Early dosage compensation of zygotically-expressed genes in *Drosophila melanogaster* is mediated through a post-transcriptional regulatory mechanism

By

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Abstract

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Many key regulators of early embryogenesis in Drosophila melanogaster are X-linked. However, the canonical, MSL-mediated dosage compensation, which involves hyper-transcription of the genes on the single X chromosome in males, is not active until the post-syncytial stage of development. A separate MSL-independent dosage compensation system active earlier in development has been described, though the mechanism through which the process functions remain unclear. In this study, we quantified transcription in living embryos at single-locus resolution to determine if early dosage compensation of the X-linked genes *buttonhead* and *giant* is sensitive to X chromosome dose. We found no evidence for a transcriptionally regulated mechanism of early dosage compensation, suggesting that the previously observed compensation of mRNA levels for these genes is achieved via a post-transcriptional regulatory mechanism.

Dedication

In loving memory of my brother, Samuel D. Blake

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Acknowledgements

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Chapter 1: Introduction

Preface

An organism's genome contains all the DNA that can be transmitted throughout generations and provides the template for building a body and sustaining life. Genes within the genome encode key products such as proteins that imbue cells with specialized functions. Together, cells of varied yet unique roles comprise the complex tissues that build bodies. Much remains unknown about how the DNA code results in the molecular assembly of complex cell and tissue types within a body. Thus, the information gleaned from studies of how the genes are regulated is fundamental to deepening our understanding of life.

Regulating gene expression is necessary for development and homeostasis

Nearly every cell within an organism contains the same genetic material, and yet the functions of the cells within that organism are impressively varied in form and function. The regulation of gene expression, or the precise determination of where, when, and how much of a gene product is available within the body, is key for assigning unique functions to a given cell type.

In general, genes are regulated at two fundamental levels which are: the synthesis of RNA from a DNA template and translation of RNA into protein. Barring the multitude of examples that demonstrate that all DNA does not get made into RNA, and all RNA does not make it into protein, the study of regulation of gene expression involves studying the molecules that mediate either of these two processes. For the purposes of this dissertation, I will focus on how this first step of reading the DNA of a given gene to synthesize RNA is regulated for directing where, when, and how much of that RNA is available for downstream processes within the body.

Precise modulation of which genes get turned on and off during development or in response to environmental stimuli is critical to the maintenance of organismal homeostasis. Regulating transcription, meaning the process through which a gene is read to synthesize RNA, is one key mechanism for modulating the amount of RNA present within a cell. In bilaterians, there are a plethora of examples of a gene's transcription being restricted to specific areas of the body during key developmental timepoints, and failures to properly regulate the gene's expression result in developmental defects (reviewed in Howard and Davidson 2004). Increasing the transcription of key genes is also necessary for responding to an environmental stimulus such as heat shock to mitigate the detrimental effects of prolonged exposure to extreme conditions that are damaging to the cell (reviewed in Pirkkala et al. 2001).

Modulating gene dose has phenotypic consequences

Another way of modulating how much RNA is present within the cell can also be a result of the number of copies of a given gene present within the genome. In the absence of

an additional gene regulatory mechanism, increasing the dose (or number of copies of a gene) within the cell generally results in a higher abundance of the resulting RNA. The effects of dosage imbalance (or a variation between the number of genes present between two organisms) drive phenotypic diversity–for example, the dose of "X Signal Elements" genes present on the X chromosome in *Drosophila* initiates the sex differentiation pathway, resulting organisms of phenotypically distinct sexes (Cline 1988). In mammals, variation in copy number of the *Fcgr3* gene predisposes humans and rats to glomerulonephritis (Aitman et al. 2006).

In many instances, failure to compensate for variations in gene dose is pathogenic or negatively impacts organismal fitness. In humans, at least 300 genes were identified as haplo-insufficient, meaning that one functional copy of a gene is not sufficient to preserve normal function; many of these are implicated in a host of developmental and metabolic disorders (Dang et al. 2008). Copy number variations (CNVs) also alter gene copy number and are implicated in what we call "genomic disorders" among which Charcot–Marie–Tooth Syndrome and Glucocorticoid-remediable aldosteronism are included (reviewed in Stankiewicz and Lupski 2002). Moreover, dosage imbalance of entire chromosomes relative to the rest of the diploid genome can result in developmental syndromes such as Down's syndrome, which is caused by having three copies of chromosome 21.

Dosage compensation is a gene regulatory mechanism that mitigates the dosage imbalance of sex chromosomes

Sex chromosomes present a widespread example of dosage imbalance, with individuals of one sex typically having half the gene dose as the other sex, specifically for sex chromosome genes. Failure to equalize dose of gene products is detrimental to organismal fitness. As a result of this biological conundrum, we observe that the sex chromosome shared between sexes (X in the case of XY system) is extensively modified in one sex, resulting in a widespread regulation of gene expression. The result is a coordinate up- or down-regulation of genes comprising the entire chromosome, resulting in a matched abundance of gene products between the two sexes. This is a process we call dosage compensation.

Although there are diverse modes for achieving dosage compensation across organisms, understanding the shared strategies for mitigating dosage imbalance informs our broader understanding of coordinate gene regulation. Studies of canonical dosage compensation pathways of three model organisms (*Mus musculus, Caenorhabditis elegans,* and *Drosophila melanogaster*) demonstrate gene regulation through directly regulating transcription of X-linked loci (Payer and Lee 2008, Cline, and Meyer 1996). From these studies and others, we know the necessary events for dosage compensation to occur. First, a molecular sensor must detect how many X chromosomes are present within the cell to then mediate a downstream targeting mechanism for the modification of the chromosome(s) of a particular sex. These modifications, which are orchestrated by a complex molecular ensemble, give rise to the

widespread regulation that corrects the dosage imbalance of the sex chromosomes between the two sexes.

Drosophila melanogaster **as a model system for studying dosage compensation**

The first observation of dosage compensation took place in *Drosophila melanogaster*. Phenotypes for partial loss-of-function alleles that encode eye pigment were being studied at varying doses, where the pigments of the eye became darker when the gene was present at higher doses. However, the pigment of eyes was identical between males and females with one eye pigment allele present on each X chromosome (Muller 1932, 1948). Therefore, there must be some mechanism equalizing the abundance of that gene product controlling eye pigment between organisms with one and two copies of the X chromosome.

This discovery sparked the genesis of a field that utilized the unique attributes of *Drosophila* to bring key discoveries to light. For example, the unique cytological properties of polytene chromosomes, which have thousands of DNA strands lined up in register, provided the first evidence that transcription was differentially regulated between sexes (Dobzhansky 1957, Mukherjee and Beermann 1965). Ingenious forward genetic screens and subsequent gene trapping experiments allowed for the identification of protein and non-coding RNA components of the dosage compensation complex (Tanaka et al. 1976, Belote and Lucchesi 1980a, Uchida et al., 1981, Hilfiker et al. 1997, Han et al. 1996, Meller and Rattner 2002). Together, the discoveries gleaned from studies of dosage compensation in this model organism in the 20th century and the turn of the millennium elucidated that equalizing of gene products in *Drosophila melanogaster* is the result of a widespread increase in the transcription of loci on the male X chromosome.

The identification and mechanism of action of key molecules that mediate canonical dosage compensation highlight critical advances in the field of gene regulation

Males absent On the First (MOF): a histone acetyltransferase that directly modifies the locus to regulate transcription

To understand how transcription is regulated, let us take a step back and consider how the entire genome's worth of DNA is stored within the nucleus of a cell. The DNA within the nucleus is highly ordered and in complex with histone proteins that comprise nucleosomes, which serve as the basic structural unit of packaged DNA. Covalent modifications to the N-terminal tails of the histone proteins within the nucleosome can be modified by gene regulatory machinery, resulting in changes to gene expression. Of the post-translational modifications made to specific histone tails, acetylation of key residues is correlated with active transcription and can result in an increase in gene expression (Zhang et al. 2021).

The discovery of a unique pattern of H4K16 acetylation (meaning, an Acetyl residue conjugated to lysine 16 on histone H4 within the nucleosome) provided the first clue in the identity of the molecule responsible for directly modulating the expression of genes on the male X chromosome. At this point, investigators knew that canonical dosage compensation was achieved through a difference in transcription, but it was unclear if it was through increasing transcription in males or decreasing transcription in females. In addition, though many of the components of the dosage compensation complex had been identified, the molecular link that explained how the assembled complex on the X chromosome influenced gene expression. It wasn't until the generation of antisera against specific acetylated lysine peptides (Turner and Fellows 1989) uncovered that the entire male X chromosome was uniquely modified with the H4K16 acetylation modification (Turner et al. 1992).

Discovery of a male sex lethal protein linked the assembly of the dosage compensation complex to the acetylation of H4K16, explaining the increased transcription from genes on male X. At this point in time, the genetic screens for proteins involved in dosage compensation had been performed on autosomes, excluding the X chromosome from these studies due to the complexity of the genetics involved in uncovering mutants. It wasn't until the X chromosome was also screened for alleles that caused male lethality that males absent on first (MOF), the acetyltransferase that modulates gene expression necessary for dosage compensation, was discovered (Hilfiker et al. 1997). Homozygous male larvae for the loss-of-function *mof* allele were not only dead (or dying), but also showed a loss of H4K16 acetylation on polytene chromosomes. Further characterization of MOF conclusively demonstrated that it was the enzyme responsible for H4K16 acetylation and the increase in transcription of genes on the male X chromosome (Smith et al. 2000, Smith et al. 2001)

MSL-2 and Sex lethal: molecules that confer the sex-specificity of the dosage compensation pathway

MSL-2 is the only member of the canonical dosage compensation complex that is not present in the maternal component and whose expression onset occurs after the maternal-to-zygotic transition stage of embryogenesis (Tomancak et al. 2002). This critical subunit is present only in males (Kelley et al. 1995, Zhou et al. 1995) and targets the rest of the MSL proteins to the X chromosome by directly binding DNA (Zheng et al. 2014), therefore bringing the MOF acetyltransferase to its H4K16 substrate (Smith et al. 2000). The observation that MSL-2 is differently regulated between males and females suggests that another, upstream molecule regulates its expression and may therefore serve as the molecular switch for sex-specific activation of the dosage compensation pathway.

The first clues as to the identity of the molecule that regulates the dosage compensation pathway came from the genetic studies of Sex lethal (Sxl), the "master-feminizing switch" that controls all aspects of the sex differentiation pathway in *Drosophila melanogaster*. Alleles of Sxl were postulated to disrupt the dosage compensation pathway (Lucchesi and Skripsky 1981, Cline 1979), where a dominant male-specific

lethal allele was ascribed to inability to activate the dosage compensation pathway, and a recessive female-specific lethal allele was due to its over-activation. The logic here stipulates that if Sxl negatively regulates a molecule involved in dosage compensation, its inappropriate expression in males inhibits the dosage compensation pathway, resulting in male-specific lethality. Autoradiography and a clever genetic assay later provided support for this hypothesis (Bernstein and Cline, 1994), linking Sxl to the dosage compensation pathway in addition to its role in sex differentiation.

Considering this discovery, investigators were on the hunt for the mechanism through which Sex-lethal controlled the sex-specific assembly of the dosage compensation complex on the male X chromosome. Sxl is an RNA-binding protein that binds poly-U sequences (Cline and Meyer, 1996), so the discovery of predicted Sxl binding sites on the 5' and 3' untranslated regions (UTRs) of MSL-2 RNA was of intrigue. When flies expressing a transgene encoding MSL-2 lacking its 5' and 3' UTRs failed to block dosage compensation in females (Kelley et al. 1997), it was clear that sex-specific regulation of the dosage compensation pathway was through direct regulation of MSL-2 by Sxl, blocking dosage compensation in females.

Given the established relationship of Sxl upstream of the MSL-complex in mediating dosage compensation, one can genetically test if the MSL complex alone controls all dosage compensation. If the MSL proteins are sufficient in mediating dosage compensation, then one would expect the loss-of-function *msl* alleles to suppress the loss of function allele of Sxl^- that gives rise to a female-specific lethality. The discovery that *msl* alleles do not rescue Sxl^- females (Skripsky & Lucchesi 1982, Uenoyama et al., 1982) made clear the possibility that molecules independent of the MSL complex may also mediate dosage compensation. Therefore, the studies on Sxl in relation to the MSL complex not only elucidated the mechanism of sex-specificity for MSL-mediated dosage compensation, but also brought forth the strong possibility that MSLs are unlikely to be the only molecules mediating dosage compensation in *Drosophila melanogaster*.

Our current understanding of Sex lethal's role in MSL-mediated dosage compensation is as follows: Sex lethal acts as the molecular sensor that senses organismal sex and differentially regulates the activation of the dosage compensation pathway in *Drosophila melanogaster*. X signal elements (XSEs) are X-linked genes that are transcribed early during embryogenesis whose abundance differentially regulates the expression of Sex lethal (Cline 1988). During female embryogenesis, the abundance of the XSEs results in the presence of Sxl protein, while Sxl is absent from males during this developmental stage. Therefore, at the onset transcription of *msl-2* at the blastoderm stage (in both sexes), the presence of Sxl in females blocks the translation of the *msl-2* mRNA (Gebauer et al. 1999, Gebauer et al. 2003), while the absence of Sxl males allows for the translation of the MSL-2 protein and the initiation of the canonical dosage compensation pathway.

Observations of MSL2-independent dosage compensation

There is one gene, *runt*, described as being dosage compensated through an MSL-2-independent pathway at the blastoderm stage of embryonic development. This gene that positively regulates Sxl expression in females during early embryogenesis (Kramer et al. 1999, Torres et al. 2009) and its dosage compensation is also dependent on Sxl and not the MSL-complex (Gergen 1987, Bernstein and Cline 1994). It is postulated that Sxl regulates *runt* abundance post-transcriptionally through binding of the 3'UTR (Kelley et al. 1995), though this hypothesis has yet to be directly tested.

Building on this single-gene observation of MSL-2-independent dosage compensation and the inability of loss-of-function *msl* alleles to rescue *Sxl*$^{-}$ female lethality, a larger question remains of whether any genes on the X chromosome prior to MSL-2 expression during early embryogenesis exhibit dosage compensation. To address this question, RNA was extracted from single embryos and subjected to mRNA sequencing to discern the abundance of X-linked transcripts of X-linked genes expressed prior to the blastoderm stage of development. Many of these genes were indeed dosage compensated, showing an equal abundance of mRNA between male and female embryos (Lott et al. 2011). These findings set the stage for further investigation on how early dosage compensation of more than one gene is achieved independently of MSL-2 (Figure 1).

My investigations of early, MSL-2-independent, dosage compensation:

I found the discovery of an early form of dosage compensation independent of MSL-2 to be intriguing and set out to understand the mechanism through which the equal mRNA abundance of key X-linked developmental regulators is achieved during early development. I begin by testing whether early dosage compensation is a transcriptionally regulated process in Chapter 2 and follow up those studies in Chapter 3 with my perspective on how future experiments might elucidate the molecules involved in early embryonic dosage compensation.

Finally, I want to briefly acknowledge that studies on the mechanisms of gene regulation in *Drosophila melanogaster* remain at the forefront of technology development in molecular biology. This is a model organism whose foundation in forward genetic screens, cytological studies, and early genomic assays have yielded a wealth of information that are further strengthened by modern-day approaches for studying gene expression. Notably, the imaging assays employed in this dissertation for studying *in vivo* transcriptional output in real time represent a significant step forward in addressing old, yet fundamental, biological questions with new technology.

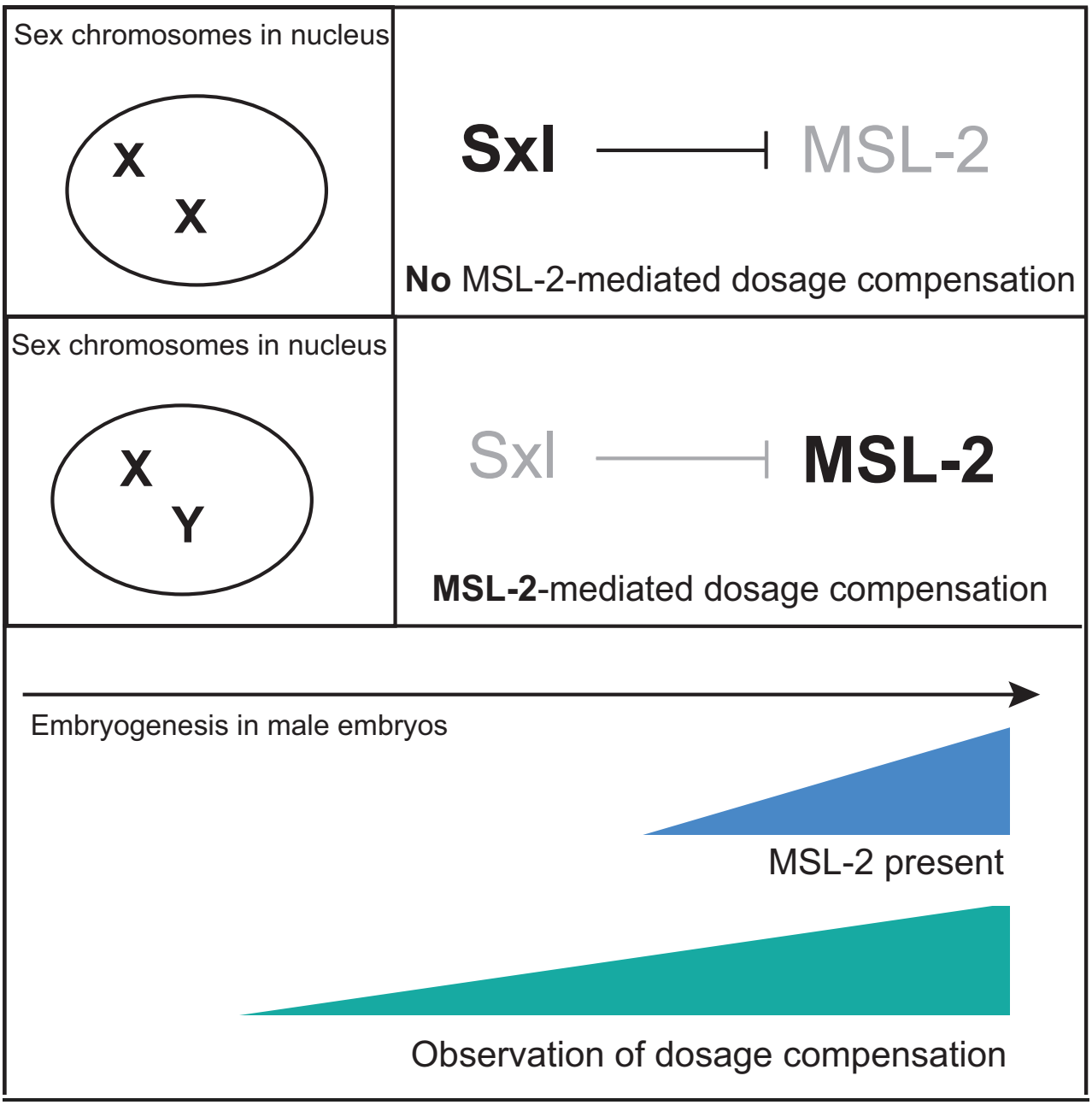


Figure 1: MSL-2 is regulated by Sxl and mediates canonical dosage compensation, but dosage compensation onset begins before MSL-2 is present in the embryo.

Chapter 2: Early Zygotic Dosage Compensation is mediated through a post-transcriptional regulatory mechanism in *Drosophila melanogaster*

Introduction

In multicellular diploid organisms with chromosomal sex determination, the functional degeneration of sex chromosome specific to the heterogametic sex (the Y in XY systems and W in ZW systems) creates a dosage imbalance for sex-chromosome encoded genes. To mitigate the detrimental impact that this dosage imbalance could have on fitness, a variety of mechanisms have evolved to equalize the abundance of gene products found on only one sex chromosome (typically the X or Z).

In the vinegar fly Drosophila melanogaster, which uses an XX/XY sex-determination system, dosage compensation is achieved by upregulating transcription of genes on the male X chromosome (reviewed in Kuroda et al. 2016). Mutations that affect this process are lethal in males (Belote and Lucchesi 1980, Lucchesi and Skripsky 1981, Hilfiker et al. 1997, Meller and Ratner 2002).

All but one of the Male Sex Lethal (MSL) proteins that mediate this process are present in the maternally deposited protein component. Male specificity is achieved through the activity of the male-specific MSL-2, which directs assembly of the dosage compensation complex to the X chromosome, resulting in acetylation of H4K16 by MOF (Hilfiker et al. 1997, Smith et al. 2000) and a subsequent doubling of transcription from X-linked genes (Smith et al. 2001, Gelbart et al. 2009).

However, an early, MSL-2 independent dosage compensation system acting on *runt* was discovered via genetic experiments (Gergen 1987, Bernstein and Cline 1994) and shown to apply more broadly by direct measurement of mRNA levels in early male and female embryos (Lott et al. 2011). It was hypothesized, based on the enrichment of putative binding sites for the master sex regulator Sxl in the 5' and 3' untranslated regions of X encoded genes, that this system was based on post-transcriptional regulation of mRNA levels (Bernstein & Cline 1994, Lott et al. 2011).

Here we use quantitative live-embryo imaging (Garcia et al. 2013, Bothma et al. 2014) to compare rates of transcription at individual X-linked loci across early development in male and female embryos. Briefly, transcription of a gene tagged with a reporter sequence (MS2 or PP7) forms stem loops that, when bound by a coat protein (MCP or PCP) conjugated to GFP, gives rise to a fluorescent signal. This allows for simultaneous direct quantification via microscopy of transcription rates at individual loci across many nuclei. The ability to quantify transcription rates of candidate genes in embryos of both sexes makes this system uniquely amenable for testing if early dosage compensation is due to increasing the rate of transcription of genes on the male X chromosome.

We focus on four X-linked genes: *giant* (*gt*) and *buttonhead* (*btd*) that had previously been shown to be dosage compensated in the early embryo, and *embryonic lethal*

abnormal vision (*elav*) and *bangles and beads* (*bnb*) whose early mRNA levels are dosage sensitive. We found that the early dosage compensation observed for these genes was not due to a doubling of transcription in males, suggesting that this process is likely regulated post-transcriptionally. In addition, we discovered an unreported doubling of the transcription rate in females for *elav*, a gene that is not dosage compensated and has no defined role in sex differentiation.

Results

Previous work identified a wide-spread dosage compensation of zygotic transcripts prior to cellularization, where 36 out of 85 X-linked genes showed less than a 1.5-fold excess of transcript abundance in female embryos relative to their male counterparts (Lott *et al.,* 2011). Many of these early-compensated genes are key developmental regulators, where differences in transcript abundance could pose profound consequences to organismal development. Because these early-compensated genes are regulated before MSL-2, the molecule that mediates canonical dosage compensation, is expressed, we sought to investigate if the early-compensation of these developmental regulators occurred through a transcriptional mechanism independent of MSL-2.

We chose two early-compensated genes (*gt* and *btd*) for further study that are essential for mediating embryonic development and whose mRNA abundance is matched in male and female embryos during the early nuclear cycles. Because not all zygotically-transcribed genes emanating from X are early-compensated, we chose two additional genes (*elav* and *bnb*) with twice as much female mRNA abundant relative to males as a point of comparison in our study. In designing this study, we made sure that all genes reached maximum transcript abundance during the first half of nuclear cycle 14 (Lott *et al.,* 2011) to mitigate any differences due to developmental stages across datasets.

We used the CRISPR-Cas9 gene editing system (Gratz *et al.,* 2015) to insert an array of either MS2 or PP7 stem loop sequences immediately upstream of the translation start codon of the endogenous gene of interest. The rationale behind choosing this insertion site was to position at the stem loops sequences at beginning of the resulting transcript, ensuring a robust reporter signal of when transcription is occurring. The MS2 or PP7 sequences were flanked by the donor and acceptor splice sites from the *hunchback* intron so that the stem loops would be removed from the mature mRNA to prevent problems with RNA localization (Heinrich *et al.,* 2017) or the translation of the endogenous protein.

We homozygosed the lines containing MS2 and PP7-tagged genes and crossed in a transgene encoding an MCP-GFP (or PCP-GFP) fusion protein that binds transcribed stem loop sequences. The binding of fluorescently conjugated coat protein to these stem loops (Bernardi *et al.,*1972) gives rise to a fluorescent puncta (what we will refer to as “spots” moving forward) in the nucleus (Figure 2B; Garcia *et al.,* 2013) whose fluorescence intensity correlates with the abundance of RNA present at the locus (Garcia et al. 2013). This system offers a spatio-temporal and quantitative characterization of transcript abundance for a gene of interest (Movies 1 & 2).

Embryos with successfully incorporated MS2-MCP (or PCP-PP7) reporter systems showed no signs of impaired organismal health and faithfully recapitulated the tagged gene's expression patterns previously reported by FISH (Figure 2B; Wimmer *et al.,* 1996, Tomancak et al. 2002). A key feature of this system that lends itself for studying dosage compensation is the ease in sexing embryos–the fluorescent spots in nuclei serve as a readout for the number of X chromosomes present. Therefore, embryos with two fluorescent spots within the same nucleus are female, while embryos with one one spot are male (Figure 2C & 2D).

We used laser-scanning confocal microscopy to acquire MCP-GFP (or PCP-GFP) movies of embryos during nc14, the last nuclear cycle before gastrulation. To ensure consistency across fields of view across these high magnification movies, we optimized data collection for patterned genes to capture all nuclei along the AP axis within the same single expression domain (Movies 1 & 2, Supplemental Figure 1). If a gene had a more ubiquitous expression domain within the anterior domain of the embryo, we positioned the anterior pole relative to the field of view consistently across all data collections. To obtain high temporal resolution and optimize signal-to-noise with minimal bleaching, we collected movies so that the acquisition time for each timepoint (or Z-stack) corresponded to intervals of 19.5s. In total, we collected 51 movies (Movies 3-51) with a minimum of three movies of each sex per genotype (see Table 1).

Embryo ID	Gene	Sex	nuclear cycle	Duration (in frames)	Total time	Movie #
M_btd_1	PP7-btd	M	14	159	51.675	Movie 1
F_btd_1	PP7-btd	F	14	172	55.9	Movie 2
M_btd_2	PP7-btd	M	14	142	46.15	Movie 3
M_btd_3	PP7-btd	M	14	162	52.65	Movie 4
M_btd_4	PP7-btd	M	14	125	40.625	Movie 5
M_btd_5	PP7-btd	M	14	141	45.825	Movie 6
M_btd_6	PP7-btd	M	14	183	59.475	Movie 7
M_btd_7	PP7-btd	M	14	99	32.175	Movie 8
M_btd_8	PP7-btd	M	14	110	35.75	Movie 9
M_btd_9	PP7-btd	M	14	94	30.55	Movie 10
F_btd_2	PP7-btd	F	14	122	39.65	Movie 11
F_btd_3	PP7-btd	F	14	194	63.05	Movie 12
F_btd_4	PP7-btd	F	14	208	67.6	Movie 13
F_btd_5	PP7-btd	F	14	123	39.975	Movie 14
F_btd_6	PP7-btd	F	14	176	57.2	Movie 15
F_btd_7	PP7-btd	F	14	166	53.95	Movie 16
F_btd_8	PP7-btd	F	14	173	56.225	Movie 17
F_btd_9	PP7-btd	F	14	119	38.675	Movie 18
M_gt_1	MS2-gt	M	14	119	38.675	Movie 19
M_gt_2	MS2-gt	M	14	148	48.1	Movie 20
M_gt_3	MS2-gt	M	14	125	40.625	Movie 21
M_gt_4	MS2-gt	M	14	114	37.05	Movie 22
F_gt_1	MS2-gt	F	14	111	36.075	Movie 23
F_gt_2	MS2-gt	F	14	143	46.475	Movie 24
F_gt_3	MS2-gt	F	14	147	47.775	Movie 25
F_gt_4	MS2-gt	F	14	139	45.175	Movie 26
M_bnb_1	PP7-bnb	M	14	102	33.15	Movie 27
M_bnb_2	PP7-bnb	M	14	122	39.65	Movie 28
M_bnb_3	PP7-bnb	M	14	122	39.65	Movie 29
F_bnb_1	PP7-bnb	F	14	160	52	Movie 30
F_bnb_2	PP7-bnb	F	14	146	47.45	Movie 31
F_bnb_3	PP7-bnb	F	14	157	51.025	Movie 32
M_elav_1	MS2-elav	M	14	133	43.225	Movie 33
M_elav_2	MS2-elav	M	14	148	48.1	Movie 34
M_elav_3	MS2-elav	M	14	128	41.6	Movie 35
F_elav_1	MS2-elav	F	14	139	45.175	Movie 36
F_elav_2	MS2-elav	F	14	159	51.675	Movie 37
F_elav_3	MS2-elav	F	14	141	45.825	Movie 38
M13_btd_1	PP7-btd	M	13	33	10.725	Movie 39
M13_btd_2	PP7-btd	M	13	31	10.075	Movie 40
M13_btd_3	PP7-btd	M	13	25	8.125	Movie 41
F13_btd_1	PP7-btd	F	13	26	8.45	Movie 42
F13_btd_2	PP7-btd	F	13	23	7.475	Movie 43
F13_btd_3	PP7-btd	F	13	23	7.475	Movie 44
M13_bnb_1	PP7-bnb	M	13	38	12.35	Movie 45
M13_bnb_2	PP7-bnb	M	13	25	8.125	Movie 46
M13_bnb_3	PP7-bnb	M	13	29	9.425	Movie 47
F13_bnb_1	PP7-bnb	F	13	50	16.25	Movie 48
F13_bnb_2	PP7-bnb	F	13	44	14.3	Movie 49
F13_bnb_3	PP7-bnb	F	13	32	10.4	Movie 50

Table 1: MS2 or PP7 live-imaging data collected in this study

We used a custom image analysis pipeline (Garcia et al. 2013, Lammers et al. 2020) to identify nuclei and extract fluorescent intensity measurements for actively transcribing X-linked loci in embryos of both sexes. A representative example of the resulting data is shown in Figure 3.

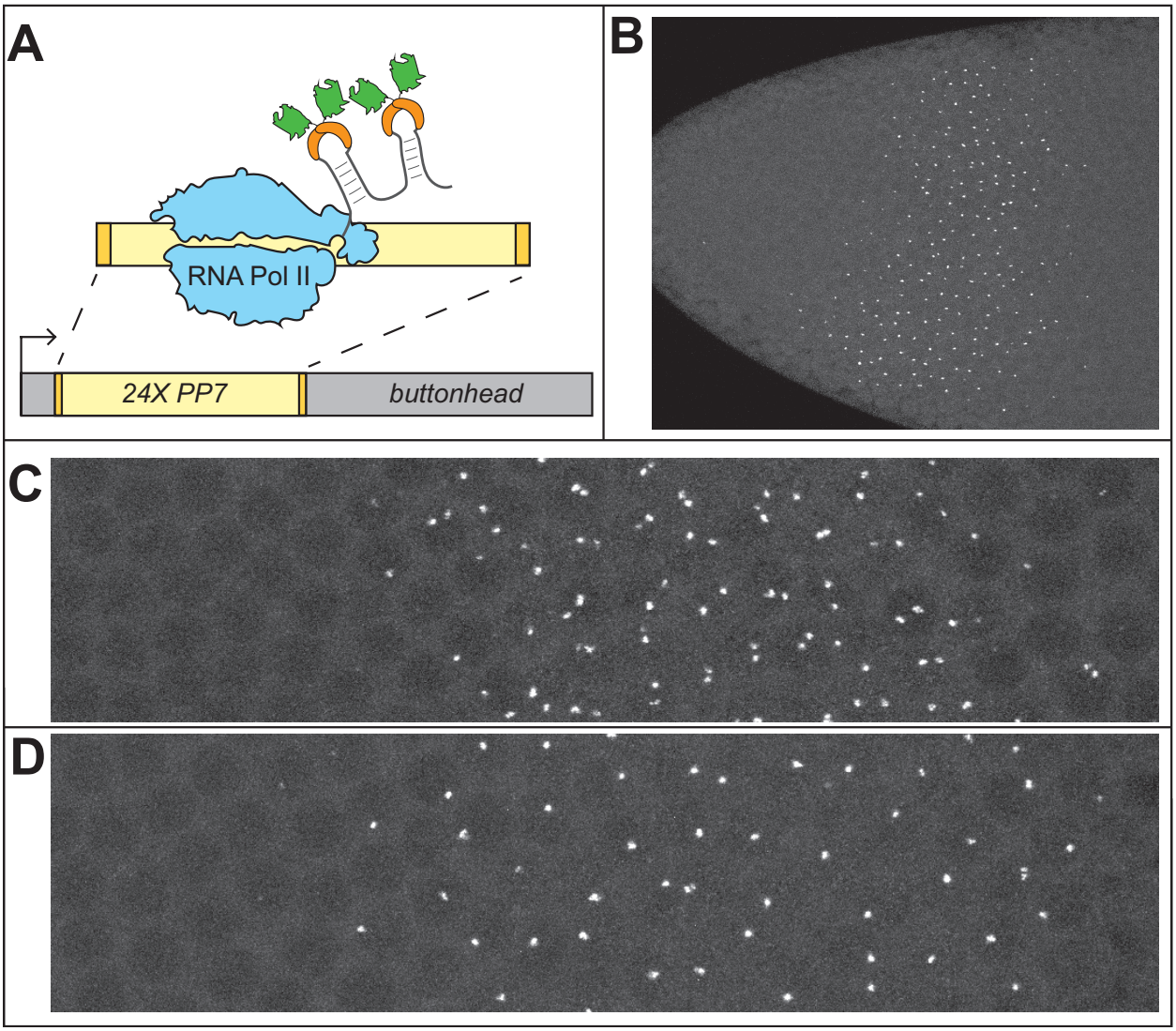


Figure 2: MS2-MCP and PP7-PCP reporter systems measure transcription rate of X-linked genes in the early *Drosophila melanogaster* embryo A) An example schematic of how the PP7-PCP reporter system works: 24 repeats of the PP7 stem loop sequence (yellow) are flanked by splice donor and accepter sites from the intron of the *hb* gene (gold) were inserted upstream of the target gene's start codon (ATG), represented here by the gene "*buttonhead*". When transcribed, the PP7 form RNA step loops that are subsequently bound by PP7 Coat Protein (orange) conjugated to GFP (green). B) The anterior portion of an embryo expression *PP7-buttonhead* (*PP7-btd*). C) Zoomed-in view of the *PP7-btd* expression domain in a female embryo, made evident by the two transcribing loci in nuclei as opposed to D) the same field of view for *PP7-btd* expression in a male embryo, where there is only one transcribing locus per nucleus.

No hyper-transcription of male X in dosage compensated genes

We compared the average fluorescence intensity (which we expect to be proportional to polymerase density on transcribing loci) of detected transcription foci from male and female X chromosomes across time during mitotic cycle 14 for both *btd* and *gt* (Figure 3A). For *btd* they are nearly identical, and for *gt*, the differences balance out over time (with slightly higher transcription from male X's early in cycle 14 and a slightly higher transcription from female X's later in cycle 14) (Supplemental Figure 1A&B). As it is possible to increase transcriptional output even with identical polymerase density on transcribing loci by increasing the fraction of loci actively transcribing, we also looked at the number of transcribing loci as a function of time in male and female embryos (Figure 3B), and, as expected, observed them to be in approximately a 2:1 ratio between females and males. These results agree with previous observations of mRNA abundance of nuclear *gt* transcripts (Zoller *et al.,* 2018) are inconsistent with early dosage compensation of *btd* and *gt* being achieved via hyper-transcription of the male X.

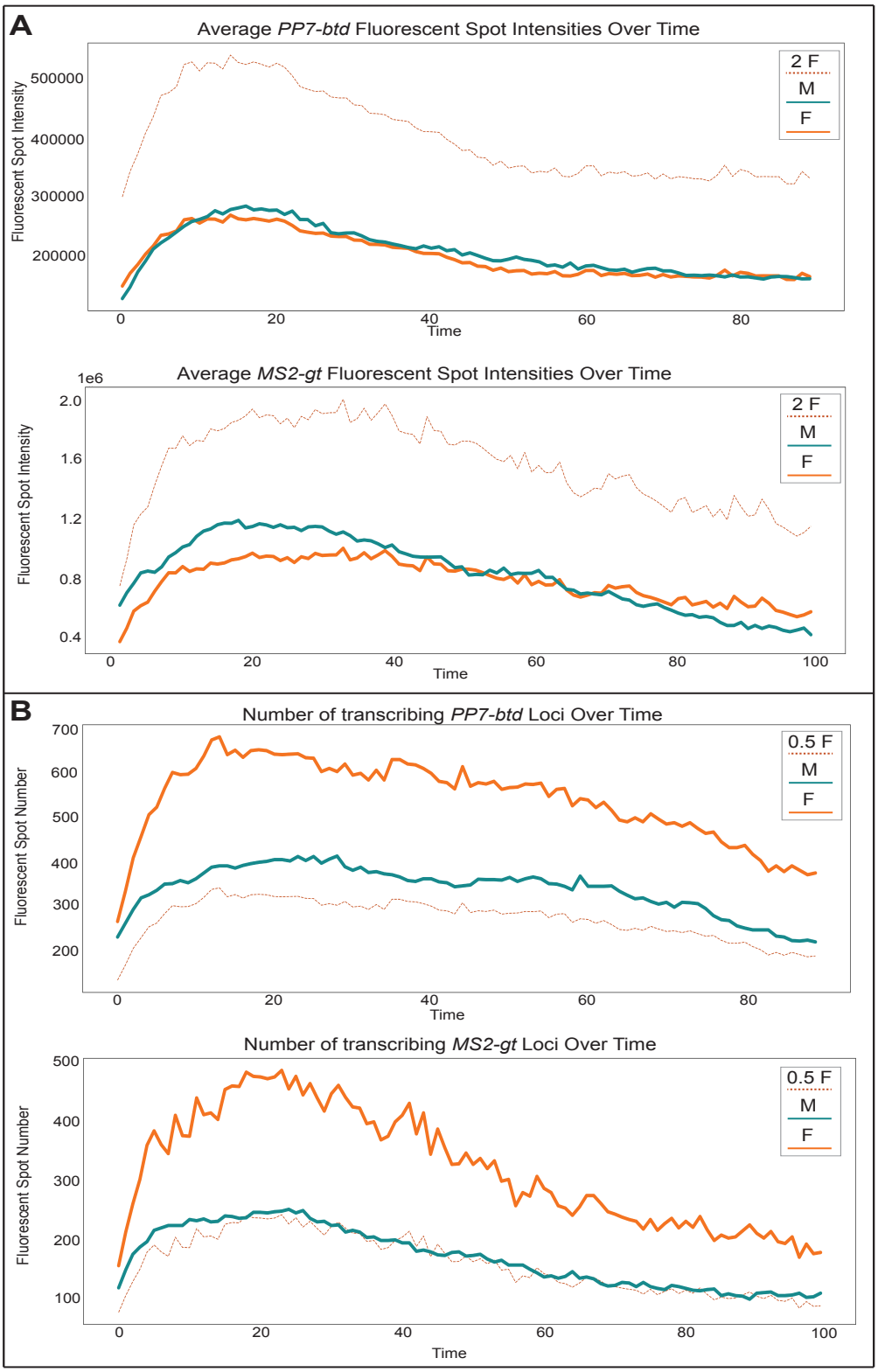


Figure 3: Transcription rate of the early dosage compensated *PP7-btd* and *MS2-gt* genes is the same in male and female embryos

A) Average distributions of fluorescent spot intensity over time (in frames, where “1” is the first instance of transcription of the locus during nuclear cycle 14) for all female

(orange) and male (dark cyan) *PP7-btd* (N=9 embryos of each sex) and *MS2-gt* (N=4 of each sex) embryos. The dashed, dark orange line represents double the average female fluorescent spot intensity for every given timepoint. B) The distribution of fluorescent spot intensities plotted over time for the dosage compensated *MS2-gt* (N=4 embryos of each sex) with total spot intensity histograms on the right. B) The number of transcribing *PP7-btd* and *MS2-gt* loci throughout nuclear cycle 14. The dashed orange line represents half of the transcribing female loci.

We made similar comparisons between average fluorescence and number of transcribing loci in the two non-compensated loci *bnb* and *elav*. These show more variation between sexes than two dosage compensated genes, with *bnb* generally higher in males, and *elav* higher in females (Figure 4, Supplemental Figure 1C&D).

Given that each gene is its own transcriptional entity, we do not expect for the parameters regulating transcription rate to be identical between sexes if early dosage compensation is indeed mediated post-transcriptionally. The nuclear compositions are different between male and female embryos, and subtle variations in transcription rate of any gene is between sexes may reflect the differences in these differing environments. This is indeed what we see for *MS2-gt* and *PP7-bnb,* with a dramatic difference in transcription rate shown for *MS2-elav.* The fact that we see subtle variations in transcript abundance between sexes in both *MS2-gt* and *PP7-bnb,* one early dosage compensated gene and one that is not, argues the variations in transcriptional abundance are independent of any dosage compensation mechanisms taking place. Moreover, these data that show that the slight differences in polymerase loading for *MS2-gt* and number of transcribing foci for *PP7-btd* between sexes do not demonstrate hyper-transcription on the male X and suggests a post-transcriptional mechanism for mediating early dosage compensation.

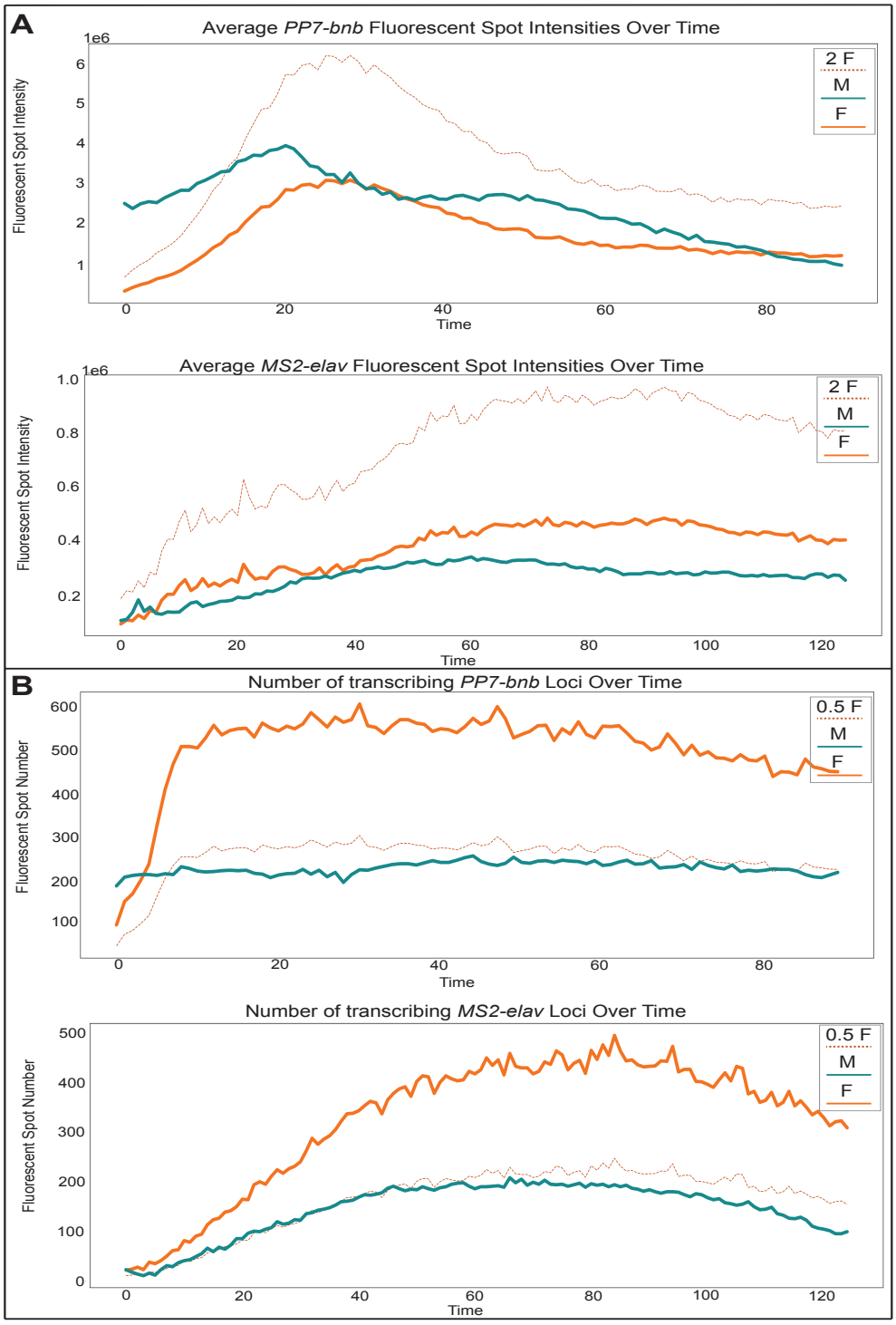


Figure 4: Transcription rate of *PP7-bnb* and *MS2-elav* is does not result in dosage compensation

A) Averages for male (dark cyan) and female (orange) *PP7-bnb* and *MS2-elav* fluorescent spot intensities plotted over nuclear cycle 14. The dashed, dark orange line represents double the average female fluorescent spot intensity for every given timepoint. B) The number of transcribing *PP7-bnb* and *MS2-elav* loci throughout nuclear cycle 14. The dashed orange line represents half of the transcribing female loci.

To more comprehensively assess if early dosage compensation is transcriptionally-regulated, we compared the average particle intensities of *PP7-btd* between sexes. In this analysis, a particle is defined as a fluorescent spot at a single locus connected through time. We limited our scope to one early dosage compensated gene because the analysis requires manual curation and is a very time-intensive process. We were once again struck by the closely matched average intensities of *PP7-btd* particles between male and female embryos (Figure 5) clearly demonstrating that early dosage compensation observed for *btd* by mRNA-sequencing is not regulated through a transcriptional mechanism.

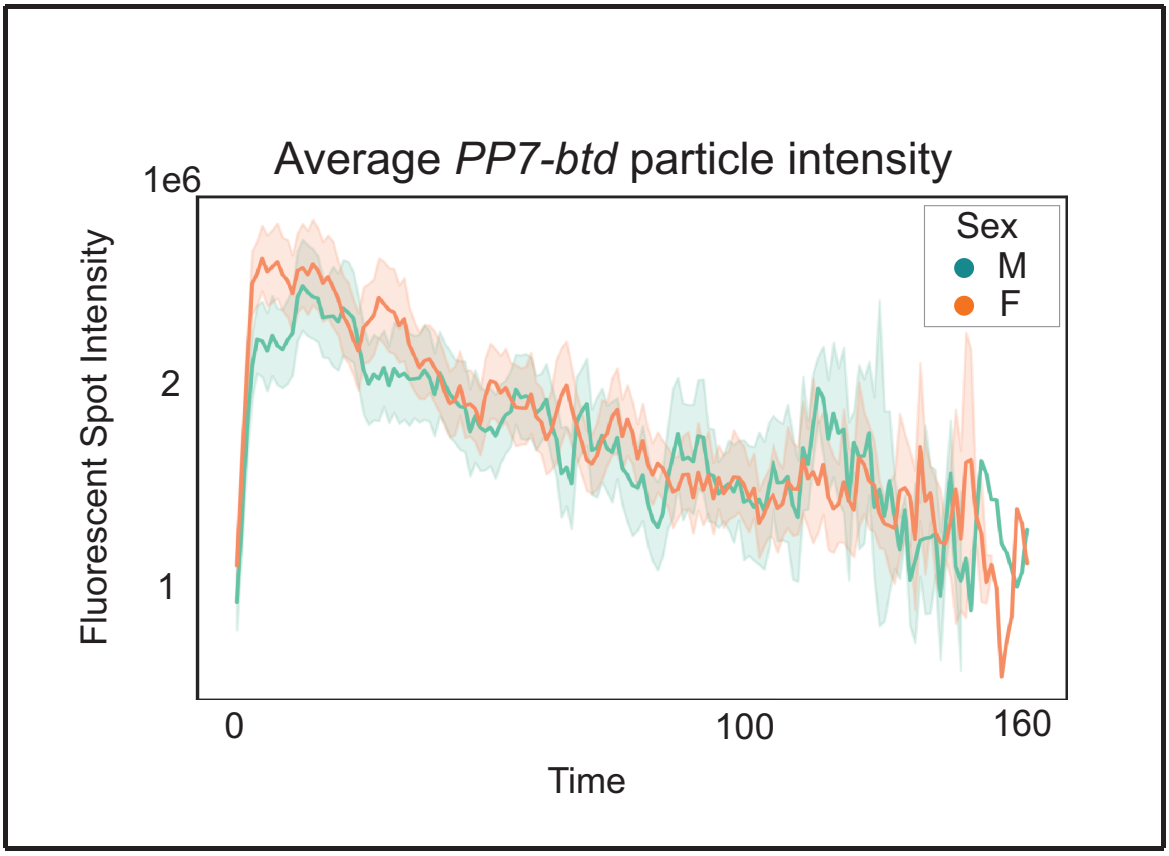


Figure 5: Transcription rate of *PP7-btd* is the same in male and female embryos The average fluorescent spot intensity for particles in male and female embryos (N=2 for each sex), where a particle is a series of fluorescent spots at one locus connected through time.

We were curious as to how the regulation of transcriptional kinetics might drive the subtle differences in transcription rate between sexes for *MS2-giant,* and *PP7-bnb*, and the more dramatic difference observed for *MS2-elav*. To address this quandary, we used a compound-state Hidden Markov Model (Lammers et al. 2020, Berrocal et al. 2020) that allows us to infer specific parameters regulating promoter state including the

rate of transcription initiation, duration of the transcriptional “burst” (meaning, a surge of expression defined by Bothma et al. 2014), and the frequency of transcriptional bursts.

First, we calculated the parameters that modulate transcription kinetics for a bulk dataset of sexed embryos for each genotype (Figure 6A). We were heartened to see that the genes with sex-specific differences in kinetic parameters regulating promoter state were in line with even the subtle differences we observed by sexed fluorescent intensity measurements. To exclude if the differences observed in the bulk data analysis were due to embryo-to-embryo variation, we calculated the same inference of promoter state for individual embryos of each sex (Figure 6D, Figure 6B-D).

These analyses suggest that modulating transcription burst duration for *MS2-gt* and *PP7-bnb* is responsible for the subtle increase in transcription rate observed at male loci during the beginning of nc14 (Figure 2A, Figure 3A, Supplemental Figure 1B&C, Figure 6C). Although the longer burst duration detected is not sufficient to drive dosage compensation, it is interesting to note which parameter of transcription kinetics may be responsible for sex-specific variations in transcription rate.

The underlying kinetic mechanism responsible for the drastic difference in the transcription rate of *MS2-elav* is more elusive. The differences noted for burst frequency in the bulk dataset were accounted for by embryo-to-embryo variation (Figure 6C). We postulate that differentially regulating transcription initiation may account for the doubling of transcription of *MS2-elav* in female loci, though there is also embryo-to-embryo variability that clearly contributes to the difference seen in the bulk dataset (Figure 6B).

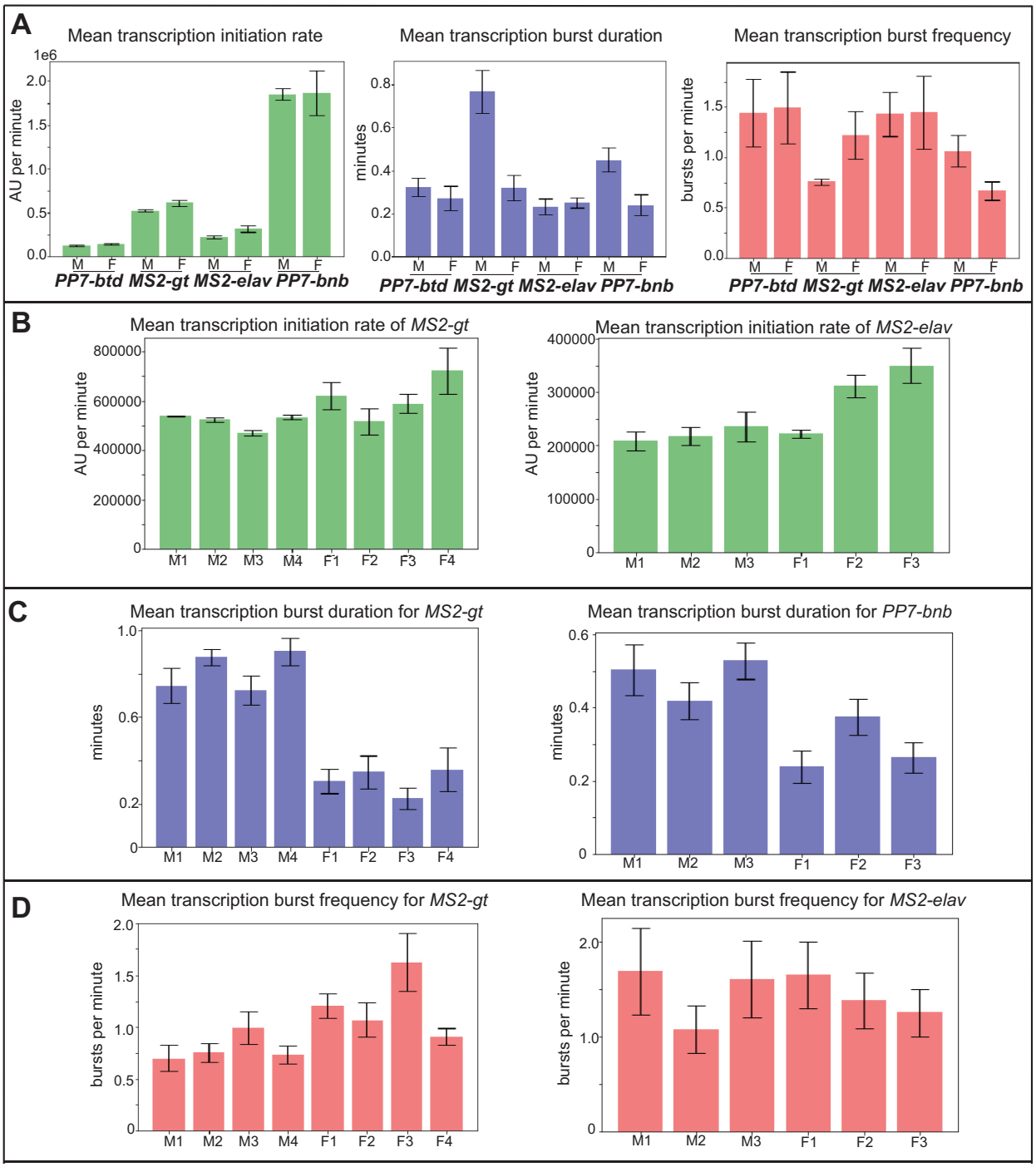


Figure 6: Analysis of transcriptional bursting dynamics reveal that differences in burst duration is responsible for the observed differences in transcription rate of *MS2-gt* and *PP7-bnb* A) Mean initiation rate, transcription burst duration, and transcription burst frequency calculations for all particles of the X-linked genes in this study. B) Mean initiation rate calculations for individual *MS2-gt* and *MS2-elav* embryos. Calculations for individual embryos were also conducted for mean burst duration in C) and mean burst frequency in D).

Discussion

Our tailoring of quantitative live-imaging methods to examine early embryonic dosage compensation in *Drosophila melanogaster* were motivated by the hypothesis that the equalizing of mRNA abundance between sexes in the absence of MSL-2 mediated by regulation of transcription rate at X-linked loci.

Instead, what our studies revealed that early dosage compensation of two key developmental regulators, *btd* and *gt* are not regulated through hyper-transcription of the male X-linked loci. These findings represent a broadening of our understanding of different ways in which the sex chromosome dosage imbalance is mitigated during early embryogenesis. Our findings offer a more dynamic way of understanding how mRNA abundance is regulated across developmental stages. Importantly, this presents a shift in our understanding of how mRNA abundance is regulated considering the decades of research whose findings posited that dosage compensation is almost exclusively a transcriptionally regulated process.

Sex lethal (Sxl), and RNA-binding protein that inhibits dosage compensation in females, may play a role in regulating the abundance of X-linked mRNAs during early embryogenesis. The regulation of X-linked *runt* abundance by Sxl is the only documented observation of an MSL-2-independent, post-transcriptional regulation of dosage compensation prior to our study (Gergen 1987, Bernstein and Cline 1994). It is possible that because the two molecules regulate each other's expression (Kramer et al. 1999, Torres et al. 2009), that observation of post-transcriptional dosage compensation by Sxl is gene-specific and therefore inapplicable to the widespread phenomenon we observe during early embryogenesis (Lott et al. 2011).

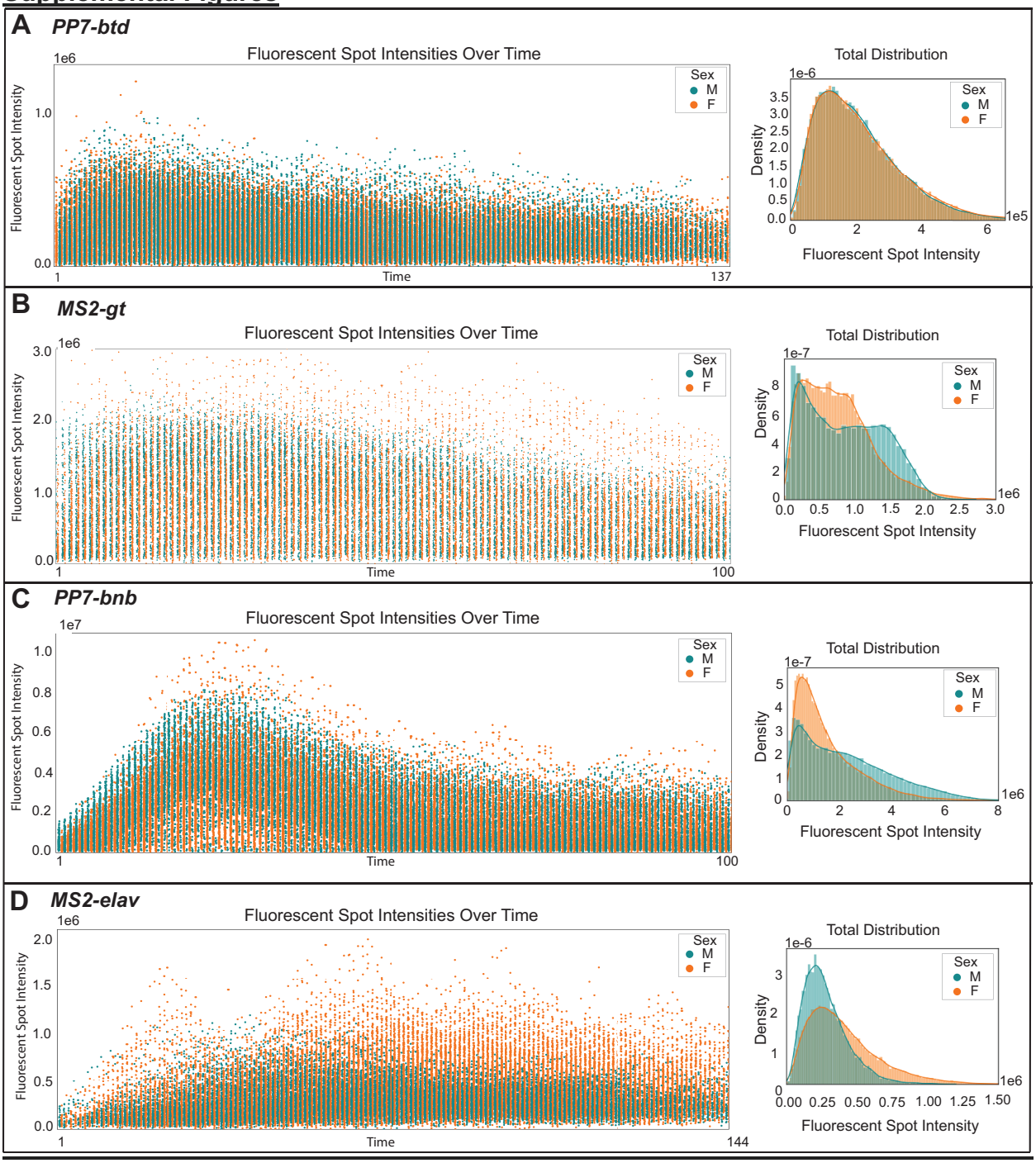
Considering our findings, the observed enrichment of predicted Sxl binding sites (poly-U tracts) in the 5' and 3' UTRs of many X-linked genes (Kelley et al. 1995) may point to one mechanism for post-transcriptionally regulating dosage compensation. There is no direct correlation between genes that are early dosage compensated and their predicted regulation by Sxl–not all genes that are early dosage compensated are predicted to be Sxl targets, and not all predicted Sxl targets experience early dosage compensation. There may however be a subset of early dosage-compensated genes whose abundance may be regulated by Sxl, a hypothesis worth exploring in future studies.

It is formally possible that regulation of transcriptional elongation may mediate early dosage compensation of *btd* and *gt*. Because the MS2 and PP7 stem loop sequences in our study are inserted immediately upstream of the translation start codon and flanked by splice donor and acceptor sites from the *hb* intron, our findings speak only to the regulation of transcription initiation. However, a recent study using MS2 and PP7 reporter systems demonstrated a concerted regulation of transcription initiation as opposed to a highly variable regulation of elongation between nuclei (Liu et al. 2020). These findings suggest that elongation is not the major contributor to transcriptional regulation, though the role of elongation during early dosage compensation using these tools warrants further study.

While the primary goal of this study was to study dosage compensation, our measurements of transcription rate at endogenous loci represent a significant advance for *in vivo* studies of mechanisms of gene regulation in the *D. melanogaster* embryo. Our use of our reporter at an X-linked loci for sexing embryos also provides a straightforward control for detecting differences in the regulation of gene expression between male and female embryos.

We hope that our data, as well as the reagents generated for the purposes of our study, can help address other open questions about gene regulation in the early *D. melanogaster* embryo. The reagents generated can be used for protein colocalization studies or can be crossed mutant alleles of interest to assess the contribution of a particular molecule in the regulation of our tagged genes. Overall, we are eager to learn how the data and reagents generated in our discovery of a post-transcriptional form of early dosage compensation make possible future learnings that deepen the community's understanding of regulating gene expression during development.

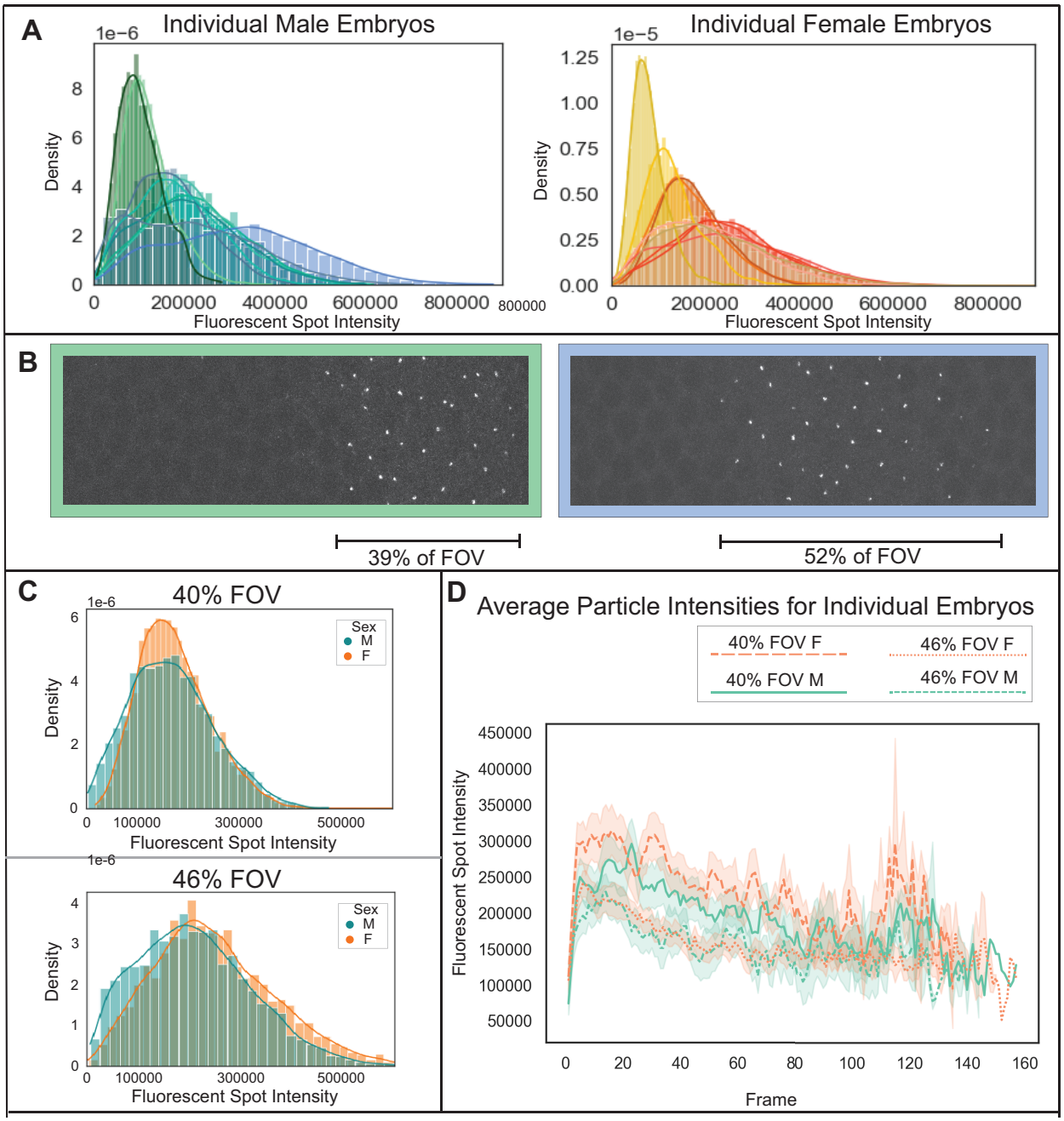
Supplemental Figures



Supplementary Figure 1: Transcription rate of early dosage compensated genes is the same in male and female embryos

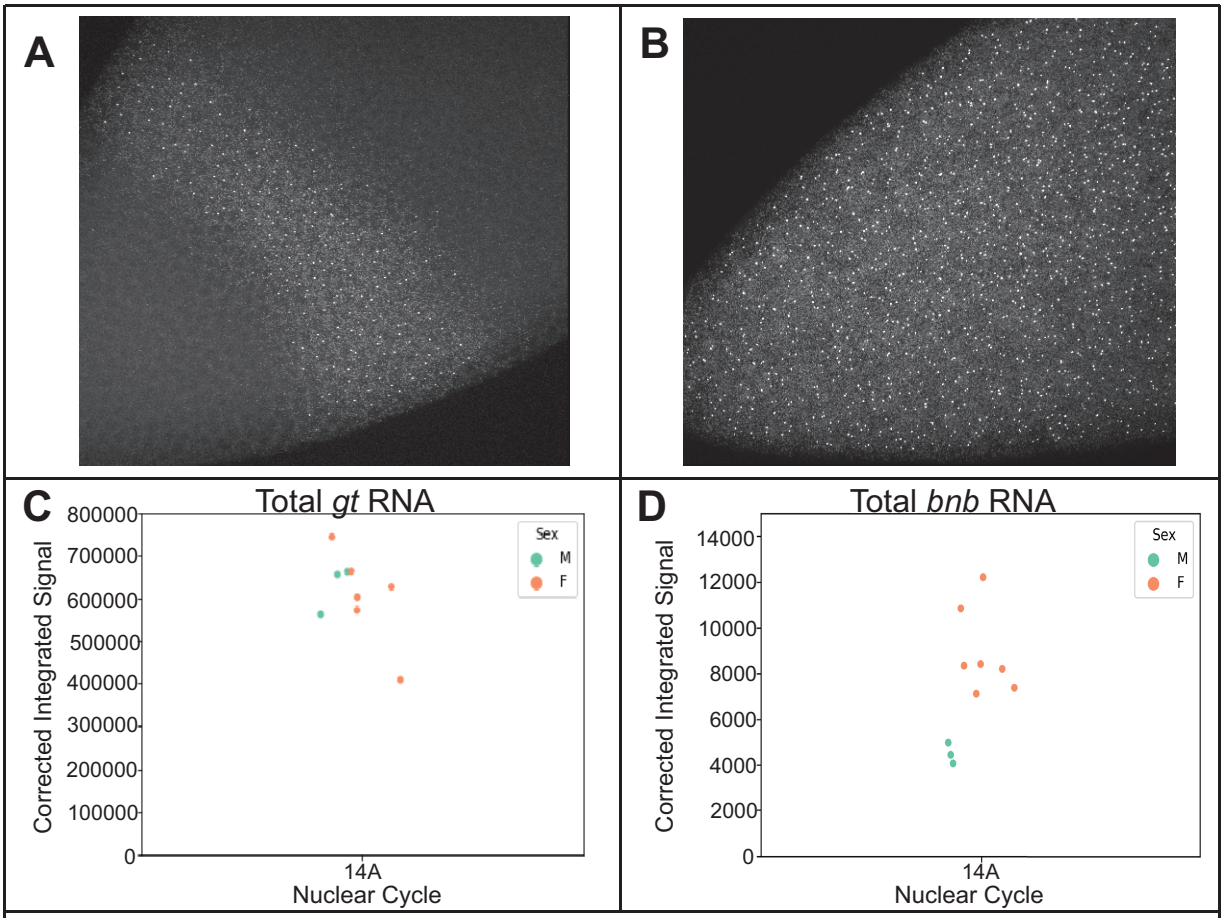
A) Distributions of fluorescent spot intensity over time (in frames, where "1" is the the first instance of transcription of the locus during nuclear cycle 14) for all female (orange) and male (dark cyan) embryos (N=9 embryos of each sex) with total spot intensity histograms on the right. B) The distribution of fluorescent spot intensities plotted over time for the dosage compensated *MS2-gt* (N=4 embryos of each sex) with total spot intensity histograms on the right. B&C) The distribution of fluorescent spot intensities

plotted over time for *PP7-bnb* and *MS2-elav*, genes that are not early dosage compensated (N=3 embryos of each sex for each genotype) with total spot intensity histograms on the right.

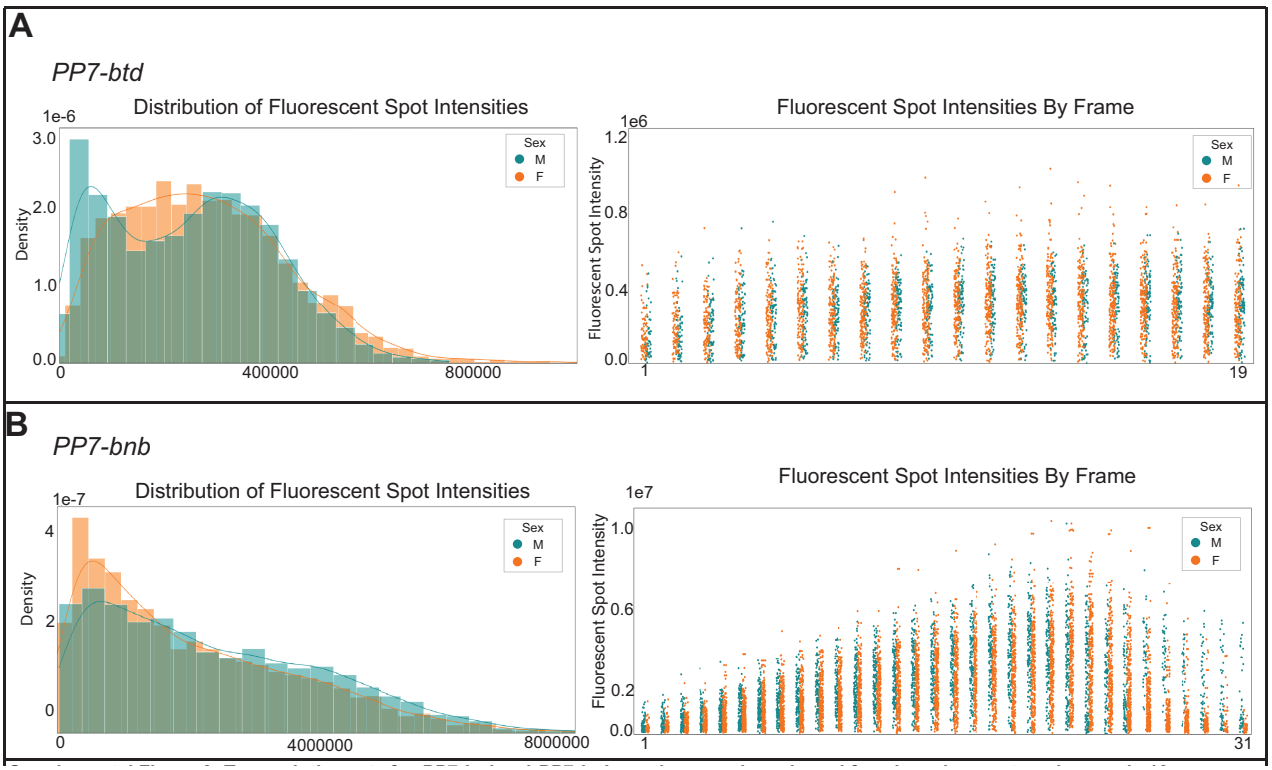


Supplementary Figure 2: Transcription rate of *PP7-btd* varies along the Dorsal-Ventral axis of the developing embryo A) Distribution of fluorescent intensity of *PP7-btd* spots in individual male and female embryos. B) Variation in the width of the *PP7-btd* expression domain along the DV axis, where the dorsal (39% of the field of view) expression domain correlates with spots of weaker fluorescence intensity (indicated by the green color that matches one of the individual histograms of a male embryo in A).

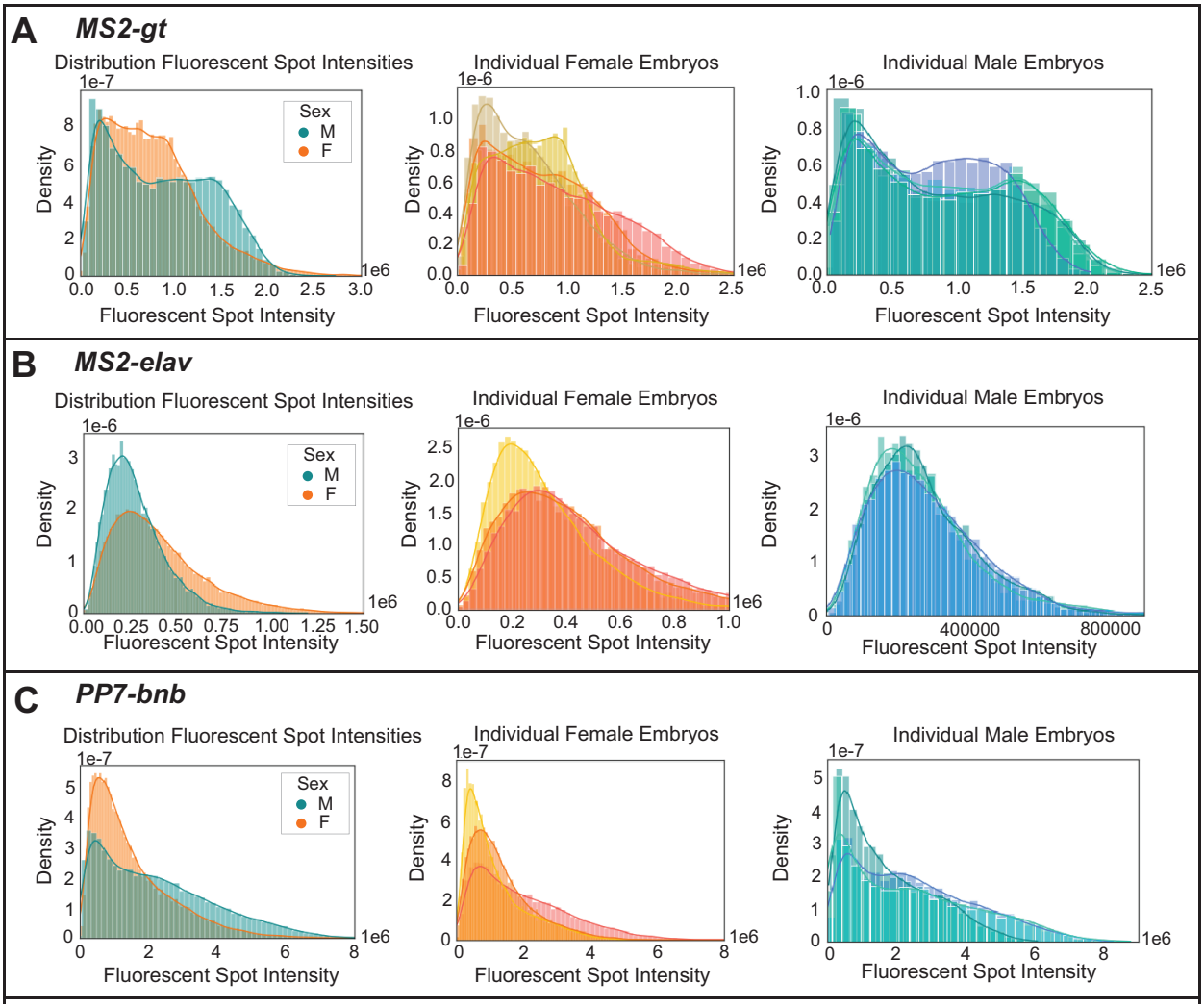
An embryo whose recorded *PP7-btd* expression is more ventral (52% field of view) has a wider distribution of spot fluorescent intensities. C) Distribution of fluorescent spot intensities of individual embryos matched by FOV, and therefore, DV position. D) Average particle intensities plotted over time for two male and female embryos on different positions on the DV axis.



Supplementary Figure 3: Validation of early dosage compensation phenotype by quantitative FISH A) A maximum intensity projection of the anterior portion of a wild type male embryo at early nc14 probed for *gt* (an early dosage compensated gene) RNA and B) a female embryo probed for *bnb* (an X-linked gene that is not early dosage compensated) RNA. Embryos were sexed by determining the number of large puncta were present within nuclei, which are dark and circular in appearance. C & D) Quantification of total probe signal for each probe in individual embryos, where the signal intensity was averaged across the expression domain with background subtraction.



Supplemental Figure 4: Transcription rate for *PP7-btd* and *PP7-bnb* are the same in male and female embryos at nuclear cycle 13 A) The total distribution of fluorescent spot intensities and the same data plotted over the course of nuclear cycle 13 for *PP7-btd* (N=3 embryos for each sex). B) The total distribution of fluorescent spot intensities and the same data plotted over the course of nuclear cycle 13 for *PP7-bnb* (N=3 embryos for each sex).



Supplemental Figure 5: Total distribution of spot intensities of *MS2-gt, MS2-elav*, and *PP7-bnb* A) Total distributions of fluorescent spot intensity for all female (orange) and male (dark cyan) *MS2-gt* embryos (N=4 embryos for each sex) and the same data plotted for individual embryos by sex. We also plotted data for *MS2-elav* in B) and *PP7-bnb* in C) in the same manner (N=3 embryos for each sex per genotype).

Chapter 3: A path toward identifying post-transcriptional gene regulatory mechanism(s) mediating early dosage compensation

Our discovery of a post-transcriptional mechanism for regulating early dosage compensation motivates subsequent investigations of the molecules mediating this mode of gene regulation. The following proposed experiments are designed to probe the specific pathways, molecules, and subcellular locations involved in early dosage compensation in the *D. melanogaster* embryo.

We first would like to understand the contribution of Sex lethal (Sxl) on early dosage compensation, given the notable enrichment of predicted binding sites in the 5' and 3' UTRs of the many X-linked that are expressed during embryogenesis. Even genes that are not predicted to be regulated by Sxl, such as *gt*, have poly-U tracts in the gene's untranslated sequences that may still bind Sxl.

To test this molecule's involvement in regulating early dosage compensation, we propose mRNA-sequencing on single embryos that are deficient in Sxl. There is a publicly available fly line generated by Tom Cline containing a Sxl overexpression allele over a Sxl deletion, meaning that one would be able to test the contribution of over- and under-expression of the molecule on early dosage compensation. Because the *Sxl* gene is X-linked and involved in sex differentiation, the only progeny of this fly line that result in fertile, adult females will always be Sxl^{OE}/Sxl^{-}, and fertile adult males are Sxl^{-}/Y. Sequencing embryos of all resulting embryonic genotypes of this cross will test the contribution of Sxl in regulating in regulating all the zygotically-transcribed, early dosage compensated genes identified in the Lott et al. study.

In parallel with the Sxl experiment, we propose a strategy for identifying which RNA degradation pathways are involved in equalizing mRNA abundance during early development. mRNA-sequencing in single embryos with key components of various RNA degradation pathways knocked down through RNAi nicely accomplishes this aim. By knocking down components of the nuclear and cytoplasmic exosome, piwi, and miRNA pathways, and other proteins associated with P-bodies, we can narrow down the candidate molecules mediating early dosage compensation. We see this strategy is a brute-force way of narrowing down how to direct further studies, and which pathways for processing mRNA act completely independently of regulating early dosage compensation.

Results of the impact of knockdowns to RNA degradation components may also yield insight into what subcellular compartment may be associated with the regulation of early dosage compensation. For example,the association between P-bodies and mRNA degradation (Beadle et al. 2022) may be involved in equalizing X-linked mRNA. If mRNA-sequencing data from RNAi knockdowns of molecules associated with P-bodies affects early dosage compensation, we propose combining probes for affected genes from the single-molecule Fluorescence *in situ* Hybridizations (smFISH experiments performed in Chapter 2) with immunofluorescence against P-body associated proteins. Colocalization of early dosage compensated mRNAs with these regulatory subcellular

structures will provide further insight into the specific molecular mechanisms of post-transcriptional early dosage compensation.

To further test where early dosage compensation may be occurring, and better determine the extent to which this is a post-transcriptionally regulated phenomenon, we propose performing total RNA-sequencing on nuclear and cytoplasmic fractions derived from single embryos. Total RNA-sequencing allows for the detection of mature and un-processed mRNA, therefore allowing a way to test if all of the early dosage compensated genes identified in the Lott et al. study are regulated through a post-transcriptional mechanism. In addition, performing RNA sequencing on nuclear and cytoplasmic fractions gives further insight into where early dosage compensation is occurring, data that can be used in combination with other experiments proposed in this chapter to narrow down the molecules mediating this regulatory process.

To test the insights gleaned from the above experiments, we propose measuring mRNA degradation in sexed, single embryos in wild type and relevant mutant backgrounds that impact early dosage compensation. By injecting embryos with a global inhibitor of transcription, one can measure what mRNA species persist over time, therefore allowing for the detection of differences in RNA degradation rate between sexes. One could also measure RNA half-life through a metabolic labelling strategy, where one can label newly synthesized RNA with a pulse of ethynyl-U or 4-thio-U. Hopefully, various components of RNA degradation pathways have been identified as potential regulators of early dosage compensation, one can also observe how RNA degradation between sexes is altered in mutant backgrounds for components of those pathways. Overall, this experiment not only tests if RNA degradation mediates early dosage compensation, but it can also directly test which molecules or pathways may be implicated in this key regulatory process.

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Appendix I: Materials and Methods

Materials and Methods:

DNA constructs:

Fly strains were tagged with MS2 or PP7 reporter sequences at the endogenous loci of X-linked genes through CRISPR-Cas9 Homology-Directed Recombination. We used the U6-gRNA protocol from flycrispr.org (Gratz et al. 2016) to clone three guide RNA sequences per gene, targeting a 100 bp region around the start codon, into pCFD5 plasmids (Port and Bullock 2016). For donor plasmids, we inserted a 24X MS2 or PP7 cassette (Garcia et al. 2013, Liu et al. 2020) between 1kb of homology arms for a gene of interest into a pUC19 vector backbone using the NEBuilder HiFi DNA Assembly protocol. We attempted to tag all target genes with both MS2 and PP7, but, due to low editing efficiency and potentially other factors, recovered only one for each gene, as described in text.

Fly line generation:

gRNA, donor, and selection plasmids (for phenotypic screening, Kane et al. 2017) were purified and sent to Rainbow Transgenic Inc. for microinjection into *y2 cho2 v1; Sp/CyO, P{nos-Cas9, y+, v+}2A* embryos.

Full details of construct and sequence information can be found in a public Benchling folder.

Progeny of injected flies with an *ebony* phenotype were molecularly screened by PCR for the insertion of the MS2 or PP7 cassettes at endogenous X-linked loci. Lines with successful transgene insertion were confirmed by Sanger sequencing.

Transcription of X-linked genes was measured by imaging embryos homozygous for the MS2- or PP7-tagged gene and a constitutively expressed *Histone-RFP; MCP-eGFP* (Garcia et al. 2013) or *PCP-eGFP* (Larson et al. 2011) allele.

Live imaging:

Image acquisition:

Sample preparation followed the procedures described in (Bothma et al. 2014, Garcia et al. 2013, Lammers et al. 2020). In brief, embryos were collected, dechorinated with 50% bleach, and mounted between a semipermeable membrane (Lumox film, Starstedt, Germany) and a coverslip while in Halocarbon 27 oil (Sigma). Data collection was performed using a Zeiss LSM 800 scanning confocal microscope (Zeiss, Jana, Germany). The MCP-eGFP or PCP-eGFP were excited with laser wavelengths of 488 nm, respectively. Data were collected at a 40X objective with oil immersion where the average laser power on the specimen was 7.3 mW and a master gain of 550V. The confocal stack consisted of 21 equidistant slices with an overall z-height of 5.97 µm and an interslice distance of 0.29 µm. The images were acquired at a frame time of 633.02 ms and a pixel dwell time of 1.03 µs. Image sizes were 78 µm x 19.5 µm, a frame size

of 1024 pixels x 256 pixels, and a pixel size of 0.08 μm. Data were taken for at least 3 embryos of each sex per genotype, and each embryo was imaged for at least the first 30 minutes of nuclear cycle 14.

Image analysis:
Live-imaging data were analyzed using a custom-written software following the protocols in (Garcia et al. 2013, Lammers et al. 2020). This software, containing MATLAB code, can be found in a public GitHub repository. In brief, this procedure involves nuclear segmentation, segmenting transcription spots based on fluorescence, and calculating the intensity of each MCP-eGFP or PCP-eGFP transcription spot inside a nucleus as a function of time.

Data analysis
To infer bursting parameters from experimental fluorescence traces, we used a compound-state hidden Markov Model described in Lammers et al. 2020 whose code can be found in a public GitHub repository.

All data analysis was done in Python using a Jupyter Notebook with custom code to generate figures. The Jupyter notebook and all data required to run it are available on Dryad and this Github link .

Quantitative RNA-FISH:
Probe design and hybridization:
Custom Stellaris® FISH Probes were designed against *gt* and *bnb* RNA by utilizing the Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (version 4.2). Single-molecule RNA-FISH protocol was followed using the *D. melanogaster* embryo protocol at the Biosearch Technologies website.

Image acquisition:
Embryos were staged at nuclear cycle 14A based on percent membrane invagination during cellularization (< 25%). Data collection was performed using a Zeiss LSM 800 scanning confocal microscope (Zeiss, Jana, Germany). A laser wavelength of 670 nm was used to excite the probe-conjugated fluors. Data were collected at a 63X objective with oil immersion with the laser power on the specimen was set to 5% and a master gain of 650V. The images were acquired at a frame time of 930.91 ms and a pixel dwell time of 1.52 μs. Image sizes were 202.8 μm x 202.8 μm, a frame size of 512 pixels x 512 pixels, and a pixel size of 0.4 μm.

The field of view was adjusted to encompass the entire expression domain of probed patterned genes, and the bounds of the z-stack for each image were determined by when the z-plane no longer detected the probe fluorescent signal.

Full details of probe sequence information can be found in a public Benchling folder. Raw and max intensity projection images can be found on Dryad.

Image analysis:
Total signal of mRNA with background correction was measured for probed genes using ImageJ. Z-slices of the original image that contained no signal were trimmed from the image before generating a 2D max intensity projection. We followed the Cooper Lab protocol for Quantitation of Total Fluorescence per Cell with Background correction (https://cooperlab.wustl.edu/). In brief, two ROIs were determined in ImageJ: one to measure signal and the other to measure background. We then averaged the mean Integrated Density of the signal (5 separate measurements encompassing the total expression domain) and of the background. The mean background is subtracted from the mean integrated density, and then divided by the area of the signal ROI to obtain the Corrected Integrated Density.

Appendix II: Additional reagents generated for future work on regulation of gene expression during *D. melanogaster* embryogenesis

As is to be expected of any exploratory research project, many more reagents are generated than get used in the final experiments. Below is a table of fly lines generated to test gene expression and protein localization that may be involved in early embryonic dosage compensation. We hope that these reagents will be of use to future trainees in their pursuit of deepening the community's understanding of gene regulation.

Generation of these fly lines followed the same methods outlined in Appendix I, with the exception that, to generate MSL-1-GFP flies, plasmids were injected into *y2 cho2 v1; P{nos-Cas9, y+, v+}3A/TM6C, Sb Tb* embryos.

Full details of construct and sequence information can be found in a public Benchling folder.

Genotype	Line	Function	Comments	Alias
+;MSL-1-NGFP	4 - 23	endogenously tagged *msl-1* with GFP on N terminus	bad signal in nuclei in heterozygous flies-- homozygose and look again	
+;MSL-1-NGFP	4 - 24	endogenously tagged *msl-1* with GFP on N terminus	bad signal in nuclei in heterozygous flies-- homozygose and look again	
MOF CmCherry;	3 - 4	endogenously tagged *mof* with mCherry on C terminus	good signal in nuclei	
MOF CmCherry;	2 - 1	endogenously tagged *mof* with mCherry on C terminus	good signal in nuclei	
MOF CmCherry;	2 - 2	endogenously tagged *mof* with mCherry on C terminus	good signal in nuclei	
Sxl NGFP;	1 - 4	endogenously tagged female-specific early exon 1 of *Sxl* with GFP on N terminus	haven't looked at embryos under scope	
Sxl NGFP;	1 - 10	endogenously tagged female-specific early exon 1 of *Sxl* with GFP on N terminus	haven't looked at embryos under scope	
Sxl NGFP;	3 - 2	endogenously tagged female-specific early exon 1 of *Sxl* with GFP on N terminus	haven't looked at embryos under scope	
Sxl NmCherry;	2 - 2	endogenously tagged female-specific early exon 1 of *Sxl* with mCherry on N terminus	haven't looked at embryos under scope	
Sxl NmCherry;	2 - 4	endogenously tagged female-specific early exon 1 of *Sxl* with mCherry on N terminus	haven't looked at embryos under scope	
Sxl NmCherry;	2 - 5	endogenously tagged female-specific early exon 1 of *Sxl* with mCherry on N terminus	haven't looked at embryos under scope	
Sxl NmCherry;	2 - 6	endogenously tagged female-specific early exon 1 of *Sxl* with mCherry on N terminus	haven't looked at embryos under scope	

Genotype	Line	Function	Comments
MS2-gt; + ; Histone-RFP, MCP-eGFP (4F)/TM3, Ser	1A	endogenously tagged *gt* with MS2	Gabriella Martini from the Garcia lab generated these flies--get TM3,Ser virgins and cross to any male in this line for imaging
MS2-sisA;	2 - 2	endogenously tagged *sisA* with MS2	see MS2 spots
MS2-sisA;	3 - 3	endogenously tagged *sisA* with MS2	see MS2 spots
MS2-sisA;	7 - 2	endogenously tagged *sisA* with MS2	see MS2 spots
MS2-sisA; Histone-RFP ; MCP-eGFP	2 - 2	endogenously tagged *sisA* with MS2	this line is ready for imaging
MS2-Xpac;	3 - 6	endogenously tagged *Xpac* with MS2	this line has not been tested to see if there are MS2 spots when crossed to MCP-GFP females
MS2 - 5' rox1;	1 -16	endogenously tagged *rox1* with MS2 on 5' end, MS2 is lacking *hb* intron splice donor and acceptor sites	this line has not been tested to see if there are MS2 spots when crossed to MCP-GFP females
MS2-elav;	4 - 1	endogenously tagged Xpac with MS2	see MS2 spots
MS2-elav;	4 - 2	endogenously tagged *elav* with MS2	see MS2 spots
MS2-elav;	4 - 3	endogenously tagged *elav* with MS2	see MS2 spots
MS2-elav; Histone-RFP; MCP-eGFP	4 - 1	endogenously tagged *elav* with MS2	this line is ready for imaging
PP7-disco;	3 - 4	endogenously tagged *disco* with PP7	see PP7 spots

Genotype	Line	Function	Comments	Alias
PP7-disco;	3 - 6	endogenously tagged *disco* with PP7	see PP7 spots	
PP7-disco;	3 - 12	endogenously tagged *disco* with PP7	see PP7 spots	
PP7-btd;	5 - 2	endogenously tagged *btd* with PP7	see PP7 spots	
PP7-btd;	5 - 4	endogenously tagged *btd* with PP7	see PP7 spots	
PP7-btd; sp/cyo; Sb/Ser	5 - 2	endogenously tagged *btd* with PP7	see PP7 spots	
PP7-btd; MCP-mCherry, His-iRFP; PCP-eGFP, MCP-mCherry	5 - 2	endogenously tagged *btd* with PP7	this line is ready for imaging	
PP7-bnb;	2 -88	endogenously tagged *bnb* with PP7	see PP7 spots	
PP7-bnb; MCP-mCherry, His-iRFP; PCP-eGFP, MCP-mCherry	2 - 88	endogenously tagged *bnb* with PP7	this line is ready for imaging	