Supplementary Experimental Methods

Stocks (with origin)

yw (WT) (Bloomington Stock Center #6598)

Δ*HisC*^{ED1429}*, UAS-2xEYFP / CyO* (gift from Alf Herzig)

Δ*HisC* ^{ED1429}*, twi-GAL4 / CyO* (gift from Alf Herzig)

Δ*HisC* ^{ED1429} was generated by (Günesdogan et al. 2010) using the DrosDel system (Ryder et al. 2004)

H3.3B^{K9R} (1) ; *CyO,twiGFP / If*

H3.3B^{K9R} (2) ; *CyO,twiGFP / If*

w^{1118} *; Df(2L)BSC110 / CyO* (Bloomington Stock Center #8835)

H3.3A^{2x1} */ CyO* (gift from Kami Ahmad)

Antibodies (species, source) (concentration IF) [concentration western]

α-H3K9me2 (mouse, Abcam 1220) (1:500) [1:2500]

α-H3K9me3 (rabbit, Active Motif 39161) (1:1000)

α-H3K9ac (rabbit, Millipore 07-352) (1:2000) [1:5000]

α-HP1-immunofluorescence (mouse, DSHB C1A9) (1:1500)

α-H3 (rabbit, Abcam 1791) [1:30,000]

CRISPR-Cas9 Mutagenesis

Forward (5'-CTTCGCGTAAGTCGACCGGAGGAA-3') and reverse (5'-AAACTTCCTCCGGTCGACTTACGC-3') oligos were annealed and inserted into pCFD3 using cloning with pCFD3 protocol from http://www.crisprflydesign.org/grna-expression-vectors/ . Homologous repair template was generated by PCR amplification of 2kb region of H3.3B using the following primers: Forward (5' - TAAGCATCTAGAATTTTCCTCTTGCCTGCACA- 3') Reverse (5'-TAAGCACCGCGGTGGTATTCCCTCCTTCCAAA-3'). *Xba*I and *Sac*II restriction enzyme digestion was used to insert amplicon into pBlueSurf (gift from Jeff Sekelsky). gRNA and repair template were coinjected into yw; nos:Cas9/CyO stock at BestGene®.

Numbers (1) and (2) in Figure S1A refer to independently derived CRISPR-Cas9 events.

Culture conditions

All stocks were maintained on standard corn media. For cross schemes see Figure S1. For cross scheme in Figure S1B 50 females and 20 males were placed in a cage at 25C and allowed to lay eggs on a grape juice agar plate. To measure the completion of embryogenesis, GFP positive eggs from a 4-hour collection were moved to a separate plate and aged 24 hours prior to counting hatching. The number of hatched eggs (observed) and the total number of eggs scored are indicated in Table 2. For all other developmental assays overnight collections were used. To measure the completion of development from egg hatching to adult eclosion, ~50 GFP positive larvae were moved to a corn media vial 48 hours after egg laying to separate Δ*HisC, UAS-2xEYFP /* Δ*HisC, twi-GAL4* mutants from their siblings. For each group of ~50 larvae the number that pupated and the number that eclosed as adults was determined and summed, as was the total number of larvae scored. Expected values for Chi-squared tests are based on observed value of *HWT* animals. For genomic or molecular analyses wing discs or salivary glands culture vials were cleared of wandering third instar larvae, and after 4-6 hours newly wandering larvae were selected.

Immunofluorescence

Salivary gland polytene chromosome spreads were performed as previously described (Cai et al. 2010). WT (yw) and $H3.3^{K9R}$ salivary glands from 3rd instar wandering larvae were prepared in matched samples. Images shown are single confocal images taken at a constant gain on a Leica TCS SP5 AOBS UV/spectral confocal laser-scanning system mounted on an inverted DM IRE2 microscope. Chromocenter immunofluorescent signal was quantified using ImageJ and the Corrected Total Cell Fluorescence (CTCF) formula (Integrated Density of the chromocenter – (Area of chromocenter X mean of fluorescence background)). Area of chromocenters varied within genotypes but the average area of WT and $H3.3^{K9R}$ chromocenters was not significantly different (data not shown). CTCF values were therefore normalized to the area of the chromocenter. To

control for variable antibody staining across days we compared each matched sample pair. For each pair, all WT values were averaged and set to 1. Each WT and H3.3^{K9R} chromocenter value was then set relative to 1 and plotted.

Sample Preparation for Sequencing

FAIRE-seq and RNA-seq samples from 3rd instar imaginal discs were prepared as previously described (McKay and Lieb 2013). Libraries were prepared with the Rubicon ThruPLEX DNA-seq kit (FAIRE) and the Nugen Ovation RNA-Seq System for Model Organisms (RNA). Sequencing was performed on an Illumina HiSeq2500.

Next-Generation Sequencing Analysis

FAIRE reads were aligned to the reference genome (dm6 Release 6.04) using Bowtie2 default parameters (Langmead and Salzberg 2012). FAIRE was performed in biological triplicate for all four genotypes (WT, H3.3A, H3.3B^{K9R}, H3.3^{K9R}). Since all replicates were highly correlated to each other (R>0.96), reads from each replicate of a particular genotype were pooled for subsequent analyses. Peaks were called on individual replicates using MACS2 with a shift size of 110bp and a cutoff of 0.01 (Zhang et al. 2008). A union peak set was generated by concatenating individual sample peaks from all replicates and merging peaks with BEDTools (Quinlan and Hall 2010). Peak overlaps between replicates were calculated with BEDTools intersect. MA plots and statistical analysis were generate using DESeq2 and raw counts at each peak (Love et al. 2014). Statistical cutoff was adjusted p value of 0.05.

RNA reads were aligned to the reference genome (dm6 Release 6.04) using Tophat default parameters (Trapnell et al. 2014). Transcripts were assembled for each genotype and merged into one assembly file. Read counts at each transcript from the merged assembly file were generated using BEDTools (Quinlan and Hall 2010) and raw counts were used for statistical analysis using DESeq2 (Love et al. 2014).

Supplementary References

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