maximum normalized counts across all tissues and conditions and c-means clustering was performed using the ppclust package (v1.1.0)[5]. MA Plots were made with ggplot2 and points were shaded using kernel density estimates calculated using the MASS (v7.3-51.4) package[6]. Heatmaps were generated using ggplot2 (v3.3.2) and patchwork (v1.1.0) in R[7-9]. Gene Ontology (GO) analysis was performed using Bioconductor packages TopGO (v2.38.1) and GenomicFeatures (v1.38.2) using expressed genes as a background set with parameters: algorithm = 'elim' and statistic = 'fisher'[10]. Similar GO terms were

collapsed based on semantic similarity using the rrvgo package in R and only the parent term was used (v1.1.1)[11]. Expressed genes were defined as genes with a normalized count value >= 10.

CUT&RUN Sequencing Analysis

Technical replicates were merged by concatenating fastq files. Reads were trimmed using bbmap (v38.75) with parameters ktrim=r ref=adapters rcomp=t tpe=t tbo=t hdist=1 mink=11. Trimmed reads were aligned to the dm6 reference genome using Bowtie2 (v2.2.8) with parameters --local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700[12]. Reads with a quality score less than 5 were removed with samtools (v1.9)[13]. PCR duplicates were marked with Picard (v2.21) and then removed with samtools. Fragments between 20 and 120bp were isolated using a custom awk script and used for downstream analyses as recommended in[14]. Bam files were converted to bed files with bedtools (v2.29) with parameter -bedpe[15]. Bedgraphs were generated with bedtools and then converted into bigwigs with ucsctools (v320)[16]. Data was z-normalized using a custom R script. MACS (v2.1.2) was used to call peaks on individual replicates and merged files using parameters -g 137547960--nomodel --seed 123[17]. As a control for peak calling, wing IgG supernatant and pellet samples were used. Wing IgG controls were *yw* CUT&RUN samples in which the primary antibody was omitted and only the mouse anti-Rabbit IgG secondary was used. To identify differentially bound regions, a union peak set was generated and RSubread (v2.0.1) was used to assign to features using parameters strandSpecific = 0, allowMultiOverlap = T and then used as input for DESeq2 (v1.26.0)[3, 4]. To identify sites that were differentially bound in each tissue irrespective of whether they were found in the supernatant or pellet samples (see Sample Preparation for CUT&RUN), we entered the DESeq2 design formula as, "~tissue + supPel". For pairwise comparisons, union peaks were subsequently filtered to contain peaks that overlapped a peak found in either sample by at least one base pair. MA plots were made as described for RNAseq. Heatmaps and average signal plots were generated from z-normalized data using the Bioconductor package Seqplots (v1.24.0) and plotted using ggplot2[18]. ChIPpeakAnno (v3.20.0) was used

to calculate distance of peaks to their nearest gene[19]. To identify clusters of EcR binding sites, the EcR peaks were resized to 5000bp, assigned to clusters, and the furthest start and end coordinate of the original peaks were used.

FAIRE sequencing analysis

Technical replicates were merged by concatenating fastq files. Reads were trimmed using bbmap (v38.75) with parameters ktrim=r ref=adapters rcomp=t tpe=t tbo=t hdist=1 mink=11. Trimmed reads were aligned to the dm6 reference genome using Bowtie2 (v2.2.8) with parameters --phred33 --seed 123 -x[12]. Reads with a quality score less than 5 were removed with samtools (v1.9)[13]. PCR duplicates were marked with Picard (v2.21) and then removed with samtools. Fragments smaller than 120bp were removed from salivary gland datasets to correct for differences in signal-to-noise between wing and salivary gland samples. The remaining processing and analysis steps were performed as described for CUT&RUN.

Motif Analysis

To identify occurrences of the EcR motif in the genome, PWMs for the EcR and Usp motifs identified by a bacterial 1-hybrid were obtained from Fly Factor Survey[20]. For the palindromic, Usp/EcR motif, the PWMs for EcR and Usp were concatenated together and the probabilities for the central, overlapping base were averaged. FIMO (v4.12.0) was run on the dm6 reference genome using parameters –max-stored-scores 10000000 --max-strand --no-qvalue --parse-genomic-coord --verbosity 4 --thresh 0.01[21]. Motif density plots were generated by counting the number of motifs from peak summits (10bp bins) and normalizing by the number of input peaks. Differential motif enrichment +/– 150bp from the summit of EcR and FAIRE peaks was performed using the 'calcBinnedMotifs' function in monaLisa[22] and the JASPAR motif database[23].

EcR knockdown in the wing and salivary gland

To knockdown EcR in the wing and salivary gland in parallel, we made use of the previously published line: *yw; vg-GAL4, UAS-FLP, Tub>>STOP>>GAL4, UAS-GFP / CyO*. Early activation of *vg-GAL4* throughout the wing primordia results in flip-out of the stop-cassette and persistent expression of *Tub-GAL4* throughout wing development. This construct is also active in the salivary gland during the first larval instar stage, which may be a consequence of the *vg-GAL4* p-element vector which has been previously reported to have a minimal promoter active in the salivary gland.

Drosophila culture and genetics

Flies were grown at 25C under standard culture conditions. Late wandering larvae were used as the –6hAPF timepoint. White prepupae were used as the 0h time point for staging +6hAPF animals. For staging –30hAPF, apple juice plates with embryos were first cleared of any larvae. Four hours later, any animals that had hatched were transferred to vials. 72 hours later, tissues were harvested. The following genotypes were used:

yw; vg-GAL4, UAS-FLP, UAS-GFP, Tub>CD2>GAL4 / CyO.

W1118; P[UAS-EcR-RNAi]104 (BDSC#9327)

yw; EcR^{GFSTF} (BDSC#59823)

w1118; Df(2R)BSC313 /CyO (BDSC#32253)

yw; + / + ; br^{disc}::tdTomato / TM6B

yw; +/+; E74_A-I::tdTomato/TM6B

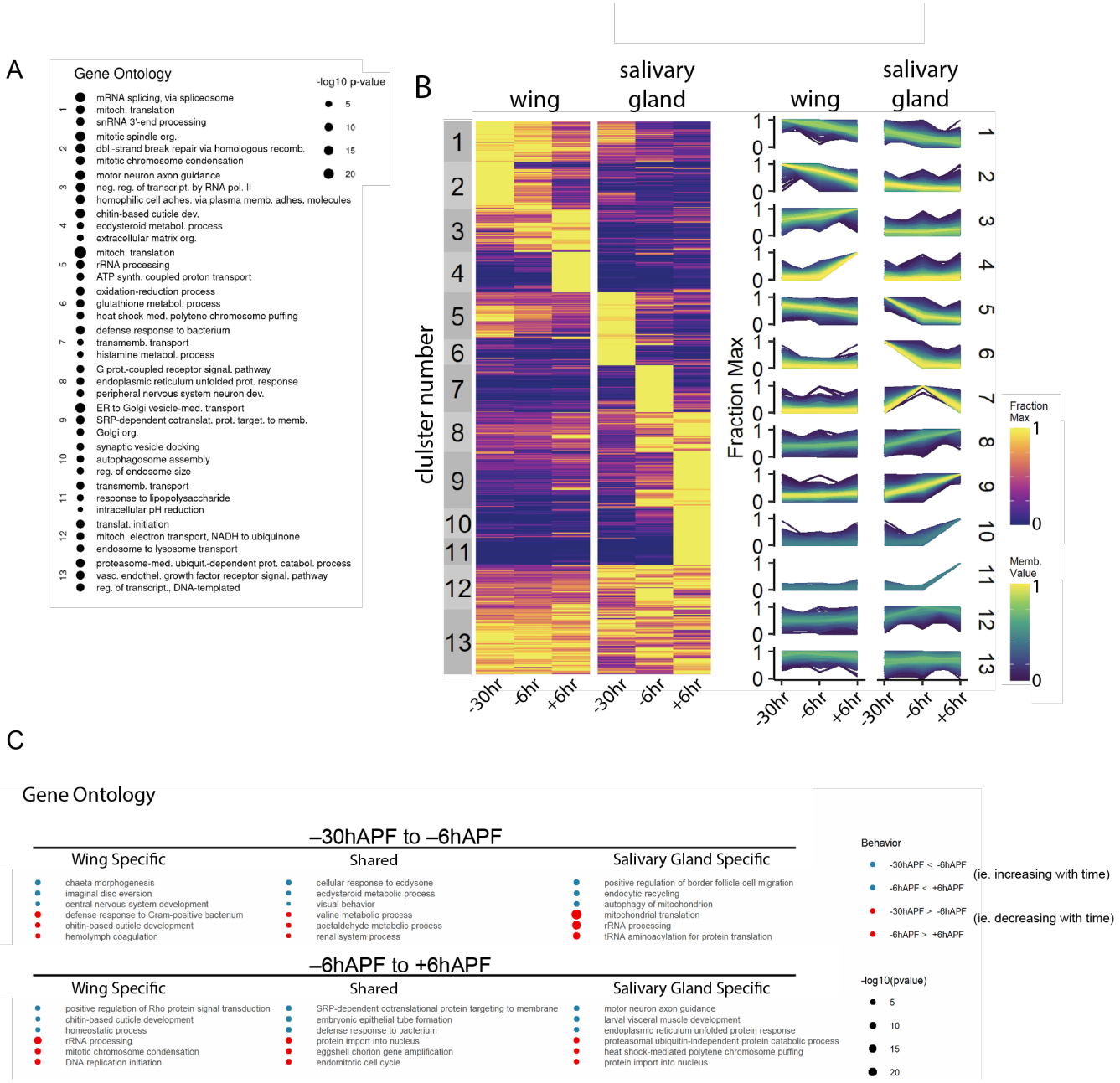
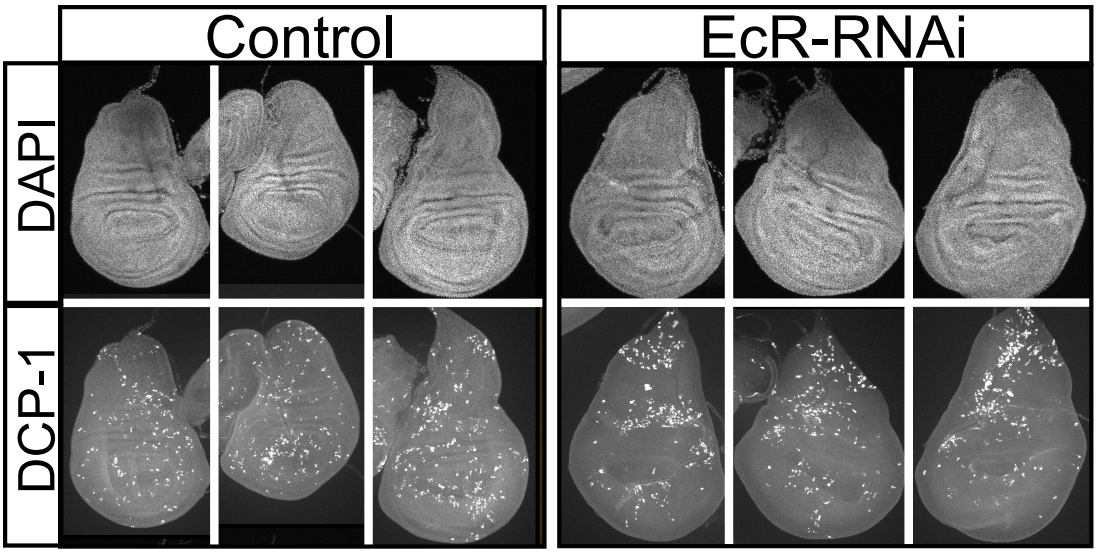


Figure S1. Gene ontology of temporally dynamic genes in wings and salivary glands. (A) Gene ontology terms for each cluster of genes depicted in the heatmap shown in Figure 1B. (B) Copy of the heatmap RNA-seq clusters shown in Figure 1B. (C) Gene ontology terms for temporally dynamic genes between adjacent time points in wild-type wings and salivary glands; these are the same genes depicted in the Venn diagram shown in Figure 1C.

A



B

	Number salivary gland nuclei	
	control	**EcR-RNAi**
mean	122.6	123.0
standard deviation	11.9	10.6

C

	DNA content and size of salivary gland nuclei				
	DAPI mean	area	perimeter	feret diameter max	feret diameter min
control	105.52	618.42	94.02	34.63	23.97
EcR-RNAi	82.54	317.67	66.37	23.10	18.01
EcRi/WT	78%	51%	71%	67%	75%

Figure S2. EcR loss of function does not increase cell death but disrupts proper polyteny. (A) Confocal images of DAPI and Dcp-1 staining from control (GAL4-only) and *EcR-RNAi* third instar wing imaginal discs. (B) Table of the number of nuclei from control (no GAL4 driver) and *EcR-RNAi* –6hAPF salivary glands (n = 12 control glands, n = 13 *EcR-RNAi* glands were scored). (C) Table of the DAPI signal (arbitrary units) and dimensions (pixels) for control (no GAL4 driver) and *EcR-RNAi* –6hAPF salivary glands (n = 47 nuclei from 9 control glands were scored, and n = 58 nuclei from 11 *EcR-RNAi* glands were scored).

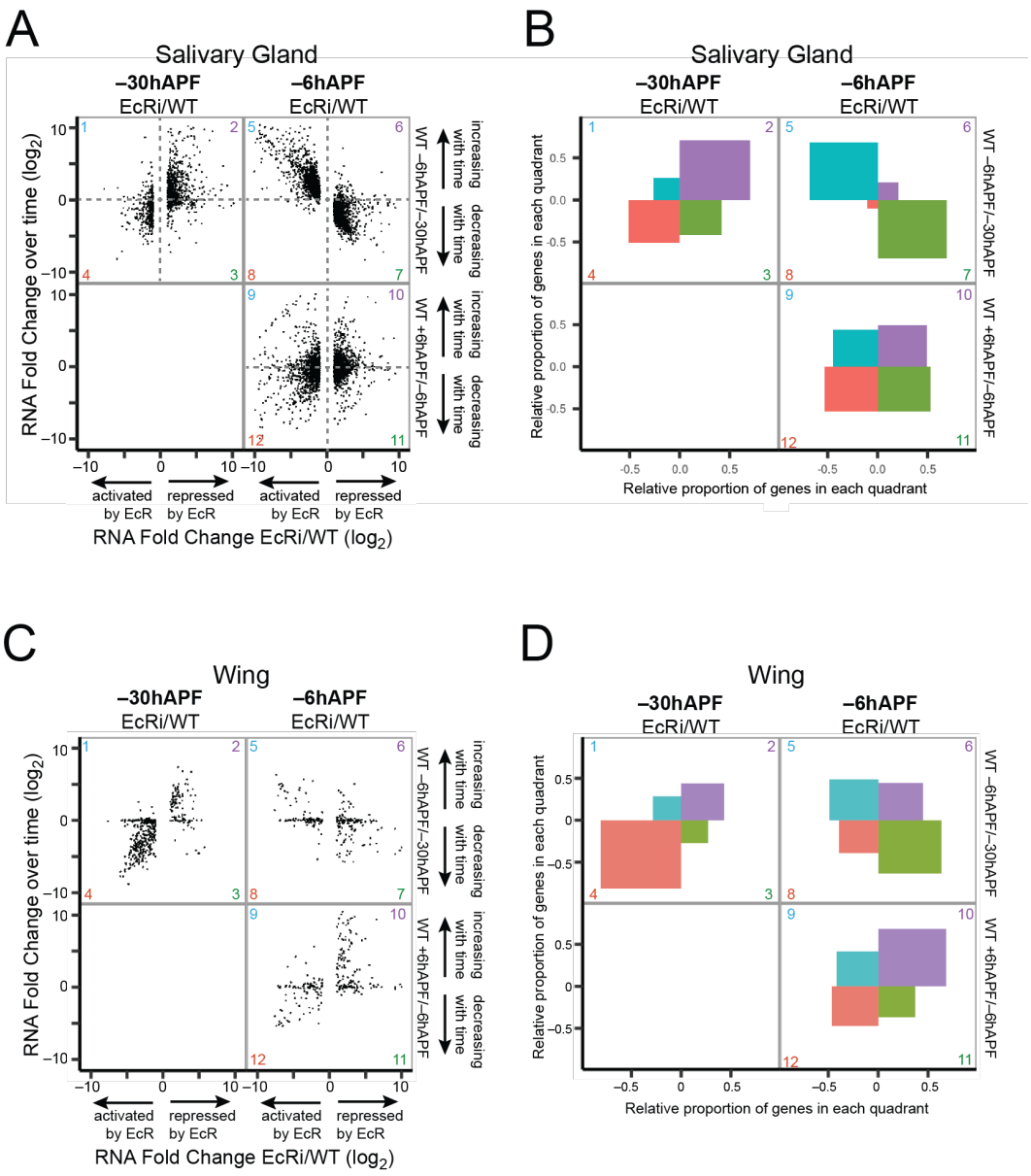


Figure S3. EcR is required for both gene activation and gene repression at –30hAPF and at –6hAPF in wings and salivary glands. (A) and (B) Copies of the scatterplots and gene proportion plots from Figure 2C, D. (C) Scatterplots of RNA-seq values for differentially expressed genes in *EcR-RNAi* wings. The ratio between *EcR-RNAi* and wild-type is shown on the x-axis for –30hAPF and –6hAPF. The ratio between adjacent wild-type stages is shown on the y-axis. (D) Plots indicating the proportion of genes located in each quadrant for the three scatterplots shown in Panel C.

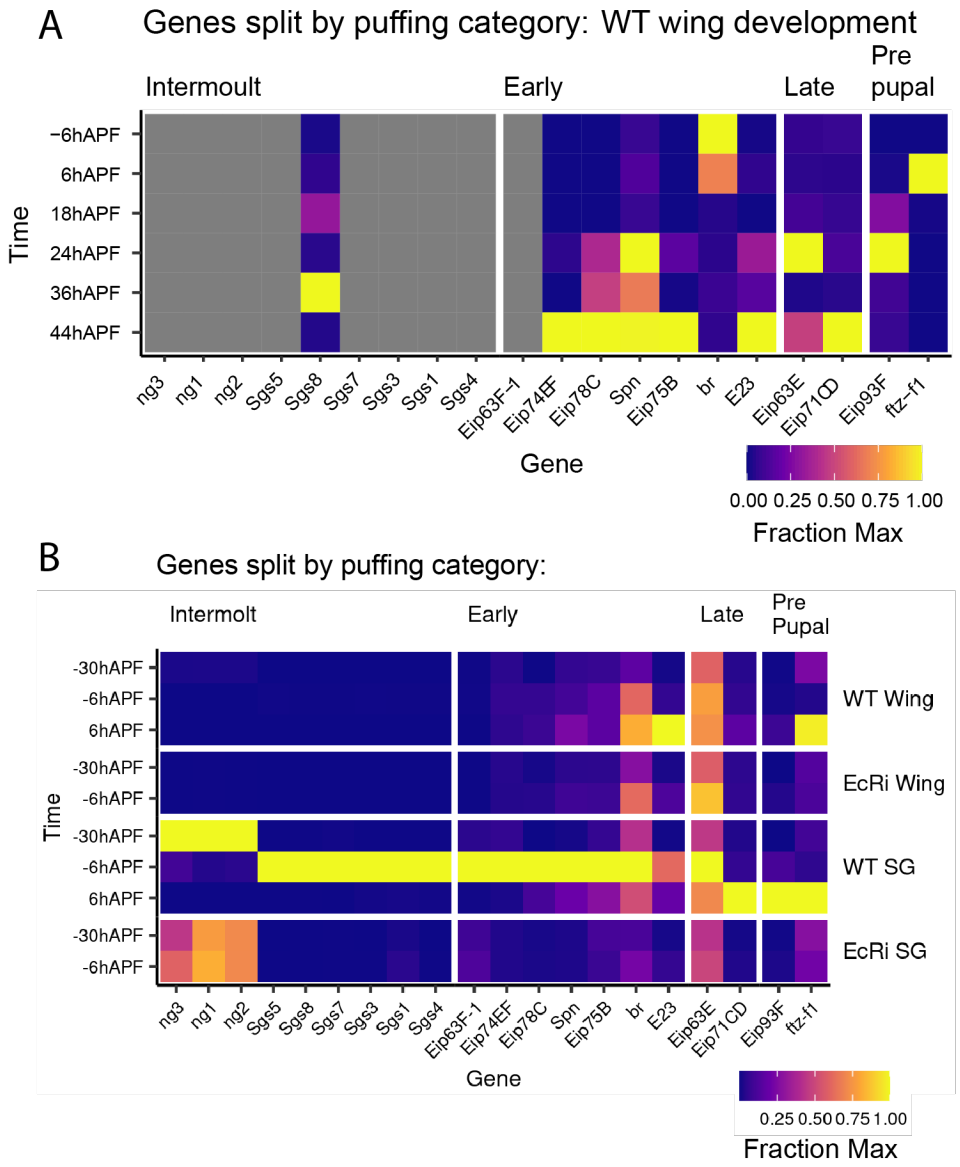


Figure S4. Expression of genes residing in puffing loci from developing wings. (A) Heatmap of RNA-seq values (fraction of max) from a wild-type wing developmental time course for select genes that exhibit ecdysone-dependent puffs in the salivary gland. (B) Heatmap of RNA-seq values (fraction of max) from wild-type and *EcR-RNAi* tissues for select genes that exhibit ecdysone-dependent puffs in salivary glands.

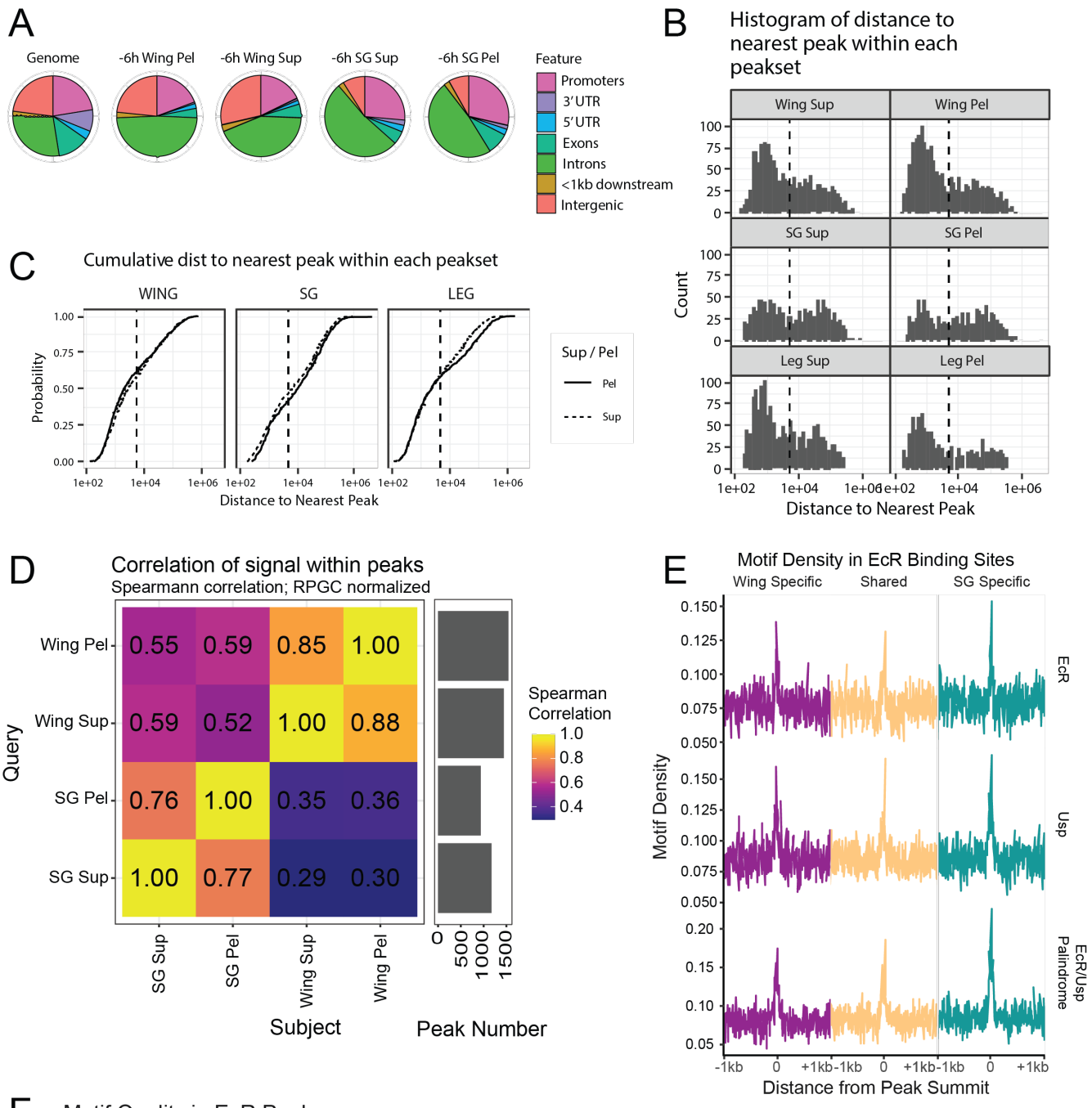
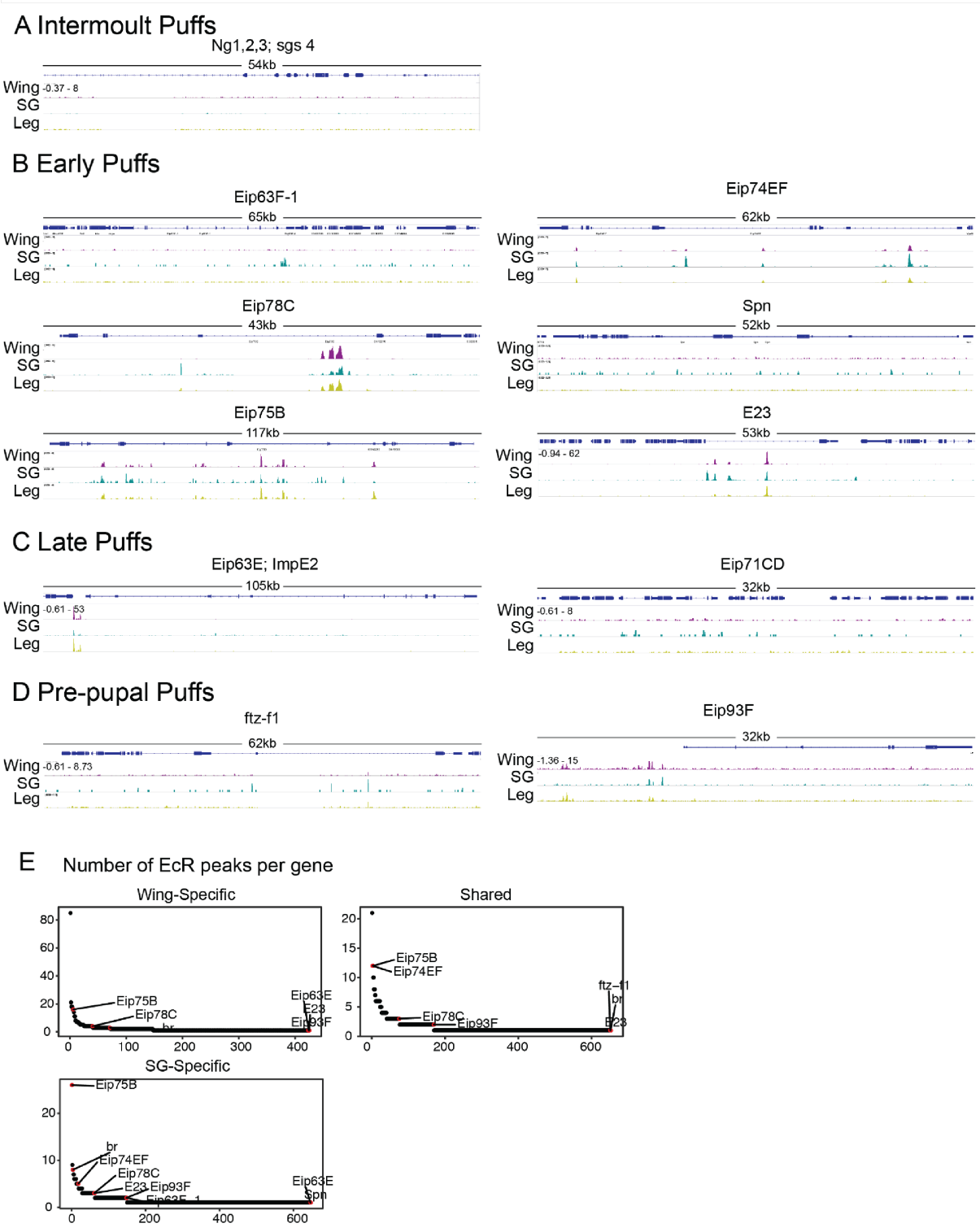

A
Genome
-6h Wing Pel
-6h Wing Sup
-6h SG Sup
-6h SG Pel
Feature
Promoters
3'UTR
5'UTR
Exons
Introns
<1kb downstream
Intergenic
B
Histogram of distance to nearest peak within each peakset
Wing Sup
Wing Pel
SG Sup
SG Pel
Leg Sup
Leg Pel
Count
Distance to Nearest Peak
C
Cumulative dist to nearest peak within each peakset
WING
SG
LEG
Probability
Sup / Pel
Pel
Sup
Distance to Nearest Peak
D
Correlation of signal within peaks
Spearmann correlation; RPGC normalized
Wing Pel
Wing Sup
SG Pel
SG Sup
Query
0.55 0.59 0.85 1.00
0.59 0.52 1.00 0.88
0.76 1.00 0.35 0.36
1.00 0.77 0.29 0.30
Spearman Correlation
Subject
Peak Number
E
Motif Density in EcR Binding Sites
Wing Specific
Shared
SG Specific
EcR
Usp
EcR/Usp Palindrome
Motif Density
Distance from Peak Summit
F
Motif Quality in EcR Peaks
EcR
Usp
EcR/Usp Palindrome
p-value (-log10)
Wing Specific
Shared
SG Specific

Figure S5. Additional properties of EcR binding sites. (A) Pie charts of the genome-wide distribution of EcR binding sites. (B) Histograms and (C) cumulative distribution plots of the distance of each EcR peak to its nearest neighbor. Peaks falling within 5kb (dotted lines) of each other define an EcR cluster. (D) Heatmap of the Spearman correlation coefficient for EcR CUT&RUN signal within EcR peaks between tissues and separated by whether the supernatant (sup) or pellet (pel) DNA was sequenced following the CUT&RUN protocol (see Methods). (E) Line plots of motif density surrounding the summit (+/- 1kb) of EcR CUT&RUN peaks. Individual EcR and Usp motifs from Fly Factor Survey were used, as well as an EcR/Usp palindrome that was generated by combining EcR and Usp motifs. (F) Violin plots of p values for sequences matching EcR, Usp individual motifs, or the EcR/Usp palindrome.



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179 **Figure S6**. EcR binds extensively near ecdysone primary response genes using a mixture of tissue-
180 specific and shared binding sites. (A-D) Browser shots of EcR CUT&RUN signal in wings and salivary
181 glands at puffing loci. (E) Ranked plots of EcR peak number at wing-specific, shared, and salivary gland-
182 specific EcR CUT&RUN peaks. Puffing genes are indicated and highlighted in red.

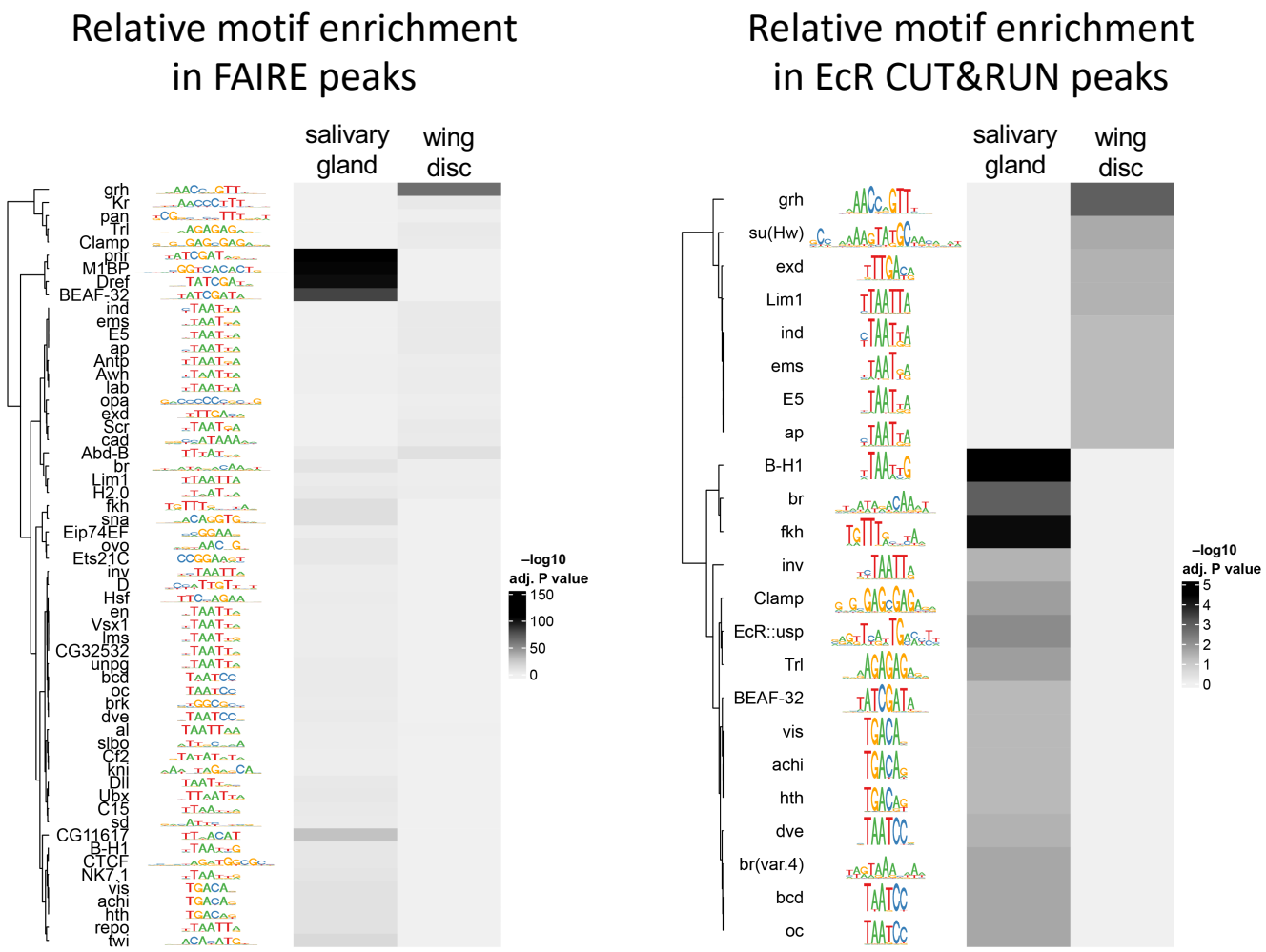

Figure S7. Relative motif enrichment in FAIRE peaks and EcR binding sites. Heatmaps of relative motif enrichment (–log10 adjusted p value) in tissue-specific FAIRE peaks (left) and tissue-specific EcR CUT&RUN peaks (right) from salivary glands and wing imaginal discs. The matching transcription factor and its motif are shown alongside. Similar motifs are hierarchically clustered by Pearson correlation.

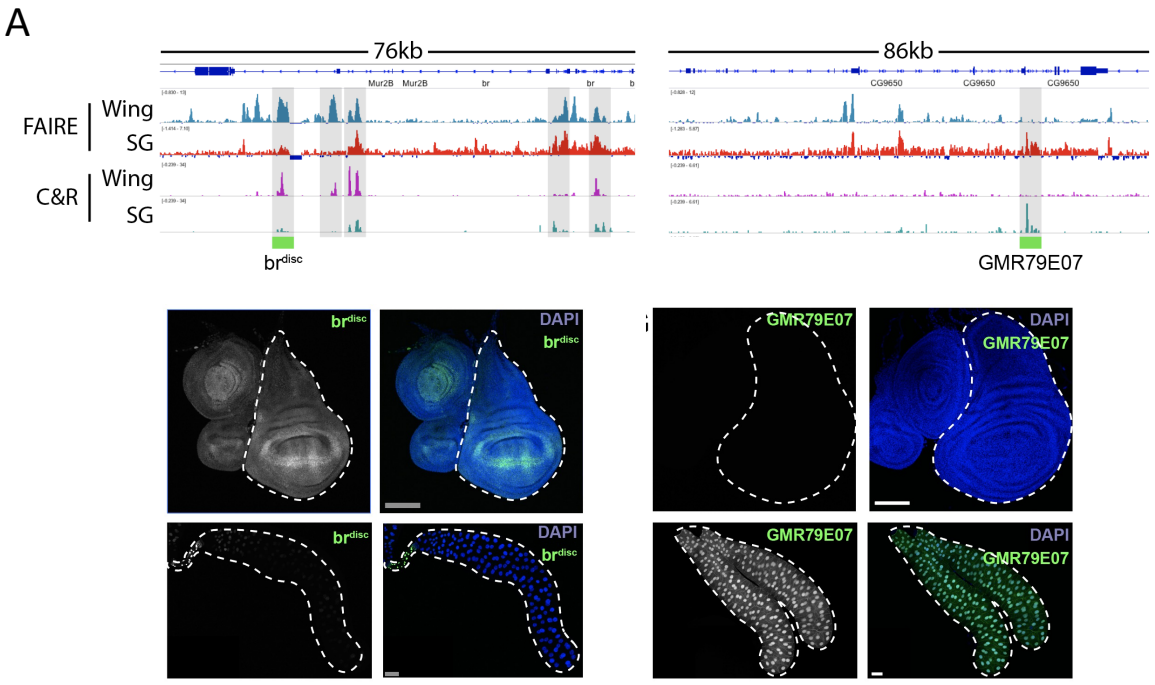
A
76kb
86kb
FAIRE
Wing
SG
C&R
Wing
SG
brdisc
GMR79E07
DAPI
B
E74 Enhancers
E74_A
wild-type Salivary Gland
EcR-RNAi Salivary Gland
wild-type wing disc
EcR-RNAi wing disc
DAPI
GFP
E74_A

Figure S8

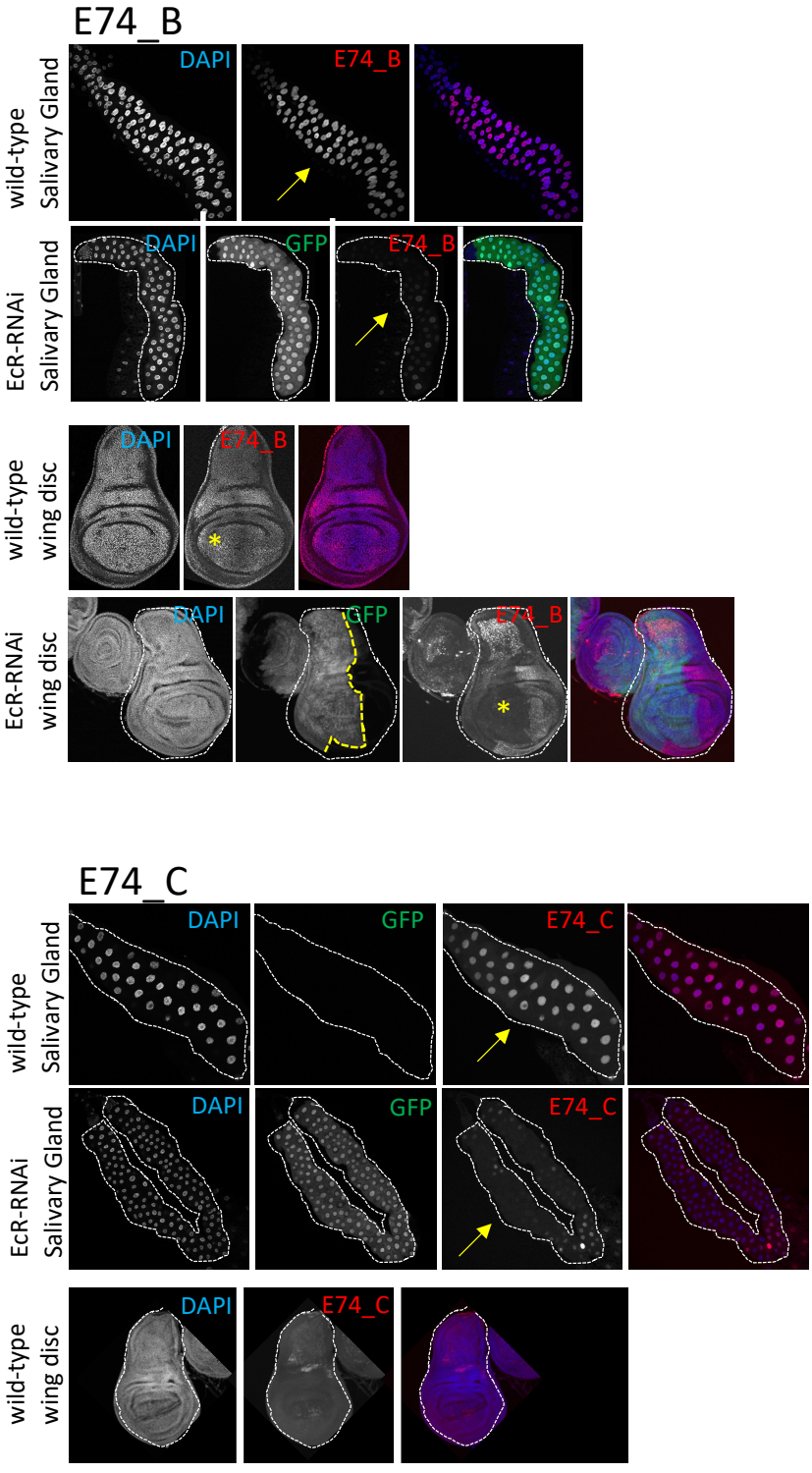

Figure S8

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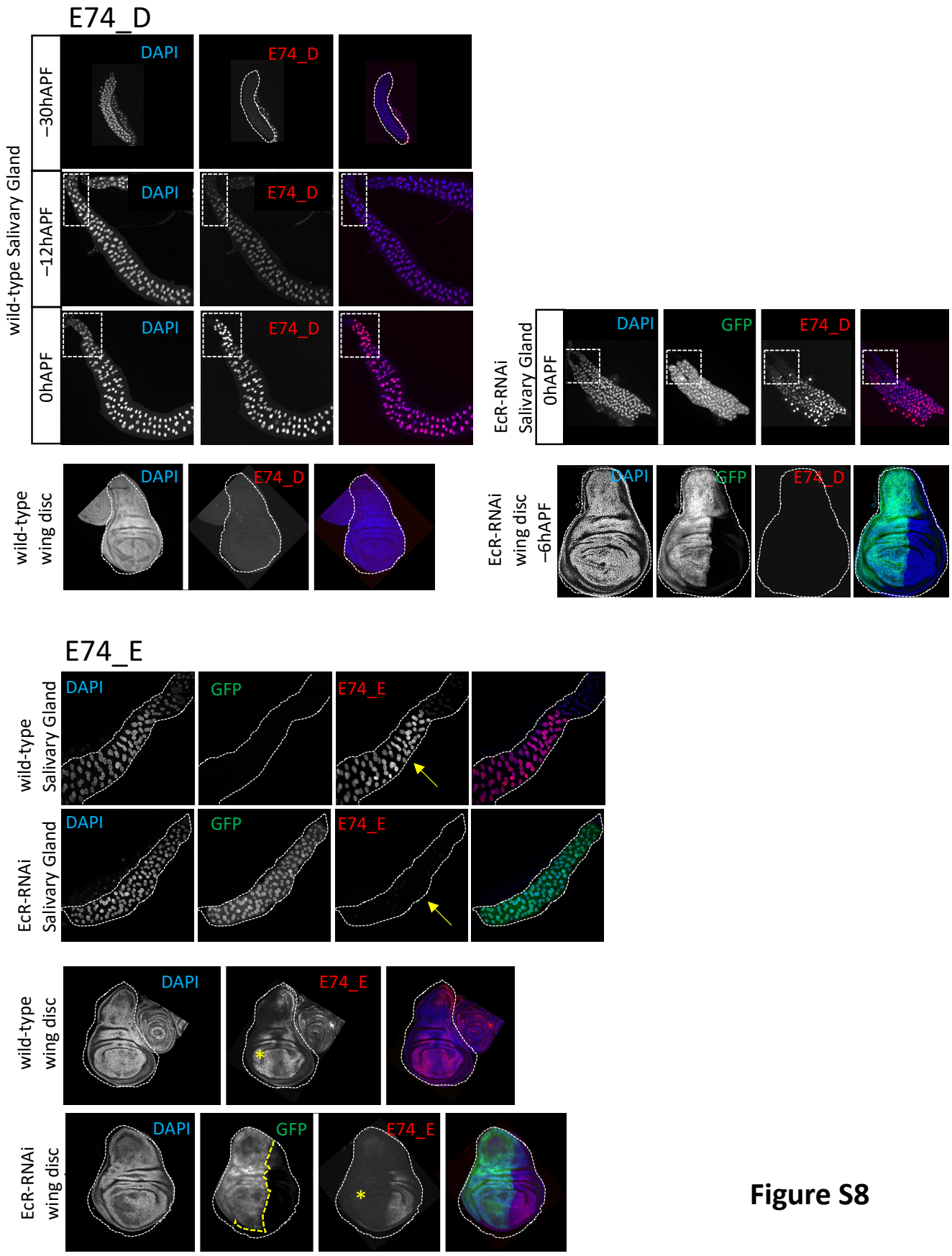

Figure S8

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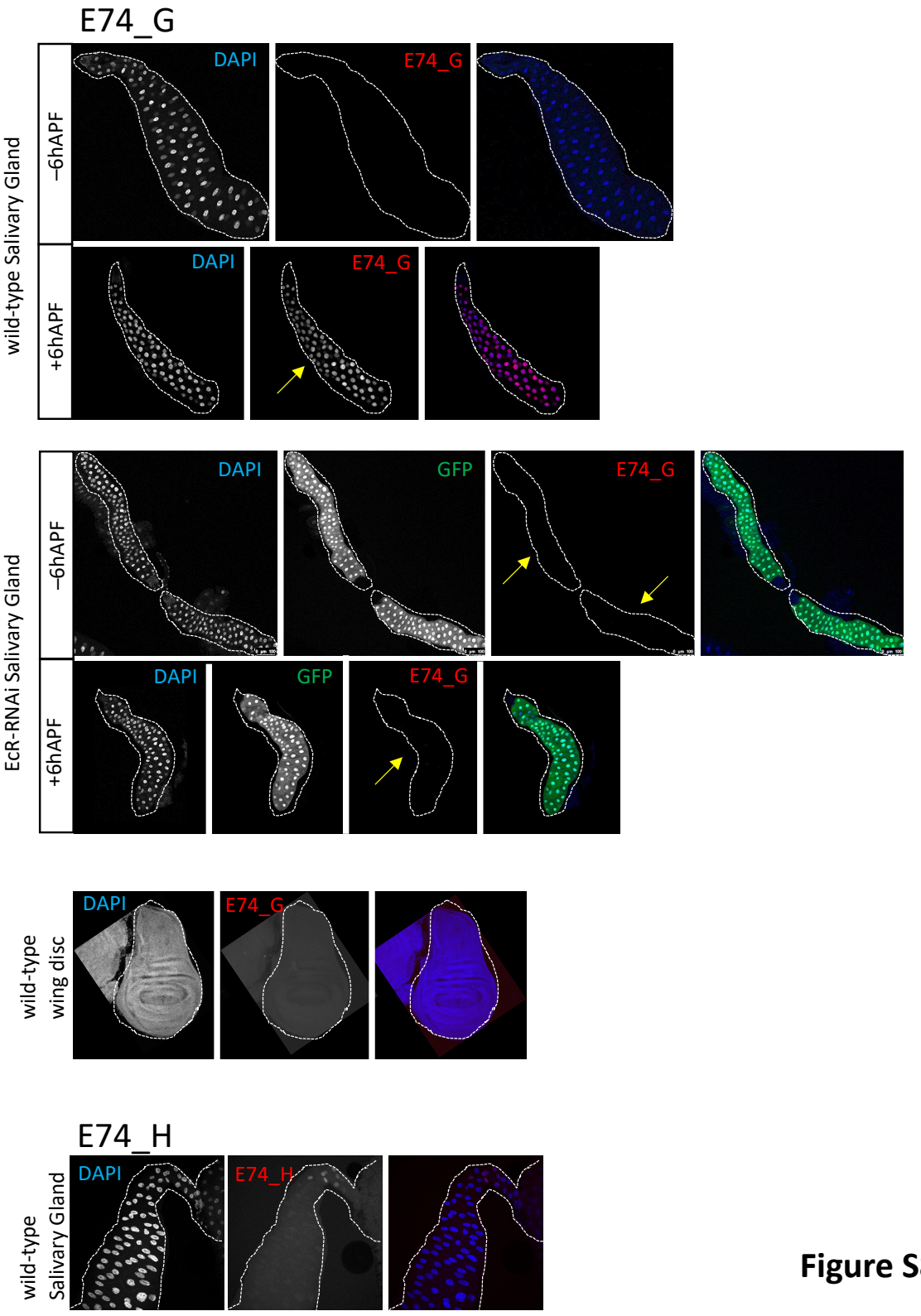

Figure S8

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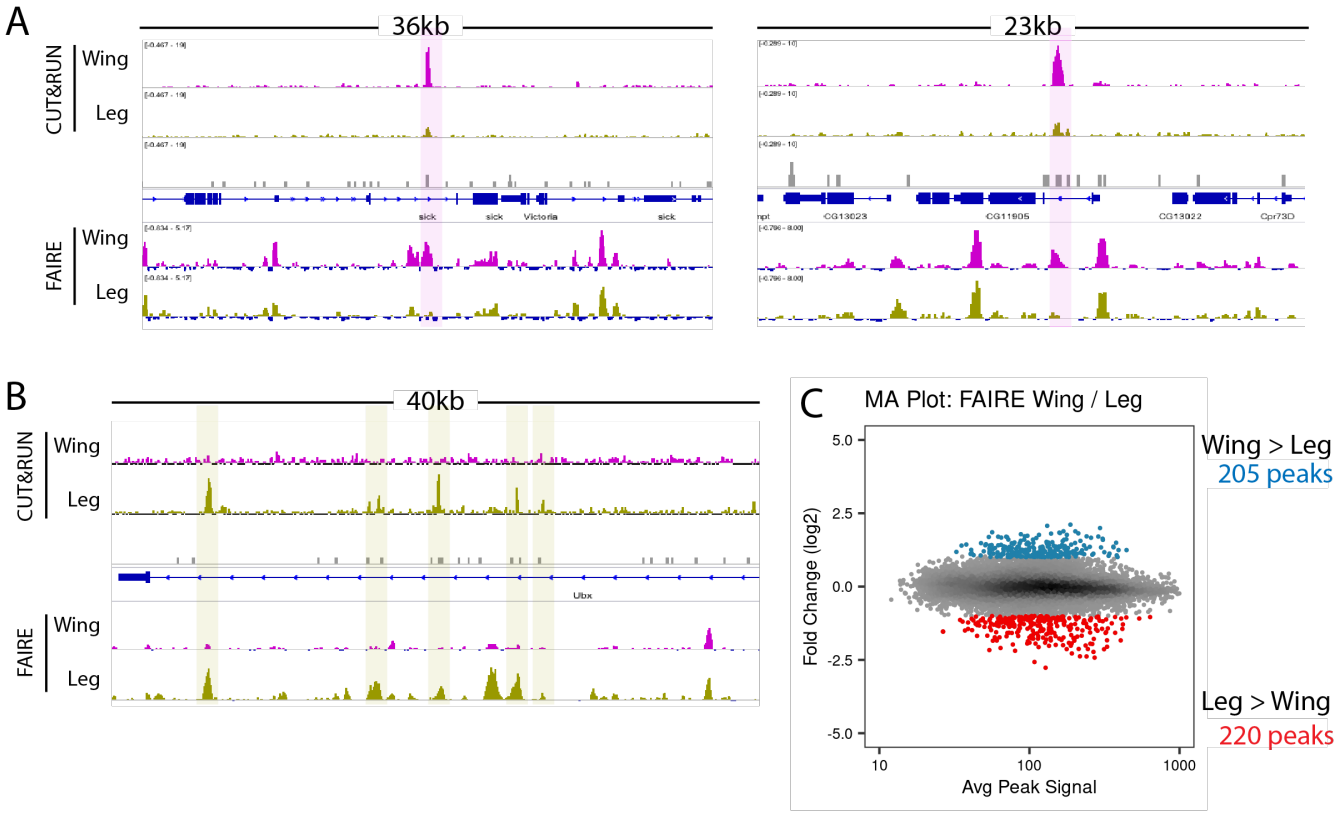
Figure S8. EcR binding sites correspond to active enhancers. (A) Browser shots of FAIRE and EcR CUT&RUN signal from –6hAPF wings and salivary glands for genomic loci surrounding the $br^{\Delta disc}$ and *GMR79E07* enhancers. Confocal images depict enhancer activity (green) and DAPI (blue) in wild-type wings and salivary glands from the same developmental stage. (B) Confocal images of *E74* enhancer activity in wild-type wings and salivary glands. Enhancer activity is visualized by tdTomato expression (red), DNA by DAPI stain (blue). For *EcR-RNAi* tissues, the expression of the GAL4 driving *UAS-EcR-RNAi* is captured by *UAS-GFP* (green). Yellow lines in wing discs indicate the boundary between *Ci-GAL4* expressing and wild-type cells. Developmental stage is –6hAPF for all images, except when indicated on the left. Yellow asterisks indicate loss of enhancer activity relative to wild-type wings. Yellow arrows indicate loss of enhancer activity relative to wild-type salivary glands. For *E74_D*, the white dashed box highlights the transition cells, which exhibit the strongest dependence on EcR for enhancer activity. In wild-type glands, *E74_D* enhancer activity increases in transition cells during the larval-to-prepupal transition; however, in *EcR-RNAi* cells, the enhancer fails to increase in activity in these cells.



208

209 **Figure S9**. Differential EcR binding between wings and legs overlaps sites of differential accessibility.

210 (A) Browser shots of EcR CUT&RUN and FAIRE signal from –6hAPF wings and legs at loci that exhibit

211 wing-specific EcR peaks. (B) Browser shots of EcR CUT&RUN and FAIRE signal from wings and legs

212 at loci that exhibit leg-specific EcR peaks. (C) MA plot of FAIRE signal in –6hAPF wings and legs.

213 Differential peaks (absolute $\log_2$ fold change > 1, adj p value < 0.05) are colored in blue and red.

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References

1. Ahmad, K., *CUT&RUN with Drosophila tissues*, in *https:/doi.org/10.17504/protocols.io.umfeu3n*. 2018.
2. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics (Oxford, England), 2013. **29**(1): p. 15-21.
3. Liao, Y., G.K. Smyth, and W. Shi, *featureCounts: an efficient general purpose program for assigning sequence reads to genomic features.* Bioinformatics (Oxford, England), 2014. **30**(7): p. 923-30.
4. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome biology, 2014. **15**(12): p. 550-550.
5. Cebeci, Z., *Comparison of internal validity indices for fuzzy clustering.* Journal of Agricultural Informatics, 2019. **10**(2): p. 1-14.
6. Venables, W.N. and B.D. Ripley, *Modern Applied Statistics with S*. Fourth ed. 2002: Springer.
7. Pedersen, T.L., *patchwork: The Composer of Plots*, in *cran.r-project*. 2020.
8. Wickham, H., *ggplot2*. 2009, New York, NY: Springer New York.
9. Wickham, H., *A Layered Grammar of Graphics.* Journal of Computational and Graphical Statistics, 2010. **19**(1): p. 3-28.
10. Alexa, A. and J. Rahnenfuhrer, *Enrichment Analysis for Gene Ontology*, in *10.18129/B9.bioc.topGO*. 2018.
11. Sayols, S., *Reduce + Visualize GO*, in *10.18129/B9.bioc.rrvgo*. 2020.
12. Langmead, B. and S.L. Salzberg, *Fast gapped-read alignment with Bowtie 2.* Nature methods, 2012. **9**(4): p. 357-9.
13. Li, H., et al., *The Sequence Alignment/Map format and SAMtools.* Bioinformatics (Oxford, England), 2009. **25**(16): p. 2078-9.
14. Skene, P.J. and S. Henikoff, *An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites.* eLife, 2017. **6**.
15. Quinlan, A.R. and I.M. Hall, *BEDTools: a flexible suite of utilities for comparing genomic features.* Bioinformatics (Oxford, England), 2010. **26**(6): p. 841-2.
16. Kent, W.J., et al., *BigWig and BigBed: enabling browsing of large distributed datasets.* Bioinformatics (Oxford, England), 2010. **26**(17): p. 2204-7.
17. Zhang, Y., et al., *Model-based analysis of ChIP-Seq (MACS).* Genome biology, 2008. **9**(9): p. R137-R137.
18. Stempor, P. and J. Ahringer, *SeqPlots - Interactive software for exploratory data analyses, pattern discovery and visualization in genomics.* Wellcome open research, 2016. **1**: p. 14-14.
19. Zhu, L.J., et al., *ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq and ChIP-chip data.* BMC bioinformatics, 2010. **11**: p. 237-237.
20. Zhu, L.J., et al., *FlyFactorSurvey: a database of Drosophila transcription factor binding specificities determined using the bacterial one-hybrid system.* Nucleic acids research, 2011. **39**(Database issue): p. D111-7.
21. Grant, C.E., T.L. Bailey, and W.S. Noble, *FIMO: scanning for occurrences of a given motif.* Bioinformatics (Oxford, England), 2011. **27**(7): p. 1017-8.
22. Machlab, D., et al., *monaLisa: an R/Bioconductor package for identifying regulatory motifs.* Bioinformatics, 2022.
23. Fornes, O., et al., *JASPAR 2020: update of the open-access database of transcription factor binding profiles.* Nucleic Acids Res, 2020. **48**(D1): p. D87-D92.