Supplemental Materials and Methods

Drosophila lines

All *Set8, H4^{K20},* and *His4r* mutant genotypes were described in Crain et al. 2022 (Crain et al. 2022). *l(3)mbt^{GM76}* and *Df^{ED10966}* were generous gifts from Ruth Lehmann. *ΔHisC^{cadillac}* is described in (Crain et al. 2024).

l(3)mbt CRISPR-Cas9 genome editing

The CRISPR-based Scarless Gene Editing System (Gratz et al. 2015) was used to generate the N-terminal epitope-tagged alleles of *l(3)mbt*. Homology arms consisted of 1 kb upstream and downstream of the *l(3)mbt* start codon were cloned into either pScarlessHD-sfGFP-DsRed (Addgene plasmid #80811) or pScarlessHD-3xFLAG-DsRed (Addgene plasmid # 80820), which were gifts from Kate O'Connor-Giles. *l(3)mbt* gRNA (GCCGTTTATGCTTAGAGCTATGG; selected using the CRISPR Fly Design protocol (Gratz et al. 2015; Bier et al. 2018) was cloned into pCFD2-dU6:2gRNA (Addgene plasmid # 49409; a gift from Simon Bullock). Co-injection of repair template and gRNA plasmids into *yw; nanos-Cas9/CyO* and screening for dsRed+ transformants was performed by BestGene (Chino Hills, CA). Excision was performed by crossing to *w*; PBac{GFP[ECFP.3xP3]=5pBlueEye.hsp70-PBac\T}* (BDSC #32175) to generate *l(3)mbt^{GFP}* and *l(3)mbt^{FLAG}* alleles and confirmed by PCR and Sanger sequencing. *l(3)mbt$^{PBac\{Scarless\text{-}ds\text{-}Red\}}$* described here is *l(3)mbt^{GFP}* prior to excision by PBac transposase, which results in a 1810kb insertion between at the l(3)mbt TSS (chr3R:27,271,759).

K-means clustering

K-means clustering was performed with a value of 6 as determined by identifying the elbow region using within-cluster sum of squares (WCSS) method.

Gene ontology (GO) analysis

GO term analysis was performed using gost function in gprofiler2 (Raudvere et al. 2019; Kolberg et al. 2020). Semantic similarity graphs were generated using rrvgo (Sayols 2023).

Transposon and piRNA analysis

Transposon and piRNA cluster expression was quantified with Salmon (Patro et al. 2017) as in the protein-coding gene analysis except transcript indexes were built using *Drosophila melanogaster* transposon (FlyBase), and piRNA cluster database (Rosenkranz 2016; Rosenkranz et al. 2022) sequences in addition to protein-coding transcripts to ensure changes in transposon or piRNA clusters were not due differences in sequences depth. Differential expression analysis was performed the same as with protein-coding genes. Results tables were filtered for transposons and piRNAs and volcano plots were generated using ggplot (Wickham 2016).

***Set8^{null}* RNA-seq comparison**

Paired-end FASTQ files from 3 replicates of *Set8^{null}* (GSE217728) were passed to the quant function within Salmon (Patro et al. 2017) for protein-coding genes, as in main text. Imported counts were normalized along with counts from *Set8^{null}* (this study) using DESeq2 with a variance stabilizing transformation. Normalized counts of *Set8^{null}* (GSE217728) and *Set8^{null}* this study were plotted using ggscatter with parameters *add = "reg.line"* and *cor.coef = TRUE* using ggpubr R package. Plotted R-squared value and p-statistic calculated via a Pearson correlation.

Supplemental Tables

Supplemental Table S1. DEGs Figure 3

Genotype	DEGs abs(log2FoldChange) > 1 & p adj < 0.01		
	Up	**Down**	**Total**
Set8^{null}	962 (64.5%)	529 (35.5%)	1491
Set8^{RG}	88 (68.2%)	41 (31.8%)	129
H4^{K20A}	43 (47.3%)	48 (52.7%)	91
H4^{K20R}	941 (77.9%)	267 (22.1%)	1208

Supplmental Table S2. DEGs Figure 3 overlap with H4K20me1

Genotype	K20me1 status	DEGs				
		Up	**Down**	**Total**	**Percent of total DEGs**	**% of H4K20me1 genes**
Set8^{null} vs Oregon-R	K20me1	18 (38.8%)	68 (61.2%)	86	5.8%	3.7%
	No K20me1	944 (71.3%)	461 (28.7%)	1405	94.2%	
Set8^{RG} vs *Set8^{WT}*	K20me1	2 (46.7%)	2 (53.3%)	4	3.1%	0.2%
	No K20me1	212 (62.2%)	129 (47.8%)	125	96.9%	
H4^{K20A} *vs* *H4^{WT}*	K20me1	NA	NA	0	0%	0%
	No K20me1	43 (42.5%)	48 (57.5%)	91	100%	
H4^{K20R} *vs* *H4^{WT}*	K20me1	20 (55.9%)	27 (44.1%)	47	3.9%	2.0%

	No K20me1	921 (82.4%)	240 (17.6%)	1161	96.1%	

Supplemental Table S6. DEGs Figure 5

Genotype	DEGs abs(log2FoldChange) > 1 & padj < 0.01		
	Up	**Down**	**Total**
l(3)mbt^{GM76}/*Df* vs Oregon-R	1519 (80.9%)	358 (18.1%)	1877
l(3)mbt$^{PBac\{Scarless\text{-}dsRed\}}$/*Df* *vs* Oregon-R	2938 (83.9%)	564 (16.1%)	3502
l(3)mbt^{WT} vs Oregon-R	1716 (92.2%)	146 (7.8%)	1862

Supplemental Table S7. DEGs Figure 5 overlap with H4K20me1

Genotype	K20me1 status	DEGs				
		Up	**Down**	**Total**	**Percent of total DEGs**	**% of H4K20me1 genes**
l(3)mbt^{GM76} vs Oregon-R	K20me1	50 (67.6%)	24 (32.4%)	74	3.9%	3.2%
	No K20me1	1469 (81.5%)	334 (18.5%)	1803	96.1%	
l(3)mbt$^{Pbac\{Scarless\text{-}dsRed\}}$ vs Oregon-R	K20me1	189 (76.2%)	59 (23.8%)	248	7.1%	10.7%
	No K20me1	2749 (84.5%)	505 (15.5%)	3254	92.9%	

l(3)mbt^{WT} vs Oregon-R	K20me1	104 (93.7%)	7 (6.3%)	111	6.0%	4.8%
	No K20me1	1612 (92.1%)	139 (7.9%)	1751	94.0%	

Supplemental References

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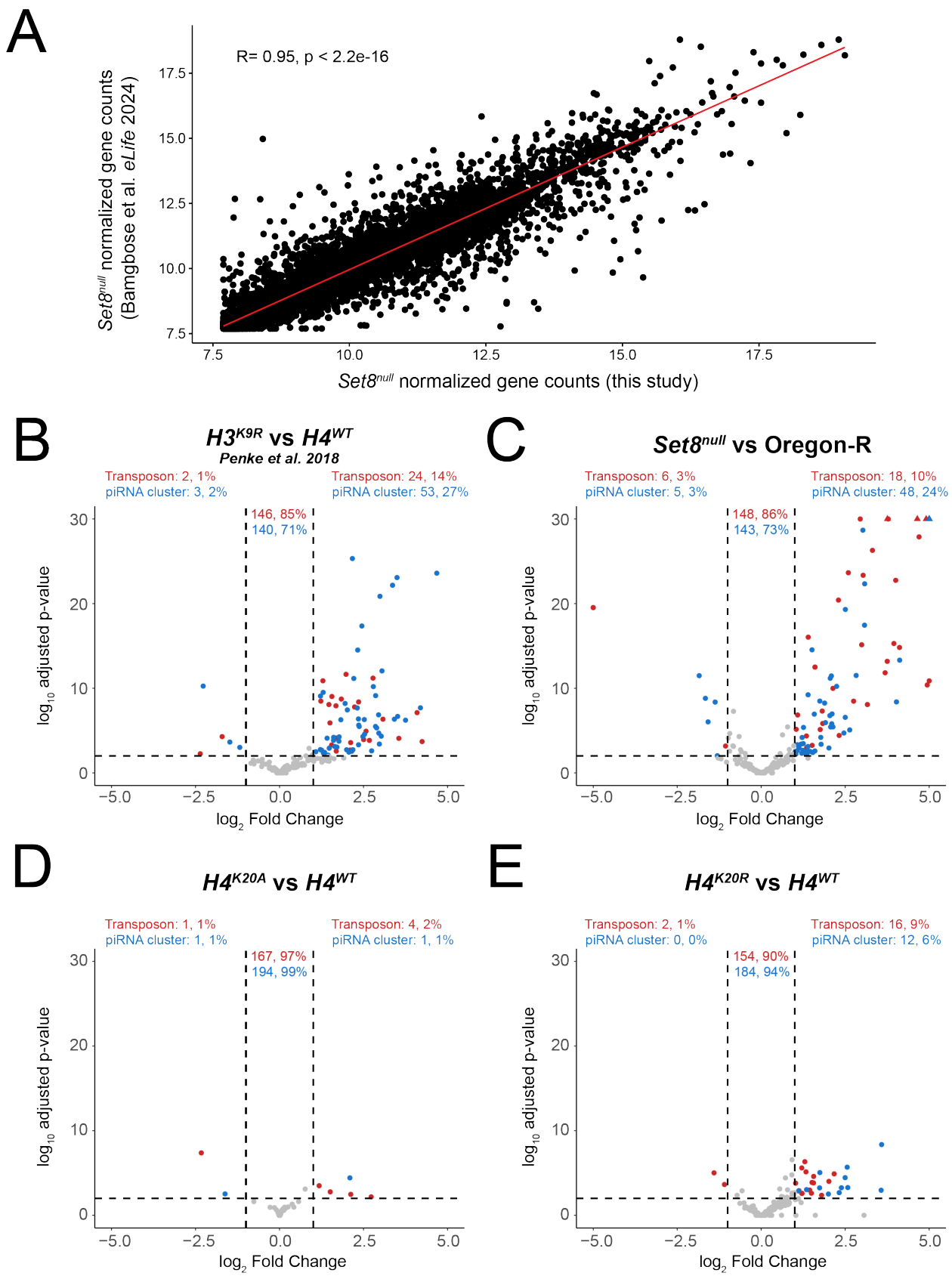
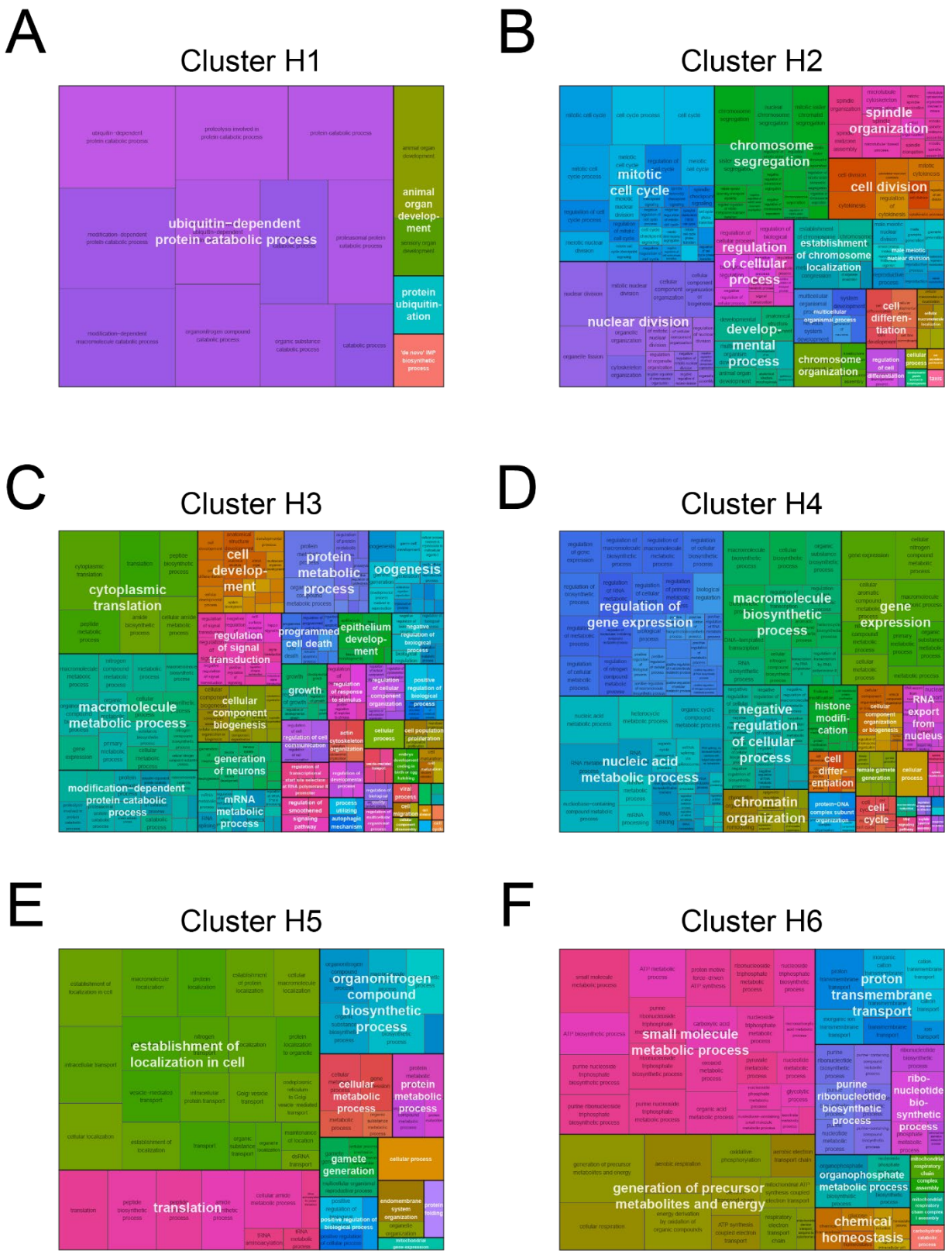
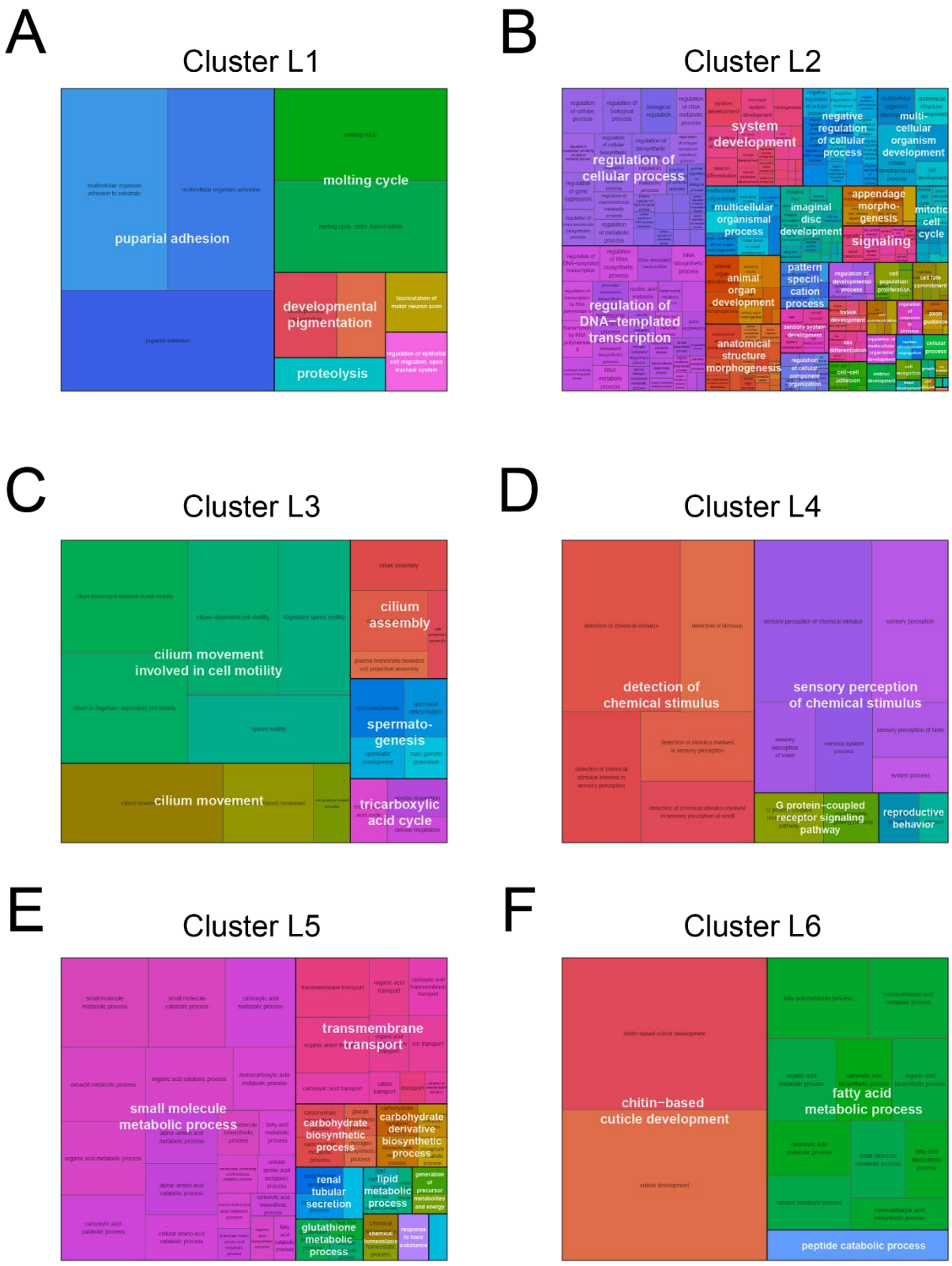

A
R= 0.95, p < 2.2e-16
Set8null normalized gene counts (Bamgbose et al. eLife 2024)
Set8null normalized gene counts (this study)
B
H3K9R vs H4WT
Penke et al. 2018
Transposon: 2, 1%
piRNA cluster: 3, 2%
Transposon: 24, 14%
piRNA cluster: 53, 27%
146, 85%
140, 71%
log10 adjusted p-value
log2 Fold Change
C
Set8null vs Oregon-R
Transposon: 6, 3%
piRNA cluster: 5, 3%
Transposon: 18, 10%
piRNA cluster: 48, 24%
148, 86%
143, 73%
log10 adjusted p-value
log2 Fold Change
D
H4K20A vs H4WT
Transposon: 1, 1%
piRNA cluster: 1, 1%
Transposon: 4, 2%
piRNA cluster: 1, 1%
167, 97%
194, 99%
log10 adjusted p-value
log2 Fold Change
E
H4K20R vs H4WT
Transposon: 2, 1%
piRNA cluster: 0, 0%
Transposon: 16, 9%
piRNA cluster: 12, 6%
154, 90%
184, 94%
log10 adjusted p-value
log2 Fold Change

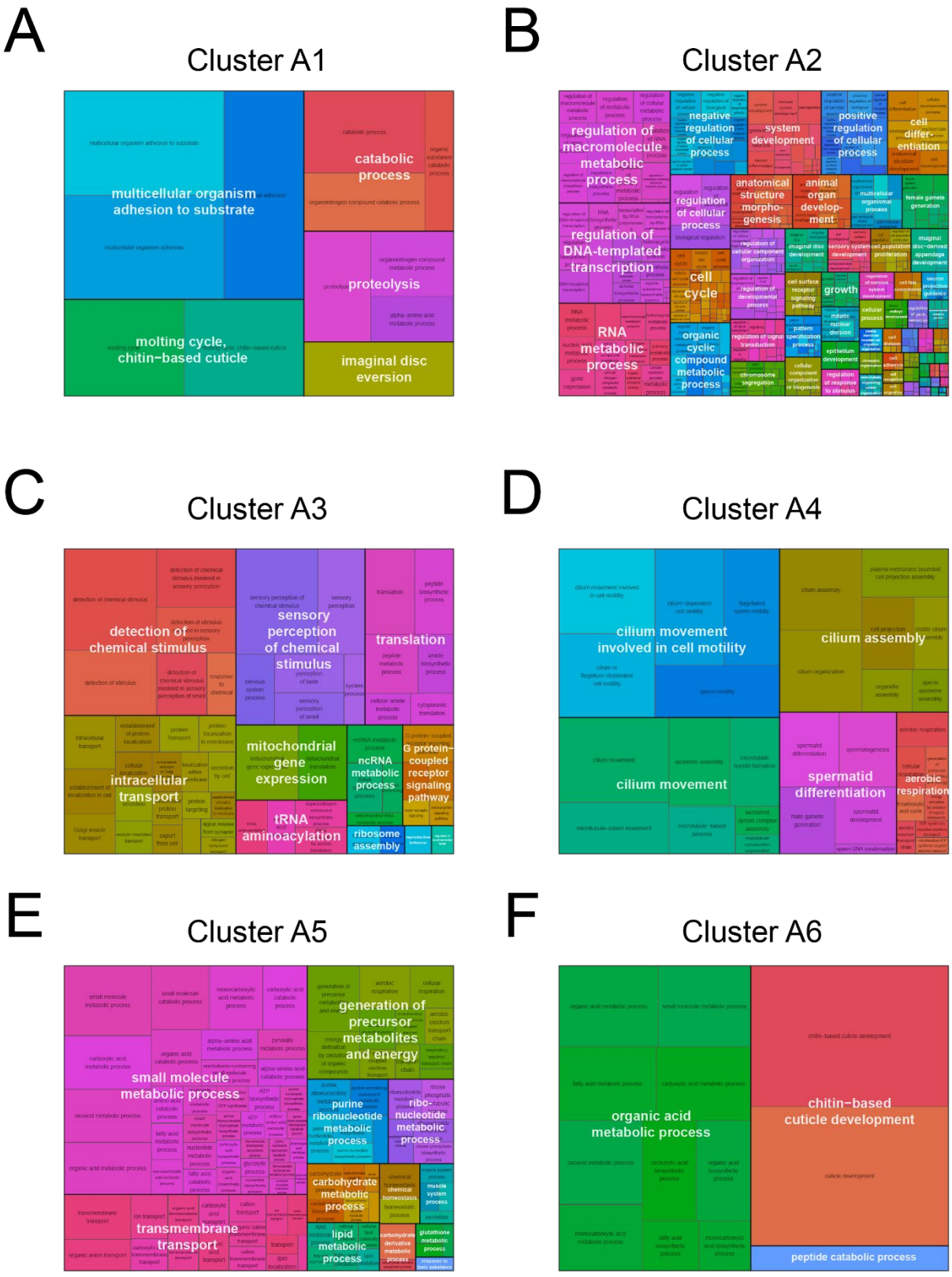
Supplemental Fig. S1. A) Comparision of protein-coding gene counts from whole larval RNA-seq in *Set8*^{null} (this study) and *Set8*^{null} (GSE217728). Each black dot indicates a single gene. Red line indicates the regression line. R-square value and p-statistic calculated using a Pearson correlation. **B-E)** Volcano plots depicting the relationship between $\log_2$FoldChange (x-axis) and $\log_{10}$ adjusted p-value (y-axis) of transposon (red) and piRNA cluster (blue) expression in indicated comparisons. Colored dots indicate significantly increased or decreased transposon or piRNA cluster expression ($\log_2$FC < -1, FDR < 0.01) relative to control and grey dots are unchanged relative to control. Text on the right and left of the plots show number and percentage of transposons or piRNA clusters that are significantly increased or decreased relative to control, respectively. Text in the middle of the plots shows number and percentage of transposons and piRNA clusters that are unchanged relative to control.



Supplemental Fig. S2. Semantic similarity tree map plot of terms from gene ontology analysis of HIGH H4K20me1 gene clusters in Figure 3.



Supplemental Fig. S3. Semantic similarity tree map plot of terms from gene ontology analysis of LOW H4K20me1 gene clusters in Figure 3.



Supplemental Fig. S4. Semantic similarity tree map plot of terms from gene ontology analysis of ALL GENES gene clusters in Figure 3.