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Eric Albanese December 8, 2024

An Ao-kay thesis:

# A cytological approach to the molecular role of *Abnormal Oocyte* (*ao*)

by

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2024

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

An Ao-kay thesis:

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*Abnormal oocyte* (*ao,* formerly: *abo*) is a *Drosophila* maternal effect gene first characterized in 1970. Loss-of-function mutations cause lethality during early embryogenesis but can be rescued with excess heterochromatin. Later characterization suggested that Ao negatively regulates histone gene expression, providing a satisfying mechanistic explanation for earlier observations. After this discovery, *ao* received little attention. We recently developed new *ao* genetic tools and discovered Ao is likely not a histone regulator in ovaries, despite localizing to the histone gene cluster. In order to determine if Ao influences histones in other tissues, we leveraged our endogenously tagged *ao* transgenes. Here, we show Ao does not localize to the histone gene cluster in larval polytene chromosomes, as previously shown, even in an overexpression environment. However, we observe Ao localization to the histone genes in blastula-stage and later embryos, indicating tissue specificity. Yet, even in embryos, Ao does not affect histone transcript levels. Further entangling our understanding of Ao, is that our recent work illustrated Ao levels were sensitive to histone gene stoichiometry. Thus, we also assess Ao localization in a reduced and histone gene environment. Our work indicates Ao localization without regulation and reopens the fifty-year-old mystery into the role of Ao with potential insights into its molecular mechanism.

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# An Ao-kay thesis:

## A cytological approach to the molecular role of *Abnormal Oocyte* (*ao*)

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## Introduction:

Histone proteins are critical to the structure and organization of the genome and are cell-cycle regulated (Duronio and Marzluff 2017). Likewise, histone mRNAs require special processing, as they are not poly-adenylated, instead terminating in a conserved stem-loop (Shopland *et al.* 2001; Marzluff *et al.* 2008). The cell's unique need for synthesis of histones during S-phase is regulated by the histone locus body (HLB): a phase-separated nuclear body that influences the transcription and processing of histone mRNAs (Liu *et al.* 2006; White *et al.* 2007; Salzler *et al.* 2013). The HLB forms at the histone gene locus in a variety of metazoans, with many conserved components. However, the constituents and mechanisms of its assembly are not well understood (McKay *et al.* 2015; Duronio and Marzluff 2017).

The histone genes of the fruit fly, *Drosophila melanogaster,* are well studied and strikingly organized. The genes are clustered at a single locus on chromosome 2L, and consist of ~100 copies of a tandemly repeating array of the five canonical histone genes (H1, H2A, H2B, H3, and H4) (Marzluff *et al.* 2008; Salzler *et al.* 2013; McKay *et al.* 2015). Some select factors, such as Muscle wasted (Mute), Multi sex combs (Mxc), and FLICE-associated huge protein (FLASH), localize exclusively to the *Drosophila melanogaster* histone locus, forming the HLB (Yang *et al.* 2009; White *et al.* 2011; Tatomer *et al.* 2016). However, other HLB factors do not exclusively localize to the histone genes (Rieder *et al.* 2017; Hodkinson *et al.* 2023). The HLB is sensitive to histone gene stoichiometry (Jimeno-González *et al.* 2015; Maya Miles *et al.* 2018; Chari *et al.* 2019), as a reduction in histone gene copy number leads to a decrease in the ability of some factors to localize, such as the negative histone gene regulator, Mute (Bulchand *et al.* 2010; McKay *et al.* 2015).

The regulation of the eponymous replication-dependent histone genes is closely tied to the cell-cycle and is highly conserved (Duronio and Marzluff 2017; Armstrong and Spencer 2021). During the cell's transition from G1 to S phase, Cyclin E dimerizes with Cyclin-dependent kinase 2 (Cdk2), activating the latter's phosphorylating catalytic site, progressing the cell cycle (Knoblich *et al.* 1994; Ohtsubo *et al.* 1995). One such target of the Cyclin E/Cdk2 complex is the HLB factor, Mxc (White *et al.* 2011). Moreover, phosphorylated Mxc is a marker of active histone expression, similar to its human homologue, NPAT (Zhao *et al.* 1998; White *et al.* 2011). Histone concentrations are themselves implicated in cell-cycle regulation, as Histone H3 has been shown to inhibit Checkpoint kinase 1 (Chk1), affecting cycle progression in the early *D. melanogaster* embryo—independent of its role in chromatin (Günesdogan *et al.* 2014; Shindo and Amodeo 2021). Thus, there is room to look for potential negative feedback systems which may ensure proper cell cycle progression and viability.

Another HLB factor candidate is the maternal effect gene *abnormal oocyte* (*ao*) (Sandler 1970; Takenaka *et al.* 2024). Embryos from *ao* mutant mothers display reduced viability, which is rescued by the subsequent addition of specific regions of heterochromatin from the 2$^{nd}$, X, and Y chromosomes (Parry and Sandler 1974; Sandler 1977; Yedvobnick *et al.* 1980; Pimpinelli *et al.* 1985; Tomkiel *et al.* 1991). Historically, homozygous *ao* mutant lines tended to develop suppressor mutations, originally believed to be duplications of rDNA as an apparent compensation mechanism (Krider and Levine 1975). However, later research was not consistent with this finding (Yedvobnick *et al.* 1980; Sullivan and Pimpinelli 1986). Ao, therefore, had a clear relationship with heterochromatin, although the mechanism by which the two interact remained a mystery.

Many years later, new research revealed that Ao targets the histone gene cluster in polytene chromosomes and acted as a negative regulator of histone expression, providing a conclusion to the–at the time–thirty-year-old mystery. Moreover, this mechanism was congruent with previous observations suggesting that the excess heterochromatin may act as a "sponge," absorbing excess histones produced in the absence of Ao (Tomkiel *et al.* 1991; Berloco *et al.* 2001). Also consistent, was the observation that the maternal effect lethality caused by Ao mutations is rescued by a reduction of the endogenous histone genes (Berloco *et al.* 2001; Takenaka *et al.* 2024).

Despite these exciting mechanistic advances, Ao has been largely overlooked for the past twenty years and key *ao* reagents, including the *ao[2]* null allele and anti-Ao antibody, were lost. We recently revisited the relationship between Ao and histone genes by engineering two novel *ao* tools: a CRISPR *ao* deletion allele and an endogenously-tagged V5 *ao* allele (Takenaka *et al.* 2024). Using these precise tools, we recapitulated many of the historical maternal effect observations yet could not replicate the impact of Ao on histone proteins nor transcripts. However, we did observe Ao localizing to the histone gene cluster in adult ovaries (Takenaka *et al.* 2024). These observations reopened what was believed to be a solved mystery.

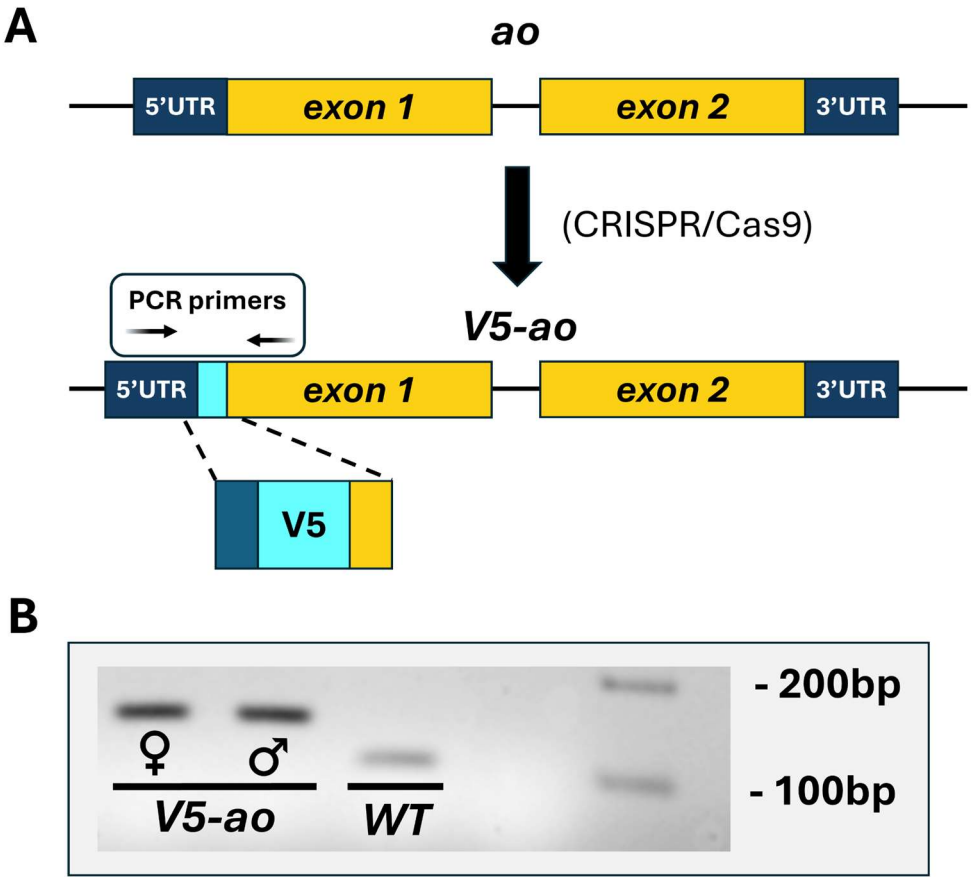
The previous literature paints a rather unique picture for Ao, which appears to have a rather idiosyncratic relationship with foundational structures of cellular function, chiefly histones and heterochromatin. Thus, to further probe the relationship between Ao and the histone genes themselves, we leveraged two endogenously tagged *ao* alleles for cytology studies. Given our recent findings, which were incongruous with historical data, and the growing mystery behind Ao's behavior, we endogenously tagged the N-terminus of the Ao locus with a V5-epitope tag. We used these animals in combination with the C-terminal tagged flies from Takenaka *et al.* (2024) to assess Ao localization in various tissues. Berloco *et al*. (2001) used an anti-Ao antibody to discover Ao localization to larval polytene clusters in both *D. melanogaster* and *D. virilis*.

Using both tagged allele reagents and a transgenic overexpression system, we did not observe Ao at the histone gene cluster in polytene chromosomes (Berloco *et al.* 2001). However, we observed Ao localizing to the histone gene cluster in blastula-stage and later embryos and ovaries, indicating tissue specificity. As the negative regulator, Mute, does not target histone loci with reduced gene content (McKay *et al.* 2015), we repeated this experiment to determine if Ao functions similarly to Mute. We found it appears to show reduced localization in similar environments to the negative regulator of histones. Our results begin to illuminate the idiosyncratic nature of Ao behavior and provide novel insights into potential feedback mechanisms within the HLB.

## Results:

N-terminus V5-Ao was successfully inserted

After the loss of the historical Ao antibody, we used a CRISPR-Cas9 system to endogenously tag the *ao* gene with a V5 epitope on the N-terminus (V5-Ao; Figure 1A & 1B). This process coincided with the creation of the C-terminus V5 epitope-tagged Ao (Ao-V5; Figure 1B & 1C), which we characterized in Takenaka *et al.* 2024.



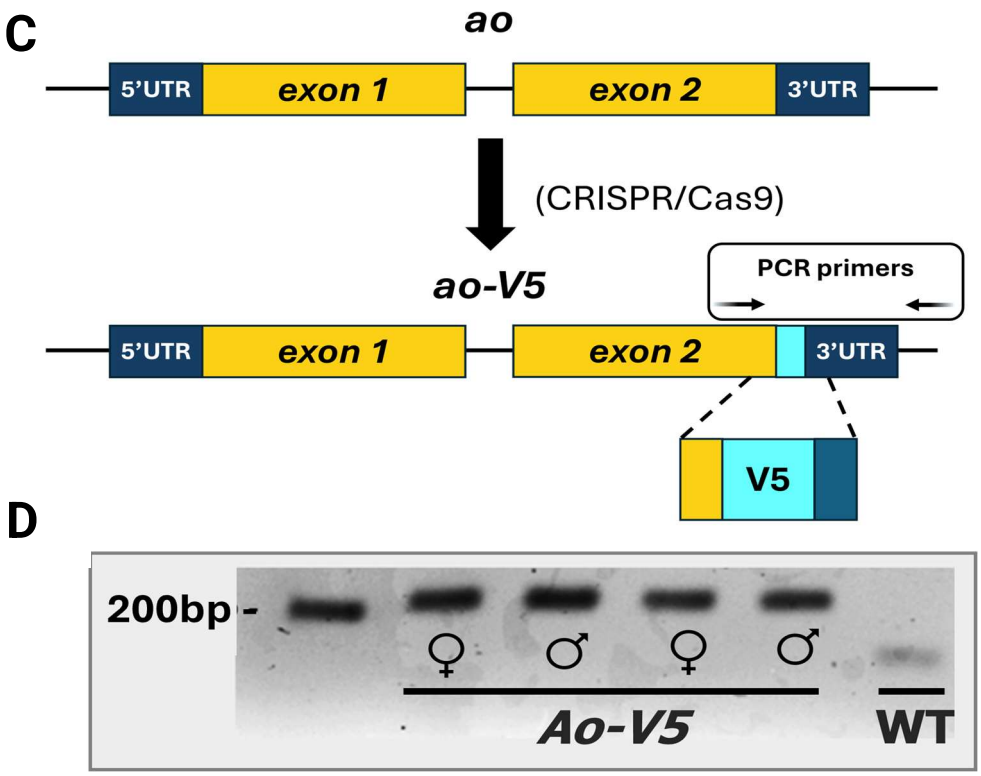



**Figure 1: Designing V5-Ao & Ao-V5.** (A) Diagram of N-terminal V5-tag insertion using CRISPR/Cas9 at the endogenous *ao* locus. The tag was inserted between the 5' UTR and the first exon of *ao*. (B) Representative PCR product of V5-Ao females and males with wild-type (*yw)* negative control. Single bands at 102 (wild-type *Ao*) and 145 base pairs (*V5-ao*) indicate homozygous animals. (C) Diagram C-terminal V5-tag insertion. (D) Representative PCR product of V5-Ao females and males with wild-type (*yw)* negative control. Single bands at 170 (wild-type *Ao*) and 212 base pairs (*ao-V5*) indicate homozygous animals.

Having dealt with the challenges of continuing scientific inquiry that has been largely overlooked for the last two decades, we hope to ensure our methods and tools may remain accessible. Therefore, we sought to use a tag with readily available commercial antibodies. We reason this should ensure our results are highly replicable by avoiding complications between different bespoke antibodies and—as history has shown—the loss of such antibodies. Commercial antibodies should have very reliable characterizations, due to their common usage. Likewise, the ability to vary our antibodies also enables an additional point of control for our results. To confirm the knock-in, we designed PCR primers to amplify the Ao gene irrespective of the presence of the V5 tag, allowing us to detect a relative shift in band position in addition to solely relying on estimated size. We observe a single band at the predicted 145 bp, confirming precise knock-in of the V5 epitope to the *ao* gene, compared to the wild-type 102 bp PCR product (Figure 1B).

Ao colocalizes with HLB-specific markers in ovaries and the early embryo

Previous work, suggesting that Ao is a negative histone regulator, localized Ao to the histone gene cluster using antibody-based techniques (Berloco *et al.* 2001). Similarly, we recently determined that Ao-V5 co-localizes in ovary nurse and follicle cells (Figure 2A) with Multi-sex combs (Mxc), a protein that exclusively targets the histone genes (White *et al.* 2011; Terzo *et al.* 2015). We found that V5-Ao also co-localizes with Mxc in ovaries (Figure 2B), consistent with Ao-V5 (Takenaka *et al.* 2024). RNA-seq data confirms enrichment of *ao* transcript in ovaries, but also in the early embryo (Brown *et al.* 2014).

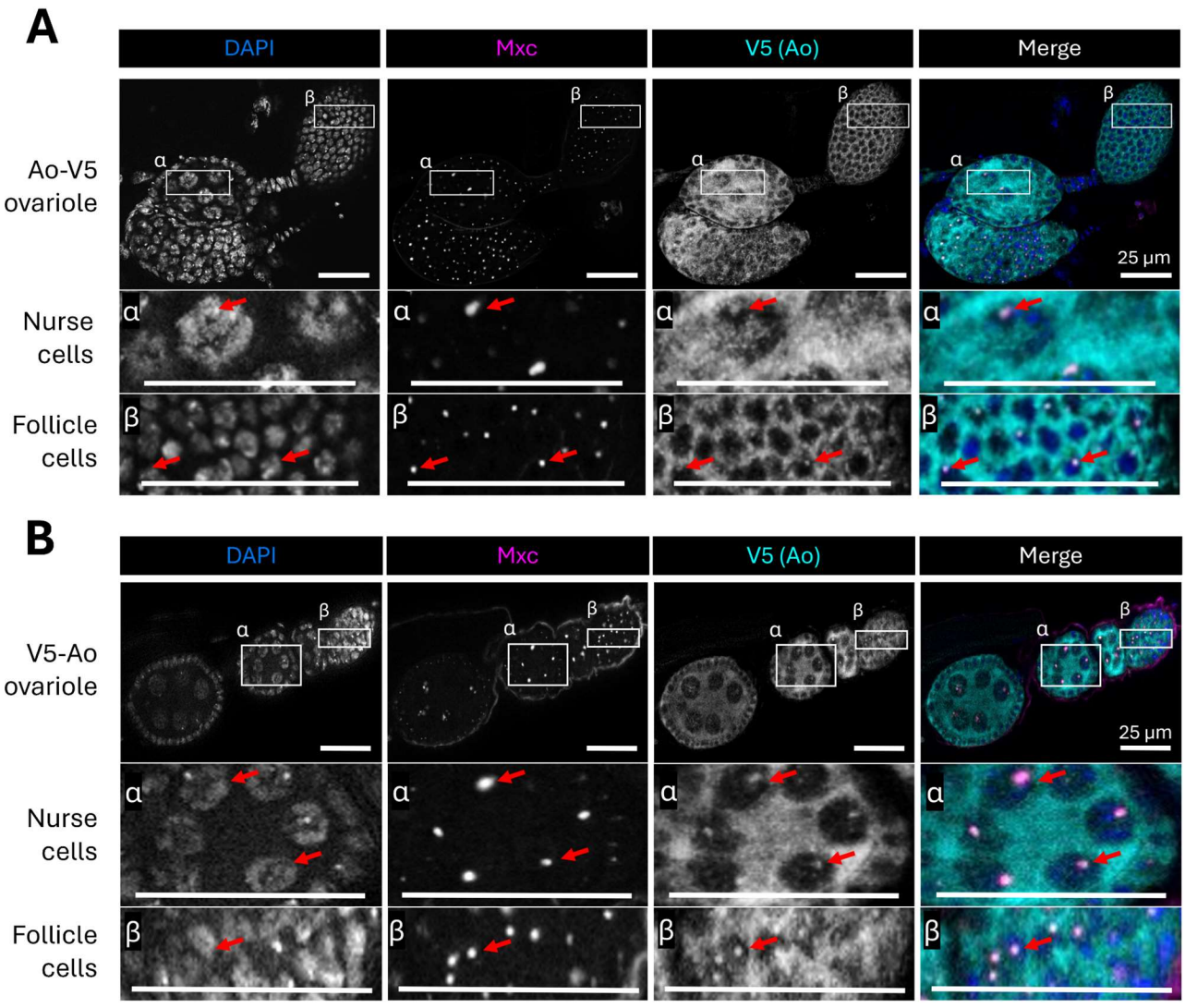



**Figure 2: Immunofluorescence microscopy of V5 tagged Ao in ovaries.** Red arrows highlight colocalization. (A) C-terminus Ao-V5 4-day virgin ovarioles. Blue (DAPI) marks DNA, magenta (Mxc) marks the HLB, cyan (V5) marks Ao. Scale bars represent 25μm. (B) N-terminus V5-Ao 4-day virgin ovarioles. Colors and scale bars are consistent with (A).

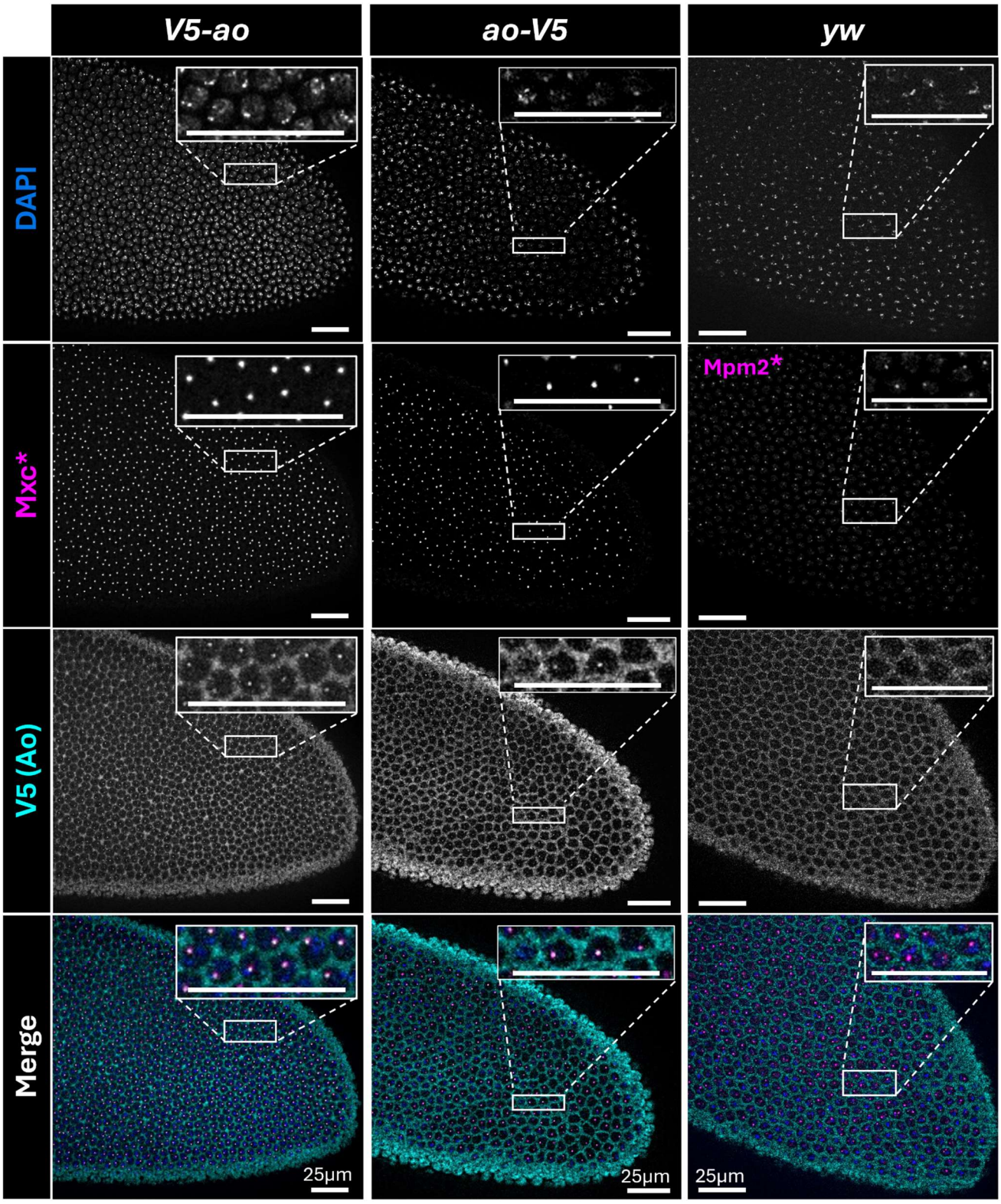



**Figure 3: Immunofluorescence microscopy of V5 tagged Ao in 1-3 hour embryos.** Red arrows highlight colocalization. Blue (DAPI) marks DNA, magenta (Mxc) marks the HLB, cyan (V5) marks Ao. Scale bars represent 25µm. Mpm2 primarily marks phosphorylated Mxc.

Therefore, we stained early 1-3 hour embryos for V5. Both V5-Ao and Ao-V5 also colocalize with Mxc in blastula stage and later embryos (Figure 3, Figure S1). Additionally, we observe there is large variation in Ao puncta, even in age-matched embryos (Figure S2).

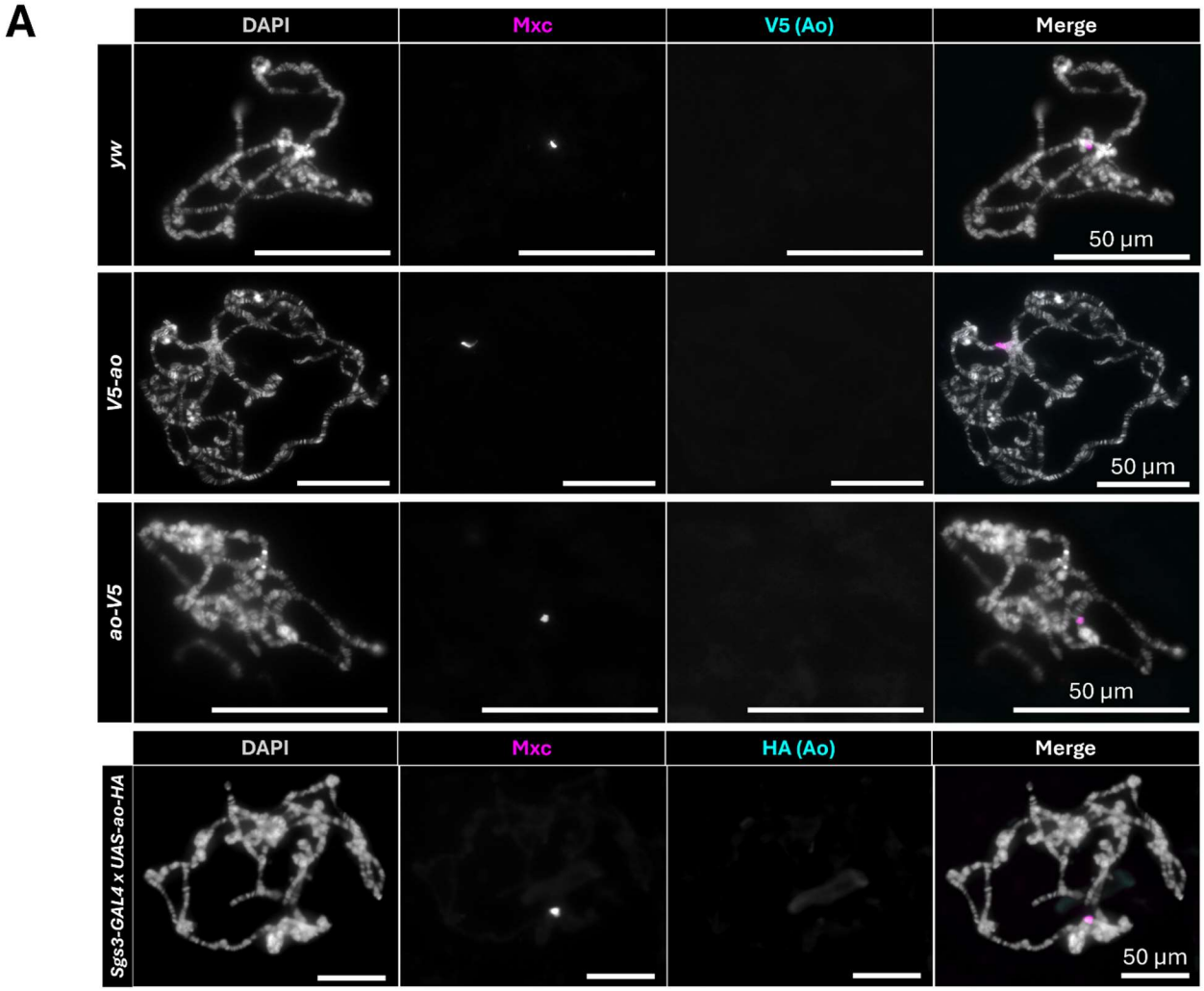
A notable weakness of this experiment is the lack of a typical control for our wild-type proxy embryos. We stained *y-, w-* flies with only a V5 tag and with a V5 tag and mpm2 (a marker of phosphorylated Mxc). However, we did not conduct a control where we used the same Mxc antibody as our endogenously tagged embryos. We can largely rule out antibody cross talk due to similar localization results with different antibodies in our later experiments and the lack of V5-puncta in the mpm2 control. Regardless, we will re-create this experiment with the optimal control for posterity's sake before publishing this work.

Ao does not localize to the histone cluster in larval salivary glands

Although previous immunofluorescence indicates Ao localizes to histone genes in larval salivary glands (Berloco *et al.* 2001; Brown *et al.* 2014), RNA-seq data indicates low levels of *ao* transcript in this tissue (Berloco *et al.* 2001; Brown *et al.* 2014). We were unable to replicate endogenous Ao localization to the histone genes in 3$^{rd}$ instar larval polytene chromosomes with either our V5-Ao or Ao-V5 (Figure 4A). In a complementary approach, we drove Ao overexpression by crossing the salivary gland *sgs*-GAL4 driver to animals carrying UAS-driven Ao-HA (Bischof *et al.* 2013). The resulting offspring showed tremendous overexpression of *ao* transcripts in salivary glands via RT-qPCR (Figure 4B). However, even with the robust overexpression, Ao still does not co-localize with Mxc on polytene chromosomes (Figure 4A), suggesting tissue specificity of Ao localization. Our findings reaffirm what we saw in Takenaka *et al.* (2024), as *ao* overexpression does not impact histone H2B transcript levels (Figure 4B).

Ao localization is proportional to histone gene stoichiometry like negative regulator of histone expression, Mute

Although Mute, a negative histone regulator, targets the endogenous histone cluster in wild-type 2-4 hour embryos, it fails to do so in age-matched embryos carrying only 24x copies of a transgenic histone array (McKay *et al.* 2015). Ao was thought to be another negative histone regulator (Berloco *et al.* 2001), although we recently determined that it likely has little effect on histone expression (Berloco *et al.* 2001; Takenaka *et al.* 2024). Ao levels do not affect histone transcript or protein levels in ovaries, but histone gene copy number does appear to affect *ao* expression: paradoxically, *ao* transcripts increase when histone copy number decreases (Takenaka *et al.* 2024).


A
DAPI
Mxc
V5 (Ao)
Merge
yw
V5-ao
ao-V5
50 µm
DAPI
Mxc
HA (Ao)
Merge
Sgs3-GAL4 x UAS-ao-HA
50 µm

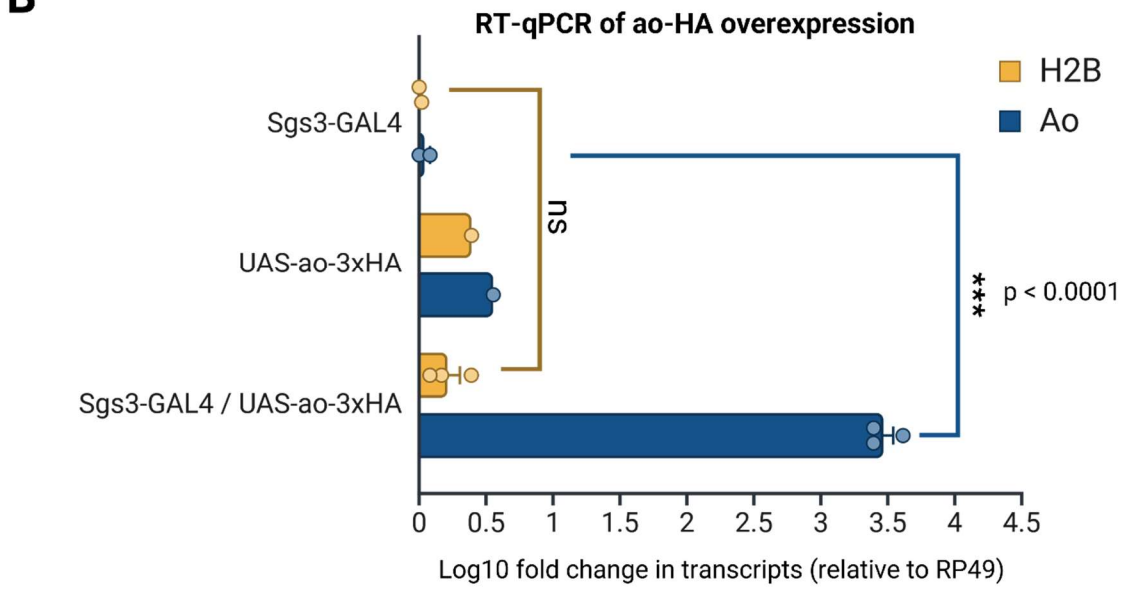

B
RT-qPCR of ao-HA overexpression
H2B
Ao
Sgs3-GAL4
UAS-ao-3xHA
Sgs3-GAL4 / UAS-ao-3xHA
ns
***
p < 0.0001
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Log10 fold change in transcripts (relative to RP49)

**Figure 4: Ao localization in larval salivary glands.** (A) Grey (DAPI) marks DNA, magenta (Mxc) marks the HLB, cyan (V5 or HA) marks Ao. Scale bars represent 50μm. Sgs3-Gal4 x UAS-ao-3xHA overexpress *ao-HA* in salivary glands, with full genotypes in Table 2. Ao does not colocalize with Mxc at endogenous or overexpressed levels. (B) Results from RT-qPCR using primers for *ao* and *H2B*, showing robust overexpression of *ao* without corresponding changes in histone H2B transcripts. RP49 is a ubiquitously expressed housekeeping gene. Statistical analyses represent unpaired two-tailed T-test for difference in means (α = 0.05) between parental (Sgs3-GAL4) flies and overexpression (Sgs3-GAL4 x UAS-ao-3xHA) offspring. Error bars indicate standard error of the mean. Only one replicate was available for the UAS lineage, so it was excluded from statistical analysis. Graph created with BioRender.com.

Thus, we investigated if Ao behaves similarly to the negative histone regulator, Mute, upon reduction of histone genes to assess for potential feedback systems. We selected embryos, as we showed consistent Ao localization to the histone cluster in this tissue. Moreover, they allow for extreme phenotypes, such as absent histone genes, to be present in living animals. We created flies carrying a deletion of the endogenous histone locus and V5-Ao, requiring a ~10 cM recombination event (Figure S3) (Crain *et al.* 2024). We rescued the homozygous histone locus deletion with a transgene carrying 12 wild-type copies of the histone gene array. The endogenous histone locus is located at on chromosome 2L (39D-39E), proximal to the chromocenter, while the 12x rescue transgene is located on chromosome 3, distal to the chromocenter (Fitch *et al.* 1990)**.** Thus, we confirmed the endogenous deletion and presence of the transgenic rescue with a single, distal Mxc band on polytene chromosomes (Figure S3). We also confirmed the flies were homozygous for V5-Ao by PCR using our genotyping primers (Figure S4, Table 1). We created three independent lines carrying this genotype. Unexpectedly, we found that these lines do not support homozygocity of the 12x rescue transgene. Homozygous adults are exceedingly rare, and we would not expect this behavior from a transgenic rescue of this kind. This may be a possible homozygous-lethal mutation somewhere on the third chromosome, but we cannot currently explain this observation. This is unlikely to affect our results. An unintended benefit, however, is that it permitted us to test a more detailed gradient of histone gene reduction.

To determine if Ao behaves similar to Mute, we stained embryos from *y-, w-* ; *V5-Abo*, $\Delta HisC^{cadillac}$ ; *12xHWT/Tm3Sb* mothers crossed to fathers of the same genotype. Embryos were of mixed genotypes: ¼ have no histone genes (0x), ½ carry 12 histone gene arrays (12x), and ¼ carry 24 histone gene arrays (24x). We genotyped embryos based on the number of Mute foci: 24x embryos have nuclei with two Mute foci (indicating homologous chromosomes carrying the histone rescue transgene); 12x embryos have only a single Mute focus per nucleus; and 0x embryos as those without clear puncta (Salzler *et al.* 2013). We determined that Ao exhibits reduced localization to the histone genes in 3–6 hour 24x embryos, and does not localize in 12x or 0x embryos of the same age (Figure 5).

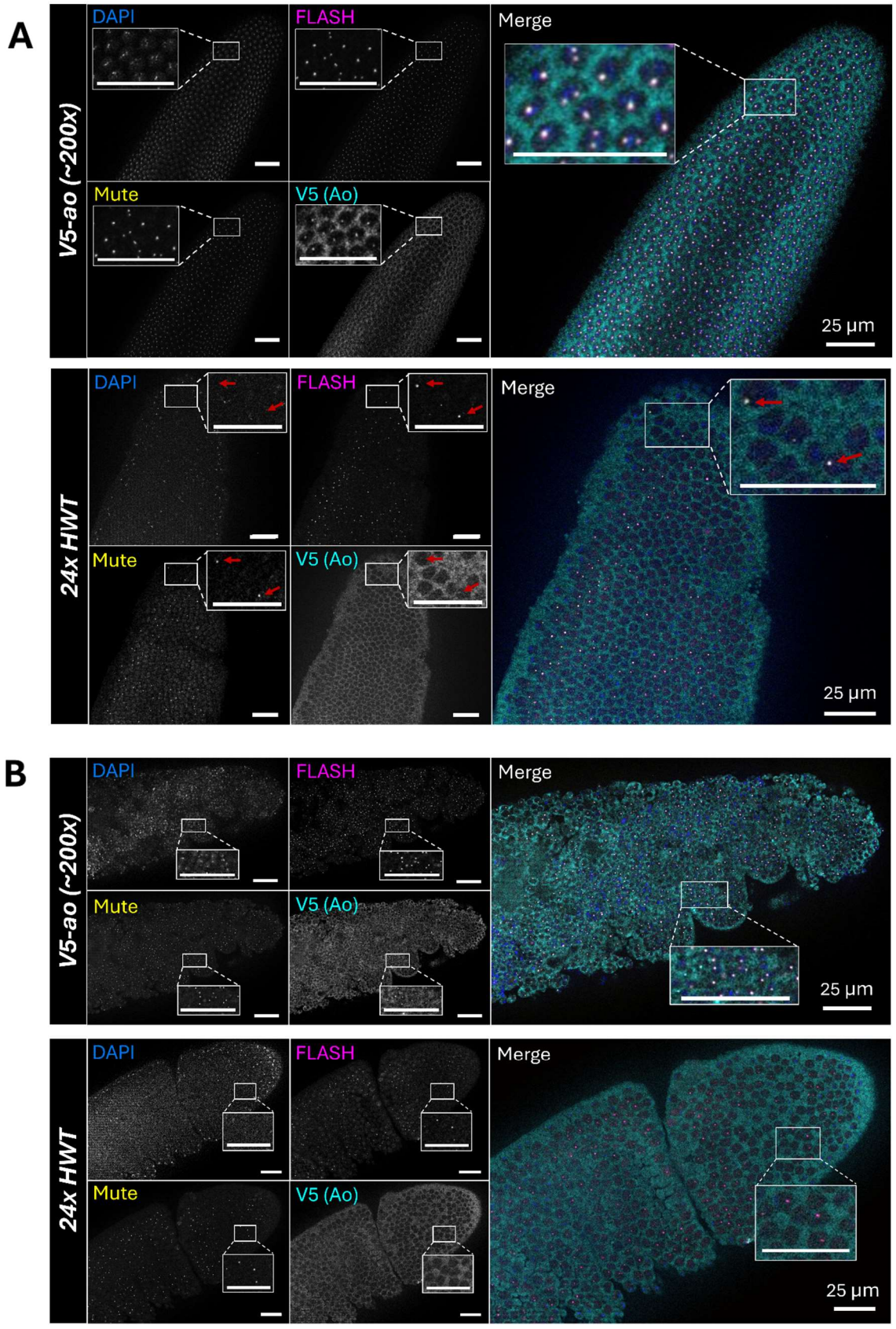

A
V5-ao (~200x)
DAPI
FLASH
Merge
Mute
V5 (Ao)
25 µm
24x HWT
DAPI
FLASH
Merge
Mute
V5 (Ao)
25 µm
B
V5-ao (~200x)
DAPI
FLASH
Merge
Mute
V5 (Ao)
25 µm
24x HWT
DAPI
FLASH
Merge
Mute
V5 (Ao)
25 µm

**Figure 5: Immunofluorescence microscopy of 3-6 hour histone transgenic embryos.** Red arrows highlight colocalization. Blue (DAPI) marks DNA, yellow (Mute) marks the HLB, as does magenta (FLASH), and cyan (V5) marks Ao. Scale bars represent 25μm. 24xHWT indicates animals homozygous for both the histone rescue transgene and endogenous deletion. (A) Early gastrulating embryos. (B) later segmenting embryos.

## Discussion:

We created a time-resistant genetic tool to assess Ao

Our successful utilization of a commercial tag maximizes our freedom to control or replicate our analysis of Ao by varying the tag's position (N- or C-terminus), its antibody, or to compare it to other tagged lines (such as our experiments with Ao-HA). We attempt to safeguard against the loss of molecular tools to assess Ao in the future, working under the assumption something like V5 will not precipitously fall out of fashion in the coming years and decades. These benefits, however, are predicated on the tag being relatively inconsequential to Ao function and behavior. These flies are able to support a permanent stock, and show no obvious phenotypic defects. Regardless, We have ongoing viability and fertility assays to confirm these observations.

Ao localization to the histone cluster is tissue specific

Once again, while different from earlier characterizations, our results appear reasonable when we take into account Ao's nature as a maternal effect gene. Larval salivary glands are a highly differentiated tissue, and we would expect *ao* transcripts to be largely depleted here, an idea further supported by previous public expression data (Brown *et al.* 2014). Since we utilized two tags with three insertion locations (Table 2), we controlled for tag-effects, finding identical results across each experiment. Even in our robust, thousand-fold overexpression, we saw no change. This could be because there is some system in place preventing excess Ao transcripts from being translated. We have two potential explanations for why our cytology differed from previous work. Berloco (2001) used a bespoke Ao antibody, which has been lost to time, where we used commercial antibodies to search for tagged Ao. The genetic background(s) of our flies also differ. However, due to the consistency between different tag locations, types, and the overexpression environment, we are confident in our results.

This sets up room for a mechanistic explanation of Ao behavior. It's possible Ao may not have a biologically relevant role in such a developed tissue as salivary glands. If Ao evolved to act only in the context of those tissues where it typically occurs—ovaries and embryos (Sandler 1970; Brown *et al.* 2014), excess protein might be akin to having oars

on an airplane: there is no need to bunch up and try to paddle. Alternatively, salivary glands only exhibit alternating S and G phases (Smith and Orr-Weaver 1991), so Ao may not be desirable at the histone genes in such a cellular context.

Utilizing this reasoning, we predicted Ao would localize nuclearly in ovaries and the early embryo. This is indeed what we show preliminarily in Takenaka *et al.* (2024) and in much more detail here, with embryos as an entirely novel tissue. Moreover, the consistency across N-terminus and C-terminus tagged Ao also assuages concerns over tag-effects. In other words, these results are markedly stronger when taken together than either C-terminus or N-terminus would be alone in a given tissue. Our findings are additionally supported by existing public expression data (Figure 2C) (Brown *et al.* 2014). On one hand, Ao localization *in these tissues* makes sense, but on the other hand, our localization results are puzzling. Under the auspices of Ao's former characterization as a negative regulator of histone genes, one may have expected localization to the histone genes, particularly in embryos whose rapid proliferation is suitable for additional regulatory needs. However, this explanation fails, in light of more updated analyses. Ao does not repress histone expression, and if anything, Ao expression may be the downstream, not upstream to histone levels (Takenaka *et al.* 2024). Taken together, Ao localization *at the histone genes* does not make sense, indicating the need for a satisfactory model to synthesize convoluted behavior. One such candidate is that Ao may not localize to the HLB randomly, but rather works as machinery for some other function, perhaps one tied to the cell cycle.

A hypothetical model of Ao function: a one-way valve for the HLB and the cell cycle

In light of Ao's difficult to understand nature, we synthesized our results with a literature review of the proteins related to Ao's ortholog DET1 (Berloco *et al.* 2001). In doing so, we formed a speculative model of Ao as part of a one-way valve for histone production and the cell cycle. This model has excellent explanatory power, and we use it to provide a satisfying explanation for what would otherwise seem to be contradictory results.

The DET1 family of proteins are highly conserved across eukaryotes with putative orthologs found in rice, mice, flies (Ao) and humans (Chory *et al.* 1989; Berloco *et al.* 2001; Pick *et al.* 2007). Furthermore, Ao's orthologs are conserved regulators of Cullin RING-based E3 ubiquitin ligase complexes (CRLs) (Wertz *et al.* 2004; Yanagawa *et al.* 2004; Bernhardt *et al.* 2006; Pick *et al.* 2007). It is believed that DET1 proteins interact with the complex via Damage-specific DNA binding protein 1 (DDB1), Cul4A, and Cul4B (Jin *et al.* 2006; Lee and Zhou 2007; Raisch *et al.* 2023). Notably, CRLs are tied to the cell cycle, often degrading cyclins and regulating cell cycle factors such as C-jun (Sherr and Roberts 1999; Wertz *et al.* 2004; Zheng and Shabek 2017). In fact, Cullin4 RING-

based E3 ubiquitin ligases in particular have both Cyclin E (CycE) and Checkpoint Kinase 1 (Chk1) as substrates, and Cul4 proteins regulate the levels of CycE in *Drosophila* (Higa *et al.* 2006; Leung-Pineda *et al.* 2009; Jackson and Xiong 2009). These are the HLBs points of information exchange from (CycE) and to (Chk1) the cell cycle, as described in the introduction (Knoblich *et al.* 1994; Ohtsubo *et al.* 1995; White *et al.* 2011; Günesdogan *et al.* 2014; Shindo and Amodeo 2021). Existing expression data further connects Ao and its orthologs, as human *hDET1* transcripts are similarly highly enriched in ovaries and the endometrium (Fagerberg *et al.* 2014; Brown *et al.* 2014). In a similar, perhaps much more coincidental vein, mutations in NPAT, the ortholog of Mxc, target of CycE/CDK2 phosphorylation are associated with neurological disease as are mutations in Cul4 E3 ligases (Imai *et al.* 1997; Zou *et al.* 2007; White *et al.* 2011; Lescouzères and Bomont 2020; Damgaard 2021).

Ao levels do not impact histone levels, but Ao levels increase as histone gene number decreases (Takenaka *et al.* 2024). Likewise, recent data suggests that histone transcripts also increase as histone gene number decreases (Koreski *et al.* 2020; Chaubal *et al.* 2023). Ao does not localize to the histone genes in polytene chromosomes from larval salivary glands, even when overexpressed (Figure 4). However, Ao does localize to the histone genes in ovaries and embryos (Figures 2 & 3). Ao does not localize to the histone genes when there are only 12x copies and shows reduced localization behavior across 24x histone gene embryos (Figure 5). We go into this idea more in-depth during the assessment of our model.

One of our foundational assumptions is that Ao, like its many orthologs in organisms ranging from rice to humans (Berloco *et al.* 2001), acts as a part of a CRL. Likewise, we predict that during Ao works with the ligase complex to mark CycE/CDK2 and Chk1 for degradation, with its levels correlating to the amount of histone transcript present. By degrading CycE/CDK2, the HLB will become inactivated, preventing re-replication events, and encouraging progress past S-phase. Degrading Chk1 also promotes progress past S-phase by preventing Chk1's inhibition of Cell division cycle protein 25 (Cdc25) (Patil *et al.* 2013). This coincides with histones H3's competitive inhibition of Chk1 (Patil *et al.* 2013; Shindo and Amodeo 2021). Here lies our second-largest assumption: that Ao levels increase with histone levels, derived from the findings from Koreski *et al.* (2020), Shindo and Amodeo *et al.* (2023), and Takenaka *et al.* (2024).

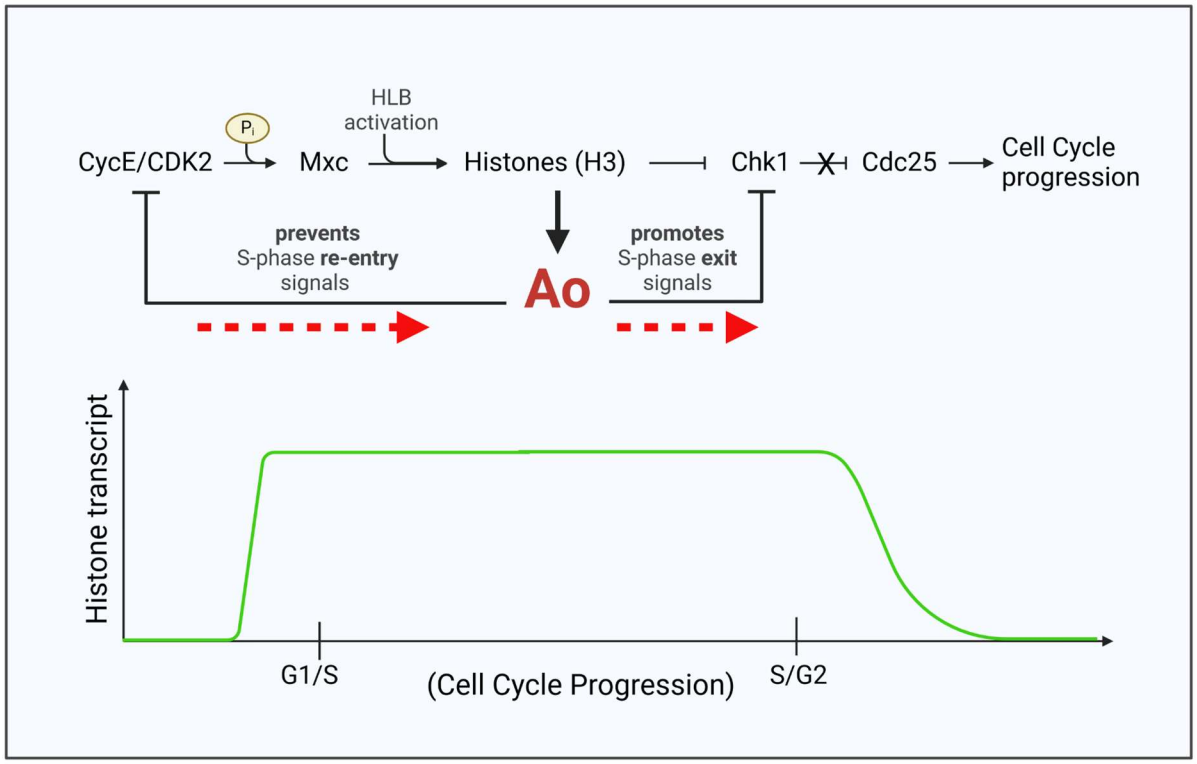



**Figure 6: Speculative model of Ao as a one-way valve for histone transcription in the cell cycle.** Created with BioRender.com.

Thus, even though Cyclin E and Chk1 are respectively upstream and downstream of histone replication during the cell cycle, Ao-related ubiquitination and degradation would promote unidirectional movement through S-phase and histone replication. Interestingly, some CRL4 complexes do show this one-way valve property. When bound to Cdt2 instead of DET1, the complex is known to degrade Cdt1, P21, Set8, acting as master regulator, preventing re-replication in S-phase (Abbas and Dutta 2011; Havens and Walter 2011). In *Drosophila*, $CRL4^{Cdt2}$ is critical in regulating Cyclin E levels around the $G_1$/S transition by degrading its transcription factor activator E2F1 (Ohtani *et al.* 1995; Shibutani *et al.* 2008; Zielke *et al.* 2011). Ao may enable the CRL4 to have a similar function in exiting S-phase within a histone-based context in *Drosophila*.

<u>An initial test of the model reveals its predictive and explanatory capabilities</u>

We hoped to see if our model could explain Ao behavior in extreme genetic conditions. A common explanation for Mute's behavior is that with fewer histone genes, the cell may have a less need for a negative regulator—much like how a slow-moving bicycle would not need much of a handbrake. This is inadequate to address Ao's similar behavior in 3-

6 hour embryos. Fortunately, Chaubal *et al.* (2023) suggests that further reduced copy numbers of histone genes may have difficulties sequestering the correct amount of required factors to create a functioning HLB. This explains both our results and those from McKay *et al.* (2015). Mute and Ao may be outcompeted by the wave of other factors converging on a patch of histone genes an order of magnitude smaller than it would be in a wild-type fly. It follows that as the cells mature and cycle less often, the flood of factors to these genes would decrease, returning Mute's capacity to localize in older 24x embryos. This is what is observed in McKay *et al.* (2015).

Returning to our predictions: If Ao is acting as a part of a E3 ubiquitin ligase complex, interfacing the input and output of the HLB with the cell cycle, it would have fairly transient interactions (supported by the variability of Ao puncta intensity we observe in aged matched embryos). Such a protein could easily be shouldered out of the way. Furthermore, with reduced histone gene count, we may expect a reduction in the need to rapidly degrade CycE/CDK2, so the cell can encourage as much active HLB formation as possible in the strenuous genetic environment. Together, these accurately predict our results. Ao lost its full capability to form puncta at the HLB, along with Mute. Thus, Ao protein behavior, not only levels, are sensitive to the stoichiometry of the histone genes. Unlike Mute, Ao does not directly affect histone levels, and Ao exhibits *reduced* localization even though Ao transcripts *increase* when histone gene count is at 24x instead of wild-type (Bulchand *et al.* 2010; Takenaka *et al.* 2024). This is yet another example of otherwise puzzling Ao behavior, yet our model provides one possible satisfactory explanation.

Along with this experiment, our speculation is congruent with our earlier data. In embryos and ovaries, the rapidly dividing cells with high Ao concentrations exhibit a need for histone gene regulation. If Ao has a cell cycle function, we would expect variation, even in age-matched embryos. We observe this. If Ao promotes movement out of S-phase, we would expect tissues that do not undergo such progression to lack Ao localization, even if we overexpress it. In one such tissue, larval salivary glands, we observe this. The model may even suggest a mechanistic reason. Cdc25, substrate of Chk1, is not needed in salivary glands endoreplication (Lilly and Duronio 2005; Zielke *et al.* 2013). Thus, there is little need for Ao in this tissue, as its altered cell cycle renders Ao's downstream targets moot.

The model is congruent with data from other research. Shindo and Amodeo (2023) observe shorter cell cycles when overexpressing H3 tails—which inhibit Chk1. Our model proposes that this may result in a concurrent increase in Ao activity in degrading Chk1 and CycE/CDK2, deactivating the HLB. This would also work to advance the cell cycle through S-phase. Of course, as one of the foundational results utilized in creating the

model, it also explains why there is no change in histone expression with a true *ao*-null allele in Takenaka *et al.* (2024). For, we speculate Ao is downstream of histone levels.

This model has obvious shortcomings. First, it provides no clear explanation for the difference in historical *ao* mutants and the *ao*-null allele. Further characterization of the mutant itself is likely to elucidate this. Most importantly, it lacks firm experimental evidence for its key assumptions. Namely, that (1) Ao interacts with the CRL4 complex in *Drosophila*, and (2) Ao levels are responsive to histone levels, not just gene copy number, and (3) Ao orthologs perform similar functions. A Co-immunoprecipitation for V5 and subsequent Western Blot for CRL factors would address our first assumption. Notably, CRLs have been shown to regulate Cyclin E in mammals, such as Cullin 1 and Cullin 3 (Singer *et al.* 1999; Koepp *et al.* 2001; McEvoy *et al.* 2007). Thus, we should expand our search to include the possibility that Ao acts with these CRLs instead of CRL4. A yeast 2 hybrid (Y2H) screen would also help address assumption (1). To address assumption (2), we can overexpress H3 tails and assess for changes in Ao levels. Finally, we can attempt to rescue *ao*-null animals with orthologous *DET1* variants to address assumption (3).

We combined our cytological approach with a wealth of previous literature info to synthesize a speculative model for the molecular mechanism of Ao. The model exhibits initial explanatory power for previously puzzling results from existing literature and for our novel results, including in the extreme genetic environment of a complete histone gene deletion. We propose a selection of future steps to begin supporting our model experimentally and eliminate our assumptions.

## Materials and Methods:

CRISPR V5 knock-in and confirmation

| Purpose | Sequence |
|---|---|
| CRISPR/cas9 guide RNA | Sense: TGCACGATTGTAGGCGCTTCTTGT<br>Antisense: AAACACAAGAAGCGCCTACAATCG |
| ssODN repair template | GACATTAGGAATAGGAACTTGGCAGAACTTTCCCAGGATCTGT CAGAATGGGCAAGCCCATCCCCAACCCCCTGCTGGGCCTGG ACAGCACCGCTTACAAGAAGCGCCTACAATCGCAGAACCTCG TGCATCTGCTGCAGAACCGCGAG |

| | |
|---|---|
| V5-*ao* primer<br>(N-term tag) | Forward: GGAACTTGGCAGAACTTTCCCAGG<br>Reverse: GTATCCGGACTCGCGGTTCTG |

Table 1. Genetic tools for V5-ao insertion

We used the CRISPR/Cas9 system to endogenously tag the N-terminus of *ao* with a V5 epitope. We cloned our guide RNA (Table 1) into pCFD5 (Addgene plasmid #73914), after selecting a guide RNA closest to the start codon of *ao* with no off-targets (Table 1) (http://targetfinder.flycrispr.neuro.brown.edu/) (Port and Bullock 2016). We designed a single-stranded oligo donor (ssODN) repair template containing the 42bp V5 tag ~50 bp upstream and ~57bp downstream of the insertion site (Table 1). To prevent re-targeting, the ssODN had a mutated PAM site. We midi-prepped the pCFD5 plasmid containing the guide RNA and lyophilized ssODN. This sample was sent to GenetiVision Inc. (Houston, TX) to be injected into *nanos Cas-9* embryos. To confirm knock-in, we performed PCR (Sigma-Aldrich RedTaq® ReadyMix™ PCR Reaction Mix) using primers specific to the *ao* locus (Table 1). We ran the products on a 2% agarose gel at 100 volts for 45 minutes in 1x TBE buffer. We also confirmed the insertion via Sanger Sequencing. This process is identical to the creation of the C-terminus *ao*-V5 in Takenaka *et al.* 2024.

Drosophila husbandry and crosses

| **Fly Stock** | **Purpose** | **Source** |
|---|---|---|
| *y-, w-* | “Wild-type” proxy | |
| *y-, w-* ; *V5-ao* | Endogenously tagged *ao* (N-terminus) | Generated via CRISPR at Genetivision (Houston TX) |
| *y-, w-* ; *ao-V5* | Endogenously tagged *ao* (C-terminus) | Takenaka *et al.* 2024<br>Generated via CRISPR at Genetivision (Houston TX) |
| *w[1118]* ; *P{w[+mC]=Sgs3-GAL4.PD}TP1* | Expression of GAL4 in salivary glands | Bloomington Drosophila Stock Center |

| | | |
|---|---|---|
| | | BDSC_6870 |
| *M{UAS-ao.ORF.3xHA.GW}ZH-86Fb* | Ectopic expression of *ao* using different GAL4 drivers | FlyORF Project #6093<br>Bischof *et al. 2013* |
| *y-, w-* ; *ΔHisC$^{cadillac}$ /CyO* ; *MRKS/Tm6B* | Endogenous histone locus deletion | Crain *et al.* 2024 |
| *y-, w-* ; *aoΔ, 3xP3-dsRed/CyO, twi-GAL4, UAS-GFP* | Endogenous *ao* locus deletion | Takenaka *et al.* 2024 |
| *y-, w-* ; *ΔHisC, UAS-YFP/CyO* ; *12xHWT/TM6B* | 12x copy wild-type histone-array rescue | McKay 2015 |
| *y-, w-* ; *If/CyO* ; *Tm3Sb/Tm6Tb* | Balancer stock | |
| *y- , w-* ; *If/CyO, twi-GAL4, UAS-GFP* ; *Tm6Tb/Tm3Sb* | Balancer stock | |

Table 2. Fly lines used in this study, their purpose, and origins.

We kept all flies at 23ºC on standard molasses-based food with supplemental Saf-instant baker's yeast. We flipped the vials every three days to avoid overcrowding of larvae. To overexpress *ao* in larval salivary glands, we utilized the Gal4-UAS system (Brand and Perrimon 1993; Bischof *et al.* 2013). We collected virgin Sgs3-Gal4 females and bred them to either male *y- , w-* flies as a control or to UAS-*ao*-3xHA flies at 23ºC (Table 2). We shucked salivary glands from F1 3$^{rd}$ instar larval offspring, which we prepared for either polytene chromosome staining or RNA extraction for RT-qPCR.

The creation of animals homozygous for both the *V5-ao* and ΔHisC$^{cadillac}$ alleles required an involved cross with an ~10 cM recombination event occurring on the second chromosome. Since recombination only occurs in female flies and females can fertilize eggs long after mating with all crosses, it is standard to select virgins for fly-crosses. A graphic of this portion of the cross can be found in Figure S5. To create this recombinant line, we crossed *V5-ao* flies with those carrying the endogenous histone-gene-deletion allele, ΔHisC$^{cadillac}$ (*y-, w-* ; *ΔHisC$^{cadillac}$/CyO* ; *MRKS/Tm6B*) (Table 2) (Crain *et al.* 2024). We collected transheterozygote virgin female offspring (*y-, w-* ; *V5-ao/ΔHisC$^{cadillac}$* ; *MRKS/Tm6B*), and crossed these to *y-, w-* ; *If/CyO* ; *Tm3Sb/Tm6Tb* males (Table 2). From this cross, we selected balanced male progeny that were positive for Act5C-dsRed (indicating *ΔHisC$^{cadillac}$*) and crossed these males with balancer females (*y-, w-* ; *If/CyO* ; *Tm3Sb/Tm6Tb*). We then screened to find recombinant animals carrying both *V5-ao* and

Δ*HisC*$^{cadillac}$ by PCR, as previously (Table 1). We then established final homozygous stocks with the genotype: *y-, w-* ; *V5-ao,* Δ*HisC*$^{cadillac}$ ; *Tm3Sb/Tm6Tb*. We crossed flies carrying with a 12-copy histone array transgene (*y-, w-* ; Δ*HisC, UAS-YFP/CyO* ; *12xHWT/TM6B*) to a balancer stock (*y-, w-* ; *If/CyO, twi-GAL4, UAS-GFP* ; *Tm6Tb/Tm3Sb*) (Table 2) (McKay *et al.* 2015). We isolated GFP positive, non-tubby larvae. Likewise, we crossed males from this selection to *y-, w-* ; *V5-ao,* Δ*HisC*$^{cadillac}$ ; *Tm3Sb/Tm6Tb* virgin female flies. The final genotype was thus: *y-, w-* ; *V5-ao,* Δ*HisC*$^{cadillac}$ ; *12xHWT*/*Tm3Sb*. Surprisingly, we could not homozygose the third chromosome, instead remaining heterozygous for the transgenic histone gene array rescue.

Immunofluorescence to assess Ao localization

| | **Antibody** | **Dilution** | **Source** |
|---|---|---|---|
| **Primary** | mouse anti-V5 | 1:500 | Invitrogen R960-25 |
| | mouse anti-HA | 1:500 | Sigma-Aldrich SAB2702217 |
| | guinea pig anti-Mxc | 1:5000 | Gift from R.J. Duronio<br>White *et. al* 2011 |
| | rabbit anti-dFLASH | 1:6000 | Gift from R.J. Duronio<br>Yang *et al.* 2009 |
| | guinea pig anti-Mute | 1:2000 | Bulchand *et al.* 2010 |
| **Secondary** | goat anti-mouse Alexa Fluor594 | 1:1000 | Thermo Fisher A-11005 |
| | goat anti-mouse AlexaFluor488 | 1:1000 | Thermo Fisher A-11001 |

| | | | |
|---|---|---|---|
| | goat anti-guinea pig Alexa Fluor488 | 1:1000 | Thermo Fisher A-11073 |
| | goat anti-guinea pig Alexa Fluor647 | 1:1000 | Thermo Fisher A-21450 |
| | goat anti-rabbit Alexa Fluor594 | 1:1000 | Thermo Fisher A-11012 |

Table 3. Antibodies, concentrations, and source

***Polytene chromosomes:***

To analyze Ao localization on polytene chromosomes, we dissected salivary glands from 3rd instar larvae and fixed them in three washes. First, we used a 4% paraformaldehyde and 1% Triton X-100 in 1X phosphate buffered saline (PBS) solution for one minute. We then transferred the glands to a 4% paraformaldehyde and 3M acetic acid solution for two minutes. Finally, we transferred the glands to a solution of one-part lactic acid, two-parts H-$_2$O, to three-parts acetic acid for 5 minutes. We placed fixed glands on a coverslip and crushed them with a slide to spread the polytene chromosomes. We plunged slides in liquid N2 and removed the coverslips. We stored the slides in 95% ethanol at -20$^o$C. Before staining, we rehydrated the slides in 1X PBS for 15 minutes, then permeabilized them in 1% Triton X-100 for 10 minutes. We blocked the glands in 0.5% BSA for one hour at room temperature. The 0.5% BSA solution was also the diluent for the antibodies. We dispensed primary antibody solution (Table 3) under a coverslip on the slide and left it to incubate overnight at 4$^o$C. The following day, we washed off the primary antibody solution with three 15-minute washes in 1X PBS. Likewise, we dispensed a secondary antibody solution and covered the sample with a coverslip (Table 3). We incubated these in the dark at room temperature for two hours. Following the incubation, we washed the slides three times for 10 minutes in 1X PBS, dried, and mounted them with ProLong Diamond Anti-Fade Mountant with DAPI (P36962 ThermoFisher Scientific), sealed the coverslip to the slide with nail polish, and stored at 4$^o$C. We imaged the overexpression polytene chromosomes with a ZEISS Axio Scope.A1 fluorescence microscope and the rest with a Keyence BZ-X810 All-in-One-Fluorescence Microscope using the “BZ-X800 Viewer” program.

***Embryos:***

We collected 1-3 hour, 0-3 hour, and 3-6 hour embryos laid on grape juice plates and began by dechorionating embryos using 100% bleach for 75 seconds. Next, we rinsed

the collected embryos with deionized water. We held the embryos in a tube containing 1X PBS-Tween (in 50mL: 5mL 10X PBS, 500µL 10% Tween-20x, 44.5 mL $H_2O$. We then washed the embryos in 1X PBS-Tween and added 223µL 1X PBS, 27 µL, 37% formaldehyde, and 1mL heptane. After vortexing the samples for 30 seconds, we left to rotate for 20 minutes at room temperature. We removed and replaced the heptane layer with methanol, and vortexed the samples again for one minute. To complete the fixation, we washed the embryos in 1mL methanol 3 times and stored them at -20°C in 0.5mL methanol.

We employed a series of baths to rehydrate the embryos, starting with 90% methanol 10% PB-tween, decreasing to 75%:25%, 50%:50%, to 25%:75**%.** Following this step, We washed the samples twice for 10 minutes in PB-Tween. We removed the washing solution and added appropriate dilutions of the primary antibody (Table 3) in blocking solution (0.1% Tween-20 and 1% BSA in 1X PBS). We let the embryos incubate overnight on a rotator at 4°C. The following day, we again washed the treated embryos in blocking solution for 5 minutes, then twice more in blocking solution for 30 minutes each. We diluted secondary antibodies (Table 3) to 1:1000 in blocking solution and incubated with embryos for 2–4 hours at room temperature while rotating in the dark. We conducted final washes three times in 1X PBS-Tween for five minutes and placed the embryos onto a coverslip using a wide-bore pipette tip. Furthermore, we used Invitrogen ProLong Diamond Anti-Fade Mountant (P36965, ThermoFisher Scientific) for final mounting. We sealed the coverslip onto the microscope slide with nail polish and imaged the embryos with a Keyence BZ-X810 All-in-One-Fluorescence Microscope using the “BZ-X800 Viewer” program.

***Ovaries:***

We collected and isolated virgin females and raised them for 4 days before dissecting ovaries. We dissected sets of 10 pairs in 1x PBS, placing each pair of ovaries on ice during the duration of the dissection. We utilized a modified version of the ovary staining protocol from the Malik Lab at the Fred Hutchinson Cancer Research Center (Kursel *et al.* 2021). After dissection, we broke apart the egg chamber, freeing the ovarioles, and placed them into a 1.7mL microcentrifuge tube with 1X PBS. We decanted off the solution and added 1 mL of shaken 1:1 paraBT:heptane (paraBT: 4% paraformaldehyde, 0.1% TritonX-100, in 1X PBS). This was left to fix for 10 minutes at room temperature on a nutator. We let the ovaries settle to the bottom of the decanted off the fixation solution, and subsequently ran 3x 5-minute washes of 0.1% Triton in 1X PBS. We blocked the ovaries using 1mL of 3% BSA in 1XPBS + 0.1%Triton for 30 minutes at room temperature on a nutator. We added our antibodies (Table 3) in this block solution to the ovaries and incubated overnight at 4°C on a nutator. The following day, we decanted off the primary solution and conducted another bout of 3x 5-minute washes in 1X PBS + 0.1% Triton. We

incubated the ovaries with secondary antibodies (Table 3) for 2 hours at room temperature in our block solution, adding DAPI to 1:1000 in the final 20 minutes of the secondary incubation. We ran an additional 3x 5-minute washes in 1XPBS + 0.1% Triton. With a wide-bore pipette tip, we transferred ~ 200µL of solution containing the ovaries onto a microscope slide. We removed excess liquid with a 20µL pipette. Furthermore, we used Invitrogen ProLong Diamond Anti-Fade Mountant (P36965, Thermo Fisher Scientific) for final mounting. We sealed the coverslip onto the microscope slide with nail polish and imaged the ovaries with a Keyence BZ-X810 All-in-One-Fluorescence Microscope using the "BZ-X800 Viewer" program. Throughout all immunofluorescence experiments, we minimized the samples' exposure to light during steps involved with the secondary antibodies.

RNA isolation and RT-qPCR

| **Primer** | **Sequence** | **Purpose** |
|---|---|---|
| V5-*ao*<br>(N-term tag) | Forward: GGAACTTGGCAGAACTTTCCCAGG<br>Reverse: GTATCCGGACTCGCGGTTCTG | PCR - Genotyping |
| *ao*-V5<br>(C-term tag) | Forward: GGAACTTGGCAGAACTTTCCCAGG<br>Reverse: GTATCCGGACTCGCGGTTCTG | PCR - Genotyping |
| *ao* | Forward: GATAAAAGTGGCAACCCGACGGAAAG<br>Reverse: CATGGGCATGGTATCGGTACCATTTG | qPCR |
| *ao*-HA | Forward: GTCTACCACGTGCACTTGTATAACCAC<br>Reverse: CATAGTCCGGGACGTCATAGGGATAG | qPCR |
| *rp49* | Forward: GCTAAGCTGTCGCACAAA<br>Reverse: TCCGGTGGGCAGCATGTG | qPCR |

Table 4. DNA primers used, sequences, and purpose

We isolated RNA from dissected salivary glands from 3rd instar larvae. We took ten pairs of salivary glands, placed them in 100 µL Trizol, homogenized, and stored them at -80°C. We later brought samples to 1mL in Trizol and rotated them for 20 minutes. Subsequently, we added 200µL chloroform to the tubes, which we shook vigorously and spun down at 20,000 RCF for 20 minutes at 4°C. We transferred the aqueous phase to a new tube and repeated the chloroform addition, shaking, spinning, and aqueous-phase removal. We added 500 µL isopropanol and 5µL glycogen to the final aqueous layer and let this incubate at -20°C overnight to precipitate RNA. We spun the samples at 20,000 RCF at 4°C for 10 minutes and washed the RNA pellets in 75% ethanol. We spun the samples again in a centrifuge for 5 minutes at 20,000 RCF. Finally, we resuspended RNA in water and stored it at -80°C until use. For RT-qPCR, we utilized a LunaScript RNA-to-cDNA conversion kit (NEB) to prepare samples. We used the RT-qPCR primers for *ao*, *ao-HA, and rp49* in table 4.

## Author Contributions:

EHA, CAS, and LER conceived experiments. EHA and CAS designed experiments. CAS generated CRISPR lines. EHA conducted all other experiments, wrote the manuscript, and created the model. EHA, LER, and CAS edited. CAS and LER supervised and provided feedback on experiments.

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## Supplemental Figures:

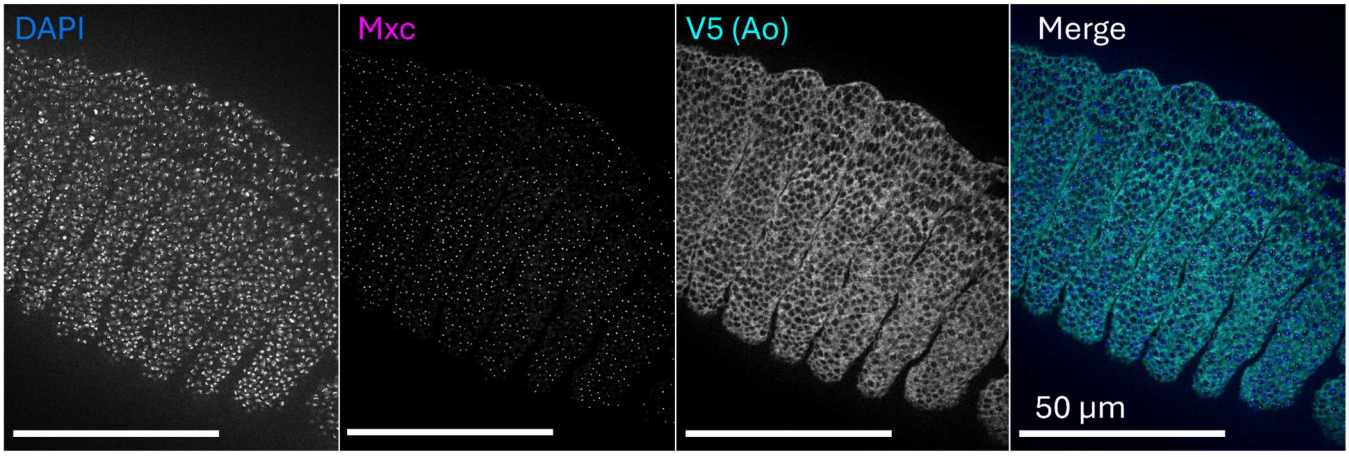



**Figure S1: V5-Ao colocalization in post-gastrulating embryo.** Blue (DAPI) marks DNA, magenta (Mxc) marks the HLB, cyan (V5) marks Ao. Scale bars represent 50µm.

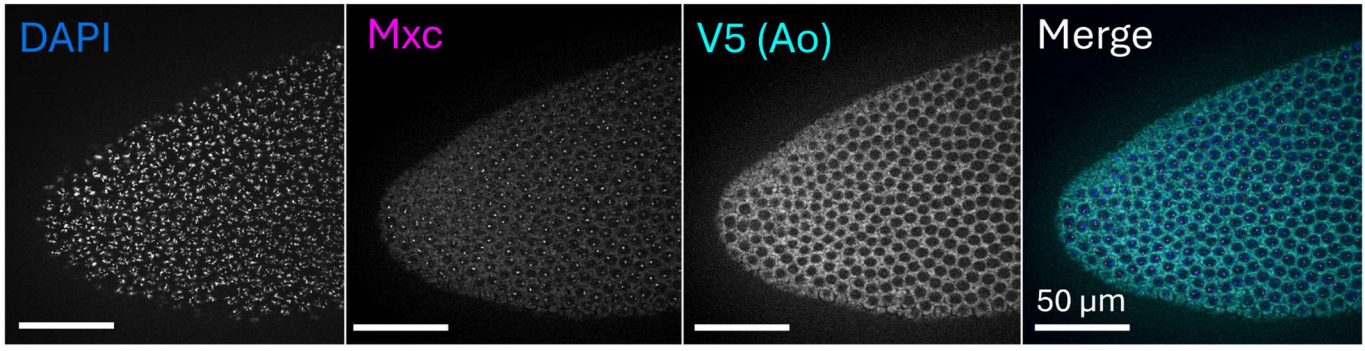
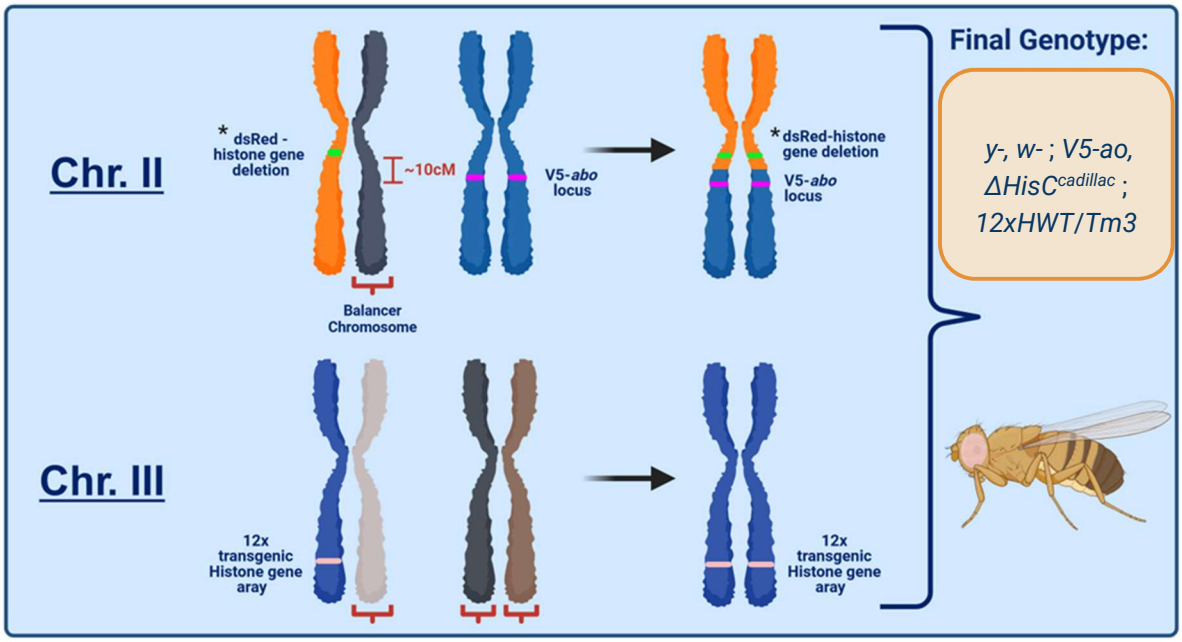
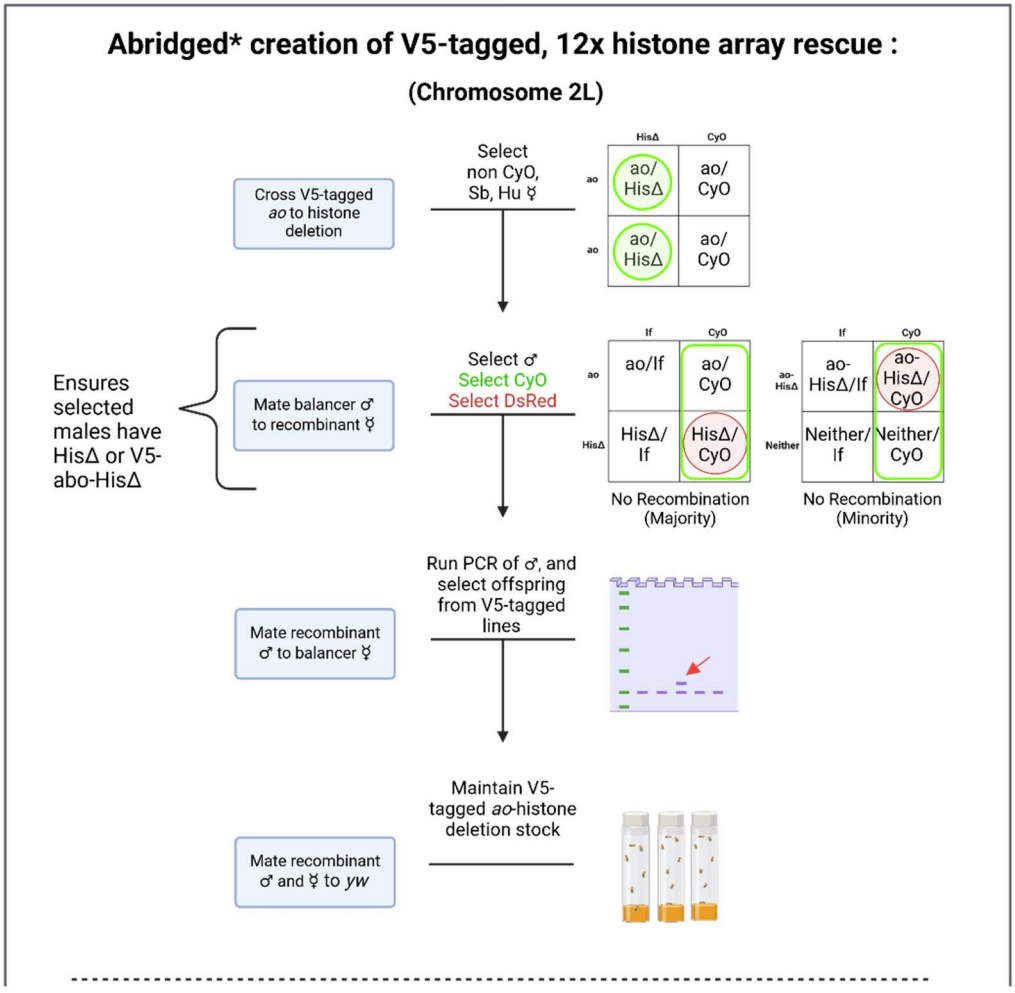



**Figure S2: V5-Ao showing minimal colocalization with Mxc in syncytial 1-3 Hour embryo.** Blue (DAPI) marks DNA, magenta (Mxc) marks the HLB, cyan (V5) marks Ao. Scale bars represent 50µm.


Chr. II
* dsRed - histone gene deletion
~10cM
V5-abo locus
Balancer Chromosome
* dsRed-histone gene deletion
V5-abo locus
Chr. III
12x transgenic Histone gene aray
12x transgenic Histone gene aray
Final Genotype:
y-, w- ; V5-ao, ΔHisC cadillac ; 12xHWT/Tm3


Abridged* creation of V5-tagged, 12x histone array rescue :
(Chromosome 2L)
Cross V5-tagged ao to histone deletion
Select non CyO, Sb, Hu ♀
HisΔ
CyO
ao
ao/ HisΔ
ao/ CyO
ao/ HisΔ
ao/ CyO
Mate balancer ♂ to recombinant ♀
Ensures selected males have HisΔ or V5-abo-HisΔ
Select ♂
Select CyO
Select DsRed
If
CyO
ao/If
ao/ CyO
HisΔ/ If
HisΔ/ CyO
No Recombination (Majority)
ao-HisΔ
ao-HisΔ/If
ao-HisΔ/ CyO
Neither
Neither/ If
Neither/ CyO
No Recombination (Minority)
Mate recombinant ♂ to balancer ♀
Run PCR of ♂, and select offspring from V5-tagged lines
Mate recombinant ♂ and ♀ to yw
Maintain V5-tagged ao-histone deletion stock

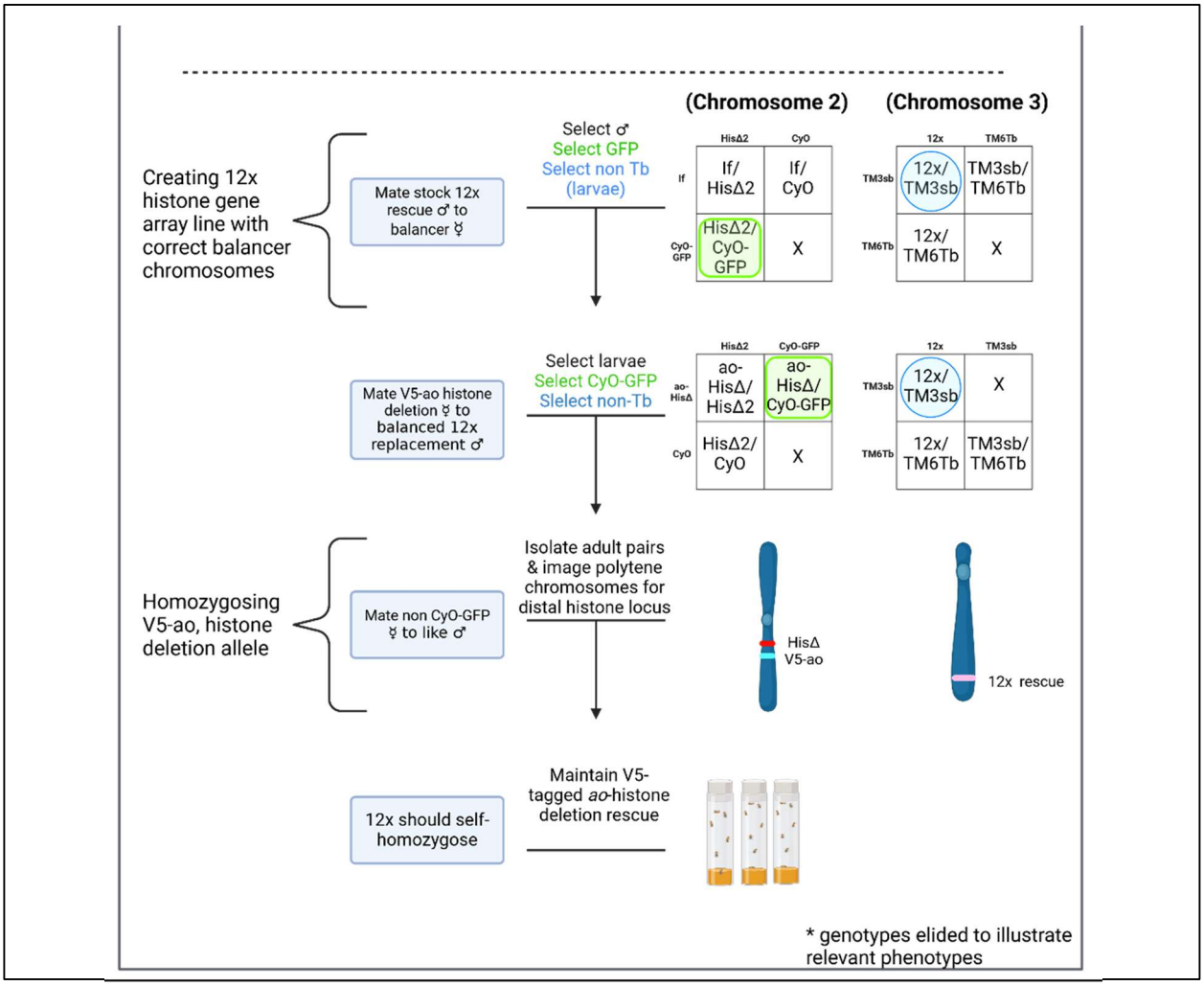



**Figure S3: Idealized histone gene deletion and transgenic rescue array and abridged schematic of recombination portion of fly husbandry.** The phenotypes described are: *CyO* (*Curly of Oster*) which is marked by curled wings; *Sb* (*Stubble*) which is marked by short, thick hairs on the animal's back; Hu (Humeral) which is marked by extra humeral bristles; *If* (*Inflated*) which is marked by rough eyes. These phenotypes were used as markers for the desired balancer chromosome(s) in flies during the cross. DsRed is a fluorescent tag associated with the histone deletion allele and results in glowing red eyes when placed under a green light. Recombination only occurs in females and females retain sperm after mating. Created with BioRender.com.

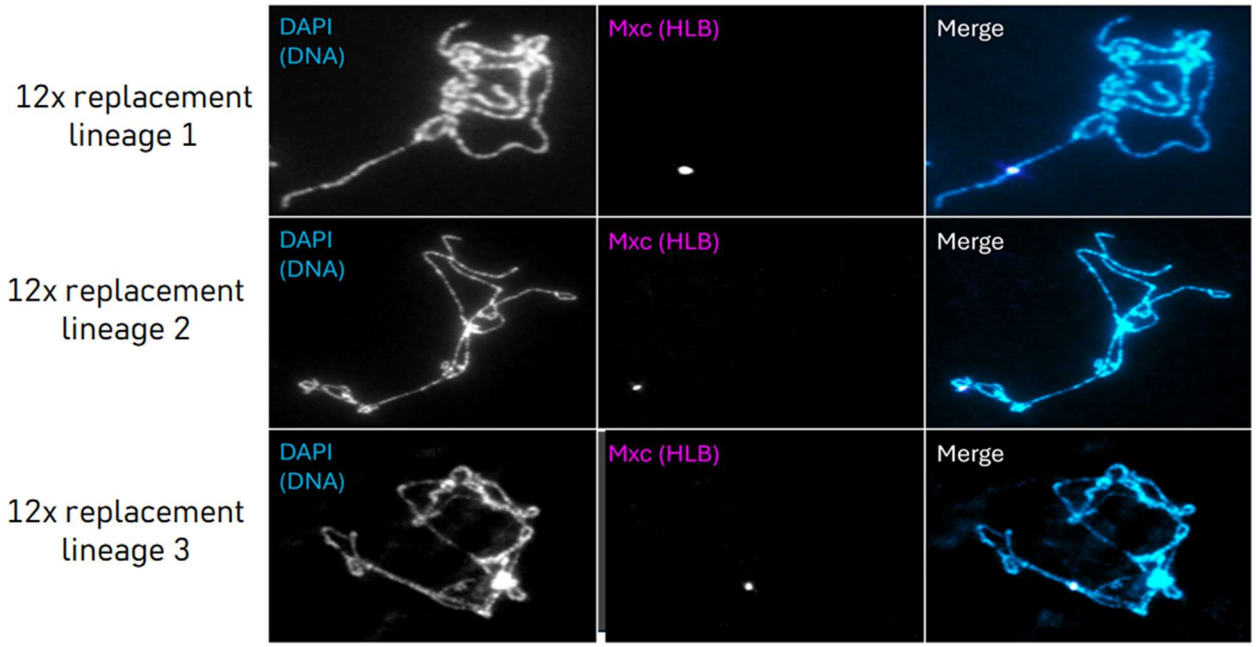



**Figure S4: Confirmation of 12x transgene insertion and endogenous locus deletion.** Mxc (magenta) exhibits a single distal band, indicating success insertion of the 12x histone transgene and the deletion of the endogenous locus. The endogenous locus is found proximal to the chromocenter.

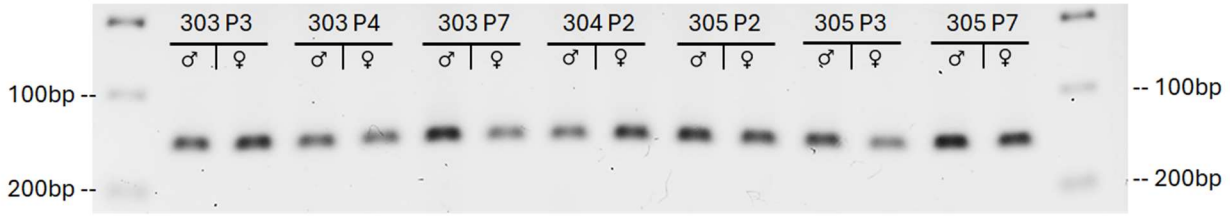
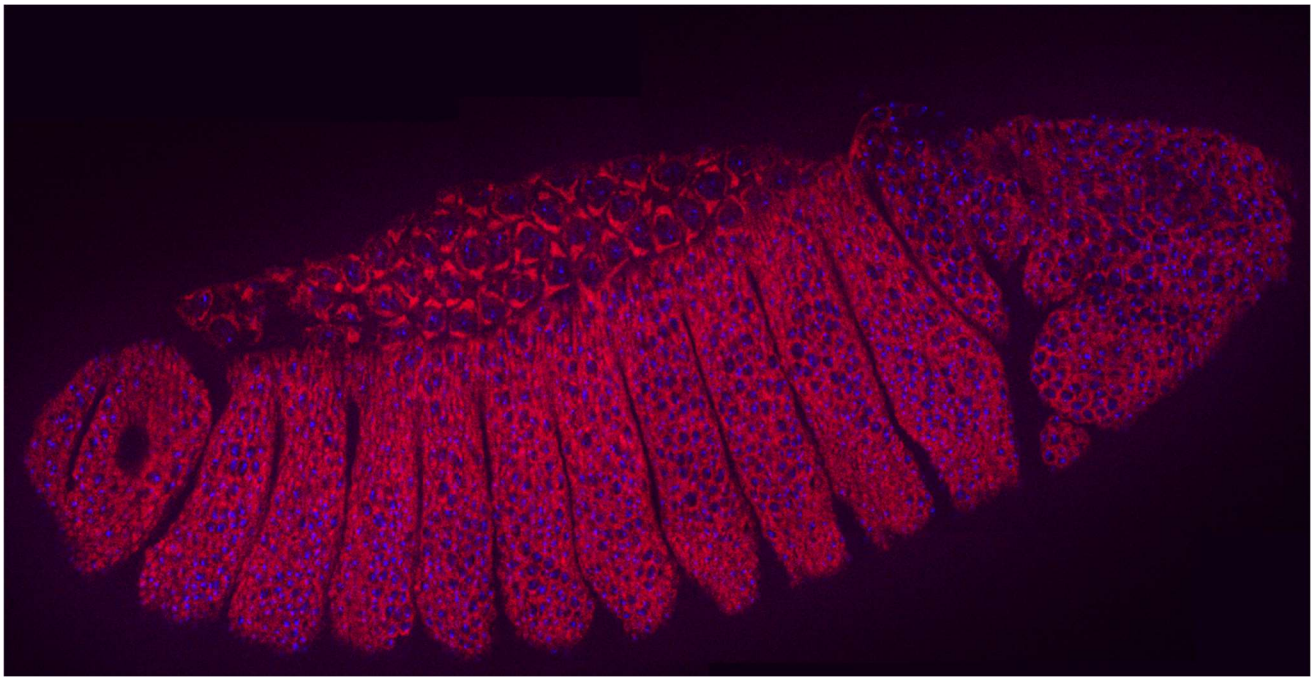
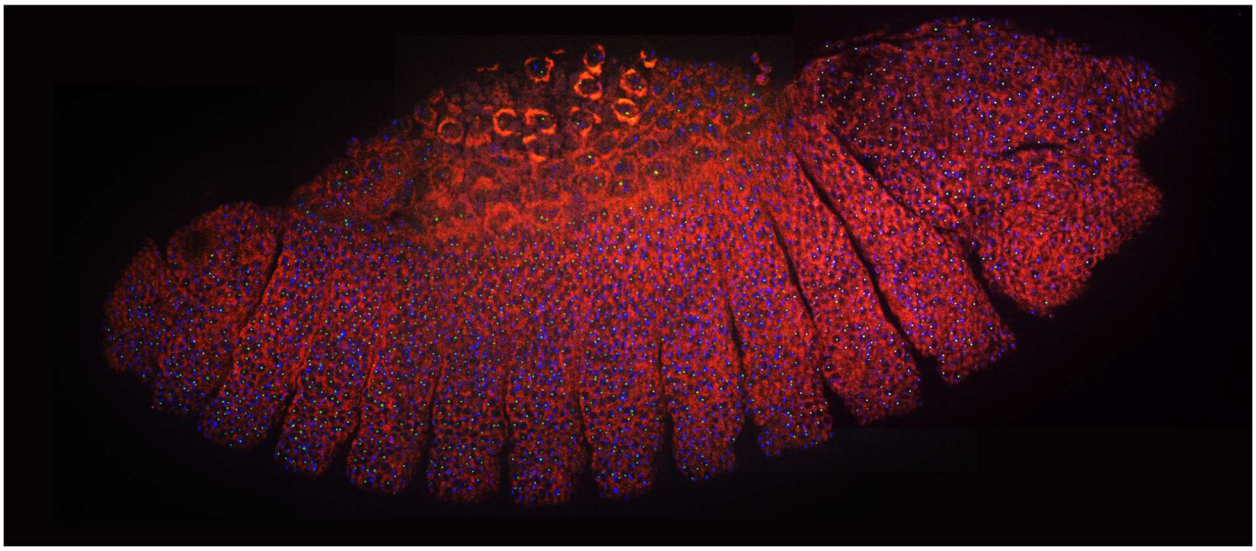



**Figure S5: PCR confirmation of V5 tag in histone rescue animals.** PCR product at 145 bp indicating V5 tag is still present and homozygous in 12x (24x) replacement animals.

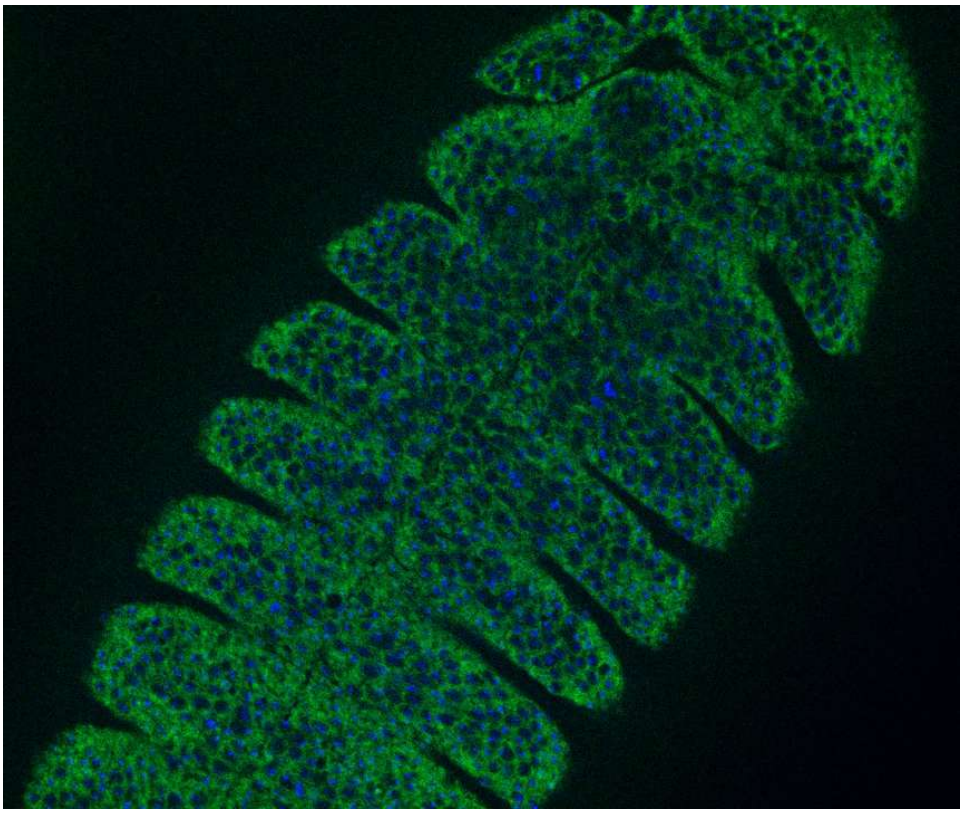
## Appendix - Enjoyable Extraneous Microscopy:



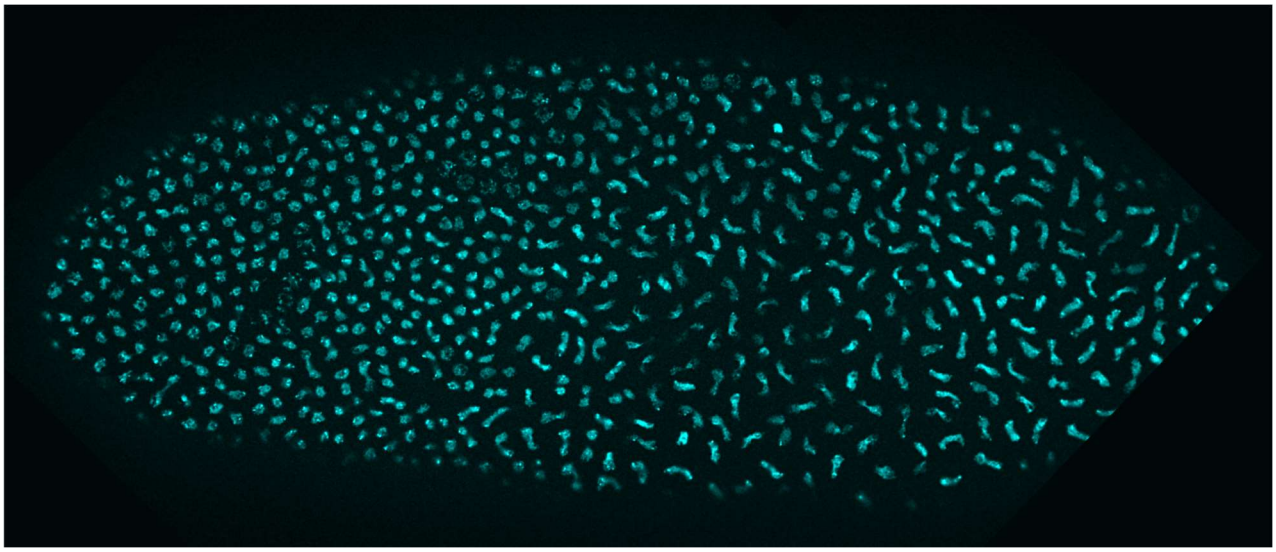
**Extra Image 1:** Dorsal view of a late-stage wild-type embryo. The anterior end faces right. V5 (red) DNA (DAPI; blue).



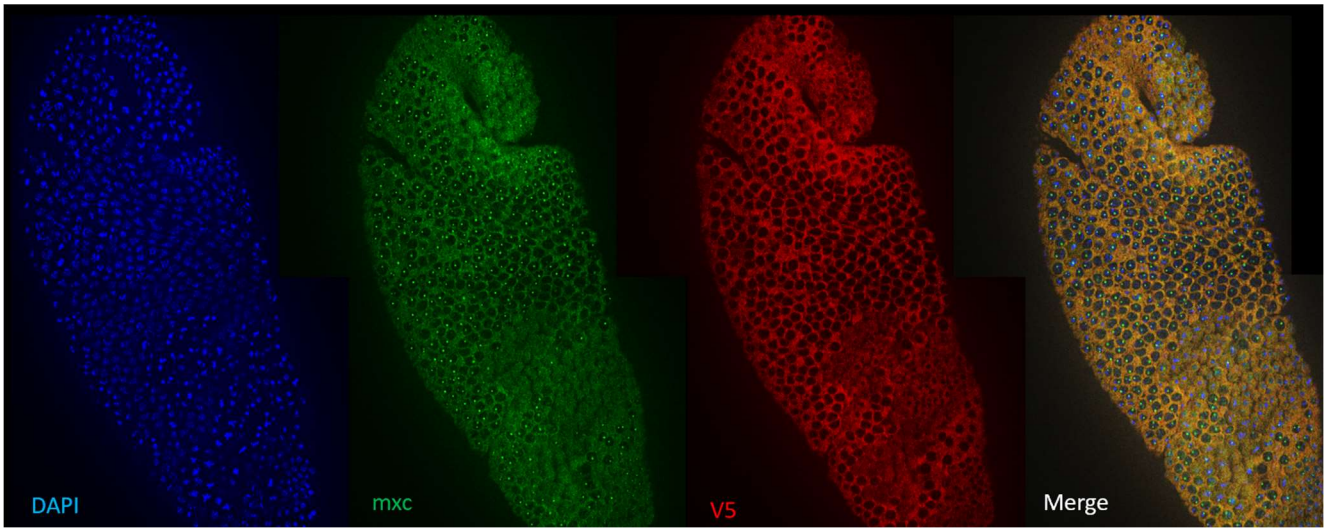
**Extra Image 2:** Dorsal view of even later-stage V5-ao embryo. Ao (V5; red), DNA (DAPI; blue), and histone genes (mxc; green).



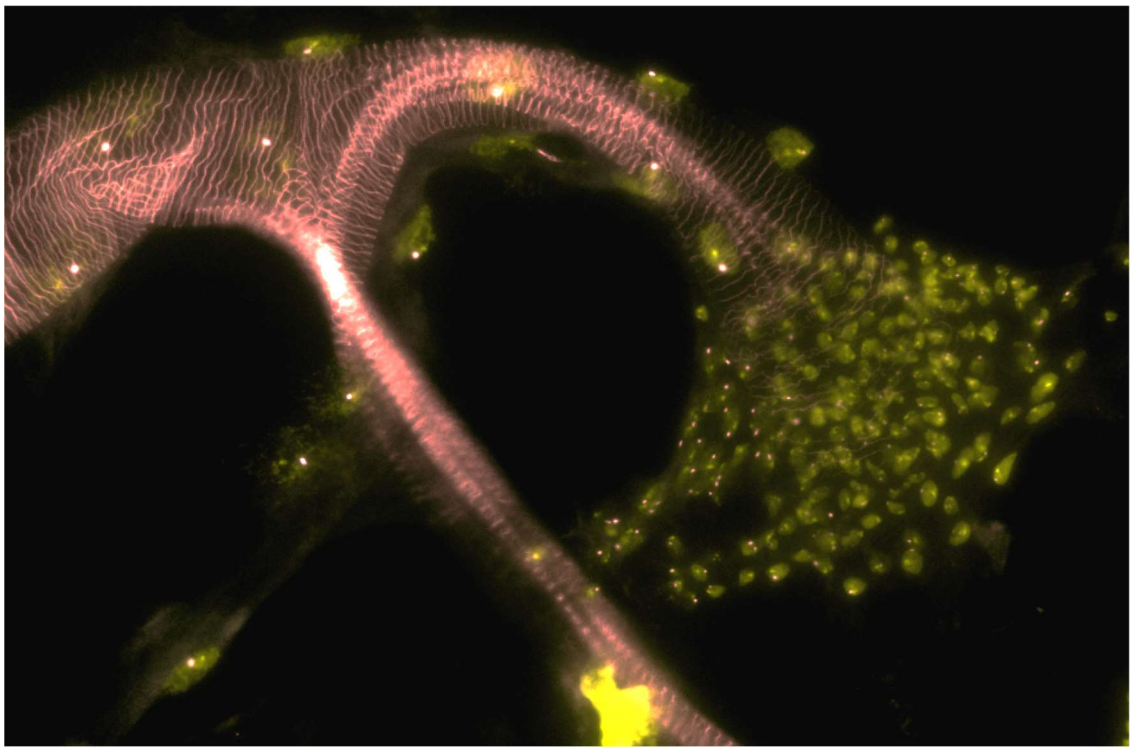
**Extra Image 3:** Ventral view of late-stage V5-ao embryo. Ao (V5; green), DNA (DAPI; blue)



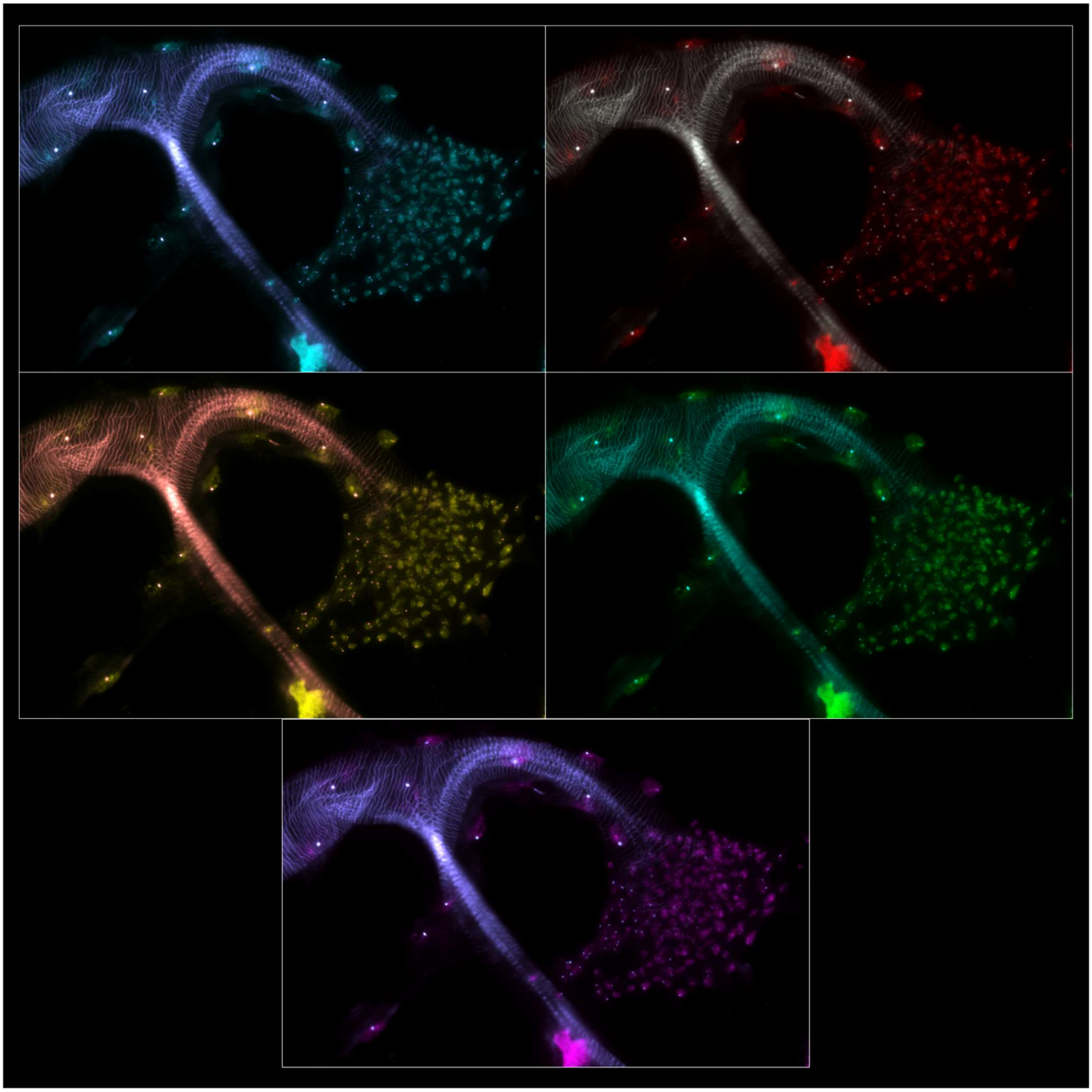
**Extra Image 4:** Syncytial (~2 hour-old) embryo undergoing a wave of mitotic divisions. DNA (DAPI; cyan).




**Extra Image 5:** Dorsal view of gastrulating embryo DNA (DAPI; blue), histone genes (Mxc; green), Ao (V5-antibody; red).



**Extra Image 6:** Suspected structural proteins of salivary gland duct and cells pouring out with histone genes marked. This was an accident where both secondary antibodies bound what appears to be a structural protein outlining where the salivary gland ducts would be. DNA (DAPI; yellow) histone genes (Mxc; red)



**Extra Image 7:** A collage of alternate colorings of extra image 6.