Primary Sex Determination in *Drosophila melanogaster* Does Not Rely on the Male-Specific Lethal Complex

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ABSTRACT It has been proposed that the Male Specific Lethal (MSL) complex is active in *Drosophila melanogaster* embryos of both sexes prior to the maternal-to-zygotic transition. Elevated gene expression from the two X chromosomes of female embryos is proposed to facilitate the stable establishment of *Sex-lethal* (*Sxl*) expression, which determines sex and represses further activity of the MSL complex, leaving it active only in males. Important supporting data included female-lethal genetic interactions between the seven *msl* genes and either *Sxl* or *scute* and *sisterlessA*, two of the X-signal elements (XSE) that regulate early *Sxl* expression. Here I report contrary findings that there are no female-lethal genetic interactions between the *msl* genes and *Sxl* or its XSE regulators. Fly stocks containing the *msl3*^{1} allele were found to exhibit a maternal-effect interaction with *Sxl*, *scute*, and *sisterlessA* mutations, but genetic complementation experiments showed that *msl3* is neither necessary nor sufficient for the female-lethal interactions, which appear to be due to an unidentified maternal regulator of *Sxl*. Published data cited as evidence for an early function of the MSL complex in females, including a maternal effect of *msl2*, have been reevaluated and found not to support a maternal, or other effect, of the MSL complex in sex determination. These findings suggest that the MSL complex is not involved in primary sex determination or in X chromosome dosage compensation prior to the maternal-to-zygotic transition.

KEYWORDS sex chromosomes; *Sxl*; X-signal element; maternal-to-zygotic transition; dosage compensation; Genetics of Sex

ONE of the first developmental decisions confronting a *Drosophila* embryo is to determine its chromosomal sex. The urgency of the decision likely reflects the need to ensure that the genic imbalances that result from having large heteromorphic X and Y sex chromosomes are compensated before they can exact deleterious effects (Vicoso and Bachtrog 2009; Conrad and Akhtar 2011; Stenberg and Larsson 2011). Genic imbalances in several X-encoded genes, known as the X-signal elements (XSEs), actually serve as the signals of chromosomal sex (Cline 1988; Erickson and Quintero 2007). The XSE products function prior to the large-scale onset of zygotic gene expression to initiate the sex determination program. Quickly engaged, the sex determination program then locks in place a mechanism that helps ensure balanced gene expression before development can be adversely affected (reviewed in Cline and Meyer 1996; Salz and Erickson 2010; Salz 2011).

The connection between sex and balanced genomic expression is the master regulatory gene *Sex-lethal* (*Sxl*) (Cline 1984). Male embryos, with one X chromosome and one set of XSEs per cell, never express *Sxl* protein and produce all the protein and RNA components of the Male Specific Lethal (MSL) complex (Cline and Meyer 1996; Salz and Erickson 2010; Salz 2011). The MSL complex then assembles and binds to the male X chromosome. There, acting in conjunction with more general chromosome buffering effects, the MSL complex helps adjust the relative expression of the male X so that it is in balance with expression from the autosomes (Conrad and Akhtar 2011; Stenberg and Larsson 2011; Sun *et al.* 2013a; Sun *et al.* 2013b; Figueiredo *et al.* 2014; Lucchesi and Kuroda 2015). In contrast, female embryos with two Xs and two sets of XSEs per cell transiently activate the *Sxl* establishment promoter, *SxlPe*, producing a brief pulse of

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Sxl protein (Keyes *et al.* 1992; Erickson and Quintero 2007). The *Pe*-derived *Sxl* protein directs a rapid transition to a stable maintenance mode of *Sxl* expression that depends on positive autoregulation of the splicing of the premessenger RNA (pre-mRNA) products of the sex nonspecific promoter *SxlPm* (Bell *et al.* 1991; Gonzalez *et al.* 2008). Once fully engaged, *Sxl* blocks the translation of key dosage compensation regulator *msl2* (Bashaw and Baker 1995; Kelley *et al.* 1995; Lim and Kelley 2012). The absence of *msl2* protein prevents the formation of the MSL complex and ensures that females also express their X chromosomes in concordance with the autosomes (Bashaw and Baker 1995; Kelley *et al.* 1995; Lyman *et al.* 1997).

The *Sxl* regulatory mechanism requires that the XSE genes be expressed differentially in early XX and XY embryos and indeed the strong XSE genes, *scute* (*sc*) and *sisterlessA* (*sisA*), encoding transcriptional activators of *SxlPe*, appear to be expressed in proportion to their gene dose (Cline 1988; Deshpande *et al.* 1995; Erickson and Quintero 2007; Lott *et al.* 2011). Curiously, this is not the case for every early expressed X-linked gene. RNA-sequencing (RNA-seq) analysis of staged single embryos has revealed that most X-linked genes undergo a partial dosage compensation prior to the time that the canonical MSL complex is thought to be active (Lott *et al.* 2011). The nature of this early zygotic dosage compensation (EZDC) is unknown, but could reflect gene-specific or more general MSL-independent "buffering" mechanisms to adjust genic balance (Stenberg *et al.* 2009; Lott *et al.* 2011; Stenberg and Larsson 2011; Philip and Stenberg 2013; Chen and Oliver 2015). Curiously, two *Drosophila* species with larger X chromosomes appear to lack this early dosage compensation (Lott *et al.* 2014).

The conventional view of sex and dosage compensation posits a temporally linear regulatory pathway in females: the XSE proteins initiate *Sxl* expression, autoregulatory splicing maintains *Sxl* activity, and *Sxl* protein thereafter prevents formation of the MSL complex ensuring an appropriate balance of X and autosomal expression. There is, however, an alternative model that upends this regulatory hierarchy. Based on reported genetic interactions between mutations affecting the *msl* complex and mutations in *Sxl*, and the XSE genes *sisA* and *scute*, as well as on correlated changes in gene expression, Gladstein *et al.* (2010) and Horabin (2012) have proposed that the MSL complex acts in early embryos of both sexes to generally elevate X-linked gene expression. With respect to sex determination, the alternative model posits that the MSL complex is part of an X-signal amplification mechanism that increases the fidelity of *Sxl* activation by elevating the concentrations of XSE proteins, both in absolute terms, and relative to maternal-supplied and autosomally expressed inhibitors. Likewise, the MSL complex facilitates the transition to stable splicing of *SxlPm* transcripts by doubling the amount of early *Sxl* protein produced in females and by doubling the levels of its *SxlPm*-derived pre-mRNA substrates (Gladstein *et al.* 2010; Horabin 2012).

The early embryonic MSL complex was also proposed to explain much of the EZDC activity observed by Lott *et al.* (2011). Initially, X-linked gene expression is elevated in both sexes but as *Sxl* protein is produced, it downregulates the production of *msl2* protein reducing the relative activity of the MSL complex in females. The net result is partial dosage compensation of X-linked genes in the period before *msl2* is fully repressed by *Sxl* protein (Horabin 2012).

Despite the appeal of a model that interconnects the beginning and ends of a regulatory pathway, and that offers a unified explanation for sex signal amplification and early zygotic dosage compensation, there are reasons to be skeptical of an early embryonic MSL function. One reason for concern is that the early embryonic MSL model has paradoxical and counterintuitive implications for the regulation of sex determination. The model postulates that the MSL complex normally elevates expression of the XSEs twofold, effectively providing females with a 4X and males a 2X concentration of XSE proteins (Gladstein *et al.* 2010). If this is so, how can it be that *msl* mutant females activate *SxlPe*, and wild-type males do not, when both should have equivalent 2X levels of XSE proteins? The proposal that *Sxl* downregulates *msl2* and the MSL complex in females before autoregulatory *Sxl* splicing is fully established (Horabin 2012) implies that the *Sxl* regulatory scheme is programmed to decrease its ability to discriminate between the male and female signals before that task is actually accomplished. This seems odd for a high-fidelity regulatory system that otherwise seems constructed to continually amplify the differences between males and females (Gonzalez *et al.* 2008). A second reason for caution is that the proposed early embryonic function of the MSL complex depends on a reported maternal effect of *msl2* (Gladstein *et al.* 2010; Horabin 2012). There is, however, no evidence suggesting that MSL2 could be deposited in eggs, nor do precisely staged single embryos contain maternally deposited *msl2* mRNA (Bashaw and Baker 1995; Kelley *et al.* 1995; Rastelli *et al.* 1995; Franke *et al.* 1996; McDowell *et al.* 1996; Lott *et al.* 2011, 2014). A third reason for doubt is that the genetic evidence used to support an early embryonic role of the MSL complex is unpersuasive. Most importantly, the critical genetic experiments presented by Gladstein *et al.* (2010) relied on nonstandard controls and on comparisons with a reference strain that may have been particularly insensitive to the biological effects measured (Cline 1988).

For these reasons, I reexamined the proposed functions of the *msl* genes in *Sxl* regulation. My data and conclusions contradict those of Gladstein *et al.* (2010) and Horabin (2012). I found that none of the *msl* genes tested exhibited the genetic interactions previously reported to occur with components of the sex determination system. I discovered that the genetic interactions previously ascribed to $msl3^1$ were due to another mutation in the strain, and that *msl3* mutations were neither required nor sufficient, for the strong female-lethal genetic interactions seen. My findings imply that the MSL complex does not participate in *Drosophila* primary sex determination. They also suggest that the MSL

complex is unlikely to underlie the little understood early zygotic X dosage compensation process (Lott *et al.* 2011).

Materials and Methods

Experiments were done at 25°, except for those with $sc^{3\text{-}1}$, which were done at 29°. Flies were raised in uncrowded conditions on a standard yeast, sucrose, cornmeal, and molasses medium. Eclosion was the criterion for viability. Mutations and chromosomes not referenced in the text are described at FlyBase. MSL complex alleles were obtained from the Bloomington *Drosophila* Stock Center: mle^{9}, $msl1^{KmB}$, $msl1^{\gamma 216}$, $msl2^{227}$, $msl2^{KmA}$, $msl3^{1}$, *Df(3L)Exel6110*, and *Df(3L)BSC224*; from James Birchler (University of Missouri): $msl2^{P17}$, $msl2^{P22}$, $msl3^{1}$, and $msl3^{MAK\text{-}1}$; from Mitzi Kuroda (Harvard University): $msl1^{L60}$, $msl3^{1}$, and *P(msl3⁺-tap)*; and from Victoria Meller (Wayne State University): mle^{1}, $msl1^{1}$, $msl1^{\gamma 269}$, $msl1^{L60}$, $msl^{undefined}$, $msl2^{1}$, $msl3^{1}$, and $rox\text{-}1^{Ex6}$. All *msl* alleles employed are thought to be null alleles or strong hypomorphs: mle^{9} (internal deletion); mle^{1} (nonsense codon after 125 aa); $msl1^{\gamma 269}$ (large internal deletion); $msl1^{L60}$ (deletion of most coding sequences); $msl1^{KmB}$ (amorph or strong hypomorph); $msl1^{\gamma 216}$ (deletion of 3′ end of gene); $msl2^{1}$ (amino acid substitution, amorph, or hypomorph); $msl2^{227}$ (early frameshift); $msl2^{KmA}$ (amorph or hypomorph); $msl2^{P17}$ (internal deletion/inversion); $msl2^{P22}$ (internal deletion/inversion); $msl2^{undefined}$ (unknown, fully male lethal); $msl3^{1}$ (amorph or strong hypomorph); $msl3^{MAK\text{-}1}$ (amorph or strong hypomorph); see FlyBase for details of sequence changes or genetic evidence of function. The sc^{71} and sc^{96} mutations are loss-of-function alleles with bristle phenotypes and reduced male viability that were recovered in a screen used to identify *sisA* null mutations (Walker *et al.* 2000). I used the null allele Sxl^{f1} in the experiments detailed here instead of the Sxl^{fP7BO} deletion allele employed by Gladstein *et al.* (2010). Control experiments showed that both null *Sxl* alleles produced equivalent results in experiments with *msl1*, *msl2*, *and msl3* mutations (data not shown). Strains not carried by the Bloomington *Drosophila* Stock Center are available upon request.

Results and Discussion

The experiments described here rely on well-defined genetic interactions among components of the primary sex determination signal and their molecular target, *Sxl* (Cline 1984, 1988, 1993). The key principle is that the dose-sensitive nature of the X-chromosome counting process means that there is an expectation that any two mutations affecting X counting will exhibit sex-specific lethal interactions between them. Females individually heterozygous for the X-signal elements *sisterlessA* (*sisA*), or *scute* (*sc*), or for *Sxl*, are fully viable, but are sensitized to the loss of another X-signal component. Females carrying a second mutation, *sc sisA*/++ or *sisA Sxl*/++, for example, suffer reduced viability because of reduced expression of *SxlPe* and a decreased ability to transition to stable autoregulatory *Sxl* pre-mRNA splicing. *Sxl*, *scute*, and *sisA* mutations also show female-lethal interactions with maternal effect loci, such as *daughterless*, that are defective for maternally supplied factors needed to control *Sxl* expression. Most experiments in this paper address female-lethal interactions that have been reported to occur between maternal components of the MSL complex and *scute*, *sisA*, or *Sxl* (Gladstein *et al.* 2010). My general strategy was to cross *msl* mutant or control mothers with males carrying an X chromosome with *Sxl* or XSE mutations and to compare the viability of the experimental daughters with their appropriate male or female siblings. While the genetic interactions between *Sxl*, *scute*, and *sisA* are well defined, the magnitudes of the lethal effects observed can vary dramatically depending on genetic background (Cline 1988). For this reason, whenever possible, viability comparisons were made between siblings or between the offspring of sibling mothers.

Table 1 Test for lethal interactions between maternal *msl* mutations and individual sex determination mutations

Maternal 2nd or 3rd chromosome genotype	Viability (%) of females of indicated X chromosome genotype compared to sibling males (no. reference)					
	Sxl^{f1}/ +		$sisA^{1}$/ +		sc^{M6}/ +	
mle^{9}	99	(99)	97	(87)	123	(100)
$msl1^{KmB}$	107	(122)	99	(80)	91	(67)
$msl2^{227}$	101	(147)	95	(94)	115	(80)
$msl3^{1}$	15	(88)	26	(95)	12.5	(183)

Homozygous *msl* females generated from stocks of genotypes: mle^{9} cn^{1} bw^{1}/*CyO*; $msl1^{KmB}$/*CyO*; $msl2^{227}$ bw^{1}/*CyO*; or $msl3^{1}$ *red/TM3,* Sb^{1} Ser^{1} were crossed to *y w* cm^{1} Sxl^{f1} ct^{6}/*Y*; *y* cm^{1} ct^{6} $sisA^{1}$/*Y*; or sc^{M6} *w/Dp(1;Y)*y^{2}*61/* (sc^{+}) males at 25°.

Neither mle, nor msl1, nor msl2 interact with Sxl or XSE mutations

The data most supportive of a role of the MSL complex in primary sex determination are the apparent female-lethal synergisms between the null Sxl^{fP7BO} allele and maternal *msl* mutations reported in figure 1A of Gladstein *et al.* (2010). The strong female-lethal effects reported for Sxl^{fP7BO}/+ progeny of $msl3^{1}$ (1.4% survival), $msl2^{1}$ (31% survival), $msl1^{L60}$ (23% survival), and even the weak effect of $maleless^{1}$ (mle^{1}) mothers (76% survival), under conditions where all female progeny would be expected to survive, hint that components of the MSL complex may be needed to ensure that *Sxl* is activated in females (Gladstein *et al.* 2010).

Because of the central importance of these genetic interactions, I reexamined the female-lethal synergisms reported between maternal *msl* mutations and *Sxl* null alleles. I also added tests for interactions with the X-signal elements, *sisA* and *scute*, which should exhibit female-lethal interactions if the MSL complex is involved in the establishment phase of sex determination (Cline 1988; Gladstein *et al.* 2010). I observed a strong female-lethal interaction between maternal $msl3^{1}$ and all three zygotic sex determination mutations (Table 1, line 4), as reported previously (figures 1A and 2A

Table 2 *mle*, *msl1*, and *msl2* show no female-lethal interactions with XSE mutations in sensitized crosses

		Progeny recovered from crosses with $sc^{3\text{-}1}$ $sisA^1$/Y males			
		Experimental daughters $sc^{3\text{-}1}$ $sisA^1$/+ +			Control sons sc^+ $sisA^+$/Y
Cross	Maternal MSL genotype	Zygotic MSL genotype	Viability (%) relative to sibling males	No.	No.
A	+ (*y w* control)	+/+	9.5	12	126
B	+ (*y w sn* control)	+/+	33	28	84
C	mle^9	mle^9/+	49	38	77
D	mle^9/*CyO*	mle^9/+	17	16	93
		CyO/+	11	10	89
E	mle^1	mle^1/+	22*	32	147
F	mle^1/*In(2L)Gla*	mle^1/+	25	33	132
		In(2LR)Gla/+	34	41	122
G	$msl1^{L60}$	$msl1^{L60}$/+	12	21	171
H	$msl1^{L60}$/ *In(2L)Gla*	$msl1^{L60}$/+	12	7	57
		In(2LR)Gla/+	12	6	51
I	$msl1^{KmB}$	$msl1^{KmB}$/+	11	7	63
J	$msl1^{KmB}$/ *In(2L)Gla*	$msl1^{KmB}$/+	10	13	125
		In(2LR)Gla/+	2.4	3	122
K	$msl2^{227}$	$msl2^{227}$/+	50	92	183
L	$msl2^{227}$/*CyO*	$msl2^{227}$/+	27	23	85
		CyO/+	25	21	83
M	$msl3^1$	$msl3^1$/+	<0.25	0	401
N	$msl3^1$/*TM3*, Sb^1	$msl3^1$/+	18	12	68
		TM3/+	12	8	65

Mothers homozygous and heterozygous for MSL mutations were siblings from crosses of the form: *msl*/*msl* ♀♀ × ♂♂ *msl*/*Balancer*. Full genotypes of stocks used as in Table 1 and Table S1, except *y w*; *y w sn*, and $msl1^{KmB}$/*In(2L2R)BcGla*^{1}. Males were: $sc^{3\text{-}1}$ *w* cm^1 ct^6 $sisA^1$/*Y*. Crosses at 29°. *Female viability in cross E was not significantly different from either the total, or the *Gla*/+, female progeny of cross F (P = 0.13 for both, Fisher's exact test).

of Gladstein *et al.* 2010). In contrast, I found that neither mle^9, nor $msl1^{KmB}$, nor $msl2^{227}$ mutants showed any indication of lethal genetic interactions with *Sxl* or the sex signal components (Table 1, lines 1–3). Because the *mle*, *msl1*, and *msl2* alleles in Table 1 are different from those used in the earlier study, I tested for lethal interactions using additional *mle*, *msl1*, and *msl2* alleles, including the three used by Gladstein *et al.* (2010). In every case, I found that female viability was unaffected by the maternal *msl* genotype (Supporting Information, Table S1).

mle, msl1, and msl2 do not enhance the female lethality of sc sisA heterozygotes

A possible explanation for the discrepancies between my findings and those of Gladstein *et al.* (2010) would be if some undefined aspect of the genetic background or experimental conditions rendered the fly lines I used less sensitive to the lethal synergism between maternal *msl* mutations and females heterozygous for a single sex determination mutation. In other words, because females heterozygous for Sxl^{f1}, $sisA^1$, or sc^{M6} mutations are fully viable, perhaps the demand for a lethal synergism with maternal *msl* alleles was too stringent a criterion. To test this possibility, I carried out sensitized crosses in which female viability was partially compromised by virtue of being heterozygous for both the $sc^{3\text{-}1}$ and $sisA^1$ alleles. Two control crosses (A and B of Table 2) illustrate an important point about the lethal interactions between the two XSE mutations—viability is sensitive to undefined aspects of genetic background (see Cline 1988). Here that difference is manifest as a threefold difference in viability between the $sc^{3\text{-}1}$ $sisA^1$/++ progeny of two lab stocks, *yellow white* (9.5% survival) and *yellow white singed* (33% survival), that would naively be expected to produce equivalent outcomes. In table 2 of his seminal paper defining the sex determination signal, Cline (1988) documented the magnitude of this variation on the related female-lethal synergism between Sxl^{f1} and $sisA^1$. Depending on the wild-type stock used, viability of Sxl^{f1} $sisA^1$/++ daughters ranged from <1% up to 79%. Because of the potential for undefined aspects of genetic background to influence results, each sensitized test performed here compared the viability of the progeny of sibling mothers either homozygous or heterozygous for the *msl* alleles.

I used $msl3^1$, the only *msl* mutation that appeared to interact with individual XSE components in the experiments of Table 1 and Table S1, as a proof of principle for the maternal-effect enhancement of *sc sisA*/++ lethality (Table 2, crosses M and N). The *sc sisA* combination exerted a considerable lethal effect on its own, as only 15% (20/133) of *sc sisA*/++ daughters of $msl3^1$/+ heterozygotes survived (cross N). As expected, the lethal impact of the XSE mutations was strongly enhanced (>50-fold) when mothers were homozygous for $msl3^1$ (0 daughters/401 sons) (Table 2, cross M). In contrast, and in agreement with the results in Table 1 and Table S1, I found that none of the other *msl* mutations

Table 3 There are no zygotic female-lethal interactions between *msl1* and *msl2, msl3* or *rox1* mutations and XSE mutations

X Chr. genotype	2nd Chr. genotype	3rd Chr. genotype	Viability (%) compared to reference ♀	No.
Cross A: *msl1^{L60} msl2/In(2LR)Gla* ♀♀ *X* ♂♂ *y sc^{71} cm^{1} ct^{6} sisA^{1}/Y; Dp(1;2)Hw^{bap} (sc^{+})/+*				
++/sc^{71} sisA^{1}	*+/Dp(1;2)sc^{+}*	*msl1^{L60} msl-2/+ +*	90	81
++/sc^{71} sisA^{1}	*+/+*	*msl1^{L60} msl-2/+ +*	14	13
++/sc^{71} sisA^{1}	*+/Dp(1;2)sc^{+}*	Gla/ +	Reference	90
++/sc^{71} sisA^{1}	*+/+*	Gla/ +	13	12
Cross B: *y sc^{96} cm^{1} ct^{6} sisA^{1} /FM7c* ♀♀ *X* ♂♂ *+/Y; msl3^{1} / Rap^{1}*				
X Chr. genotype		3rd Chr. genotype	Viability (%) compared to reference ♀	No.
sc^{96} sisA^{1}/++		*msl3^{1}/+*	32	24
sc^{96} sisA^{1}/++		*Rap^{1}/ +*	26	17
FM7c/ + +		*msl3^{1}/+*	114	75
FM7c/ + +		*Rap^{1}/ +*	Reference	66
Cross C: *y w rox1^{ex6} + +/ + + + cm^{1} ct^{6}* ♀♀ *X* ♂♂ *sc$^{3\text{-}1}$ w cm^{1} ct^{6} sisA^{1}/Y*				
X Chr. genotype[a]			Viability (%) compared to reference ♂	No.
rox1^{ex6} sc$^{3\text{-}1}$ sisA^{1}			80	103
rox1^{+}/sc$^{3\text{-}1}$ sisA^{1}			57	75
rox1^{ex6}/Y			Reference	129
rox1^{+}/Y			102	131

Full genotypes of crosses as shown. *Dp(1:2)Hw^{bap}* is a duplication providing *sc^{+}* function (Cline 1988). It dominantly expresses ectopic abdominal bristles. Cross C at 29°.
[a] Only flies nonrecombinant for the *w-ct* interval were scored.

enhanced the lethality of *sc$^{3\text{-}1}$ sisA^{1} /++* heterozygotes (Table 2, crosses C–L).

There are no zygotic lethal interactions between msl and XSE mutations

The results of the crosses shown in Table 1, Table 2, and Table S1 provide no support for the claim (Gladstein *et al.* 2010; Horabin 2012) that maternal *mle*, *msl1*, or *msl2* mutations affect the assessment of chromosomal sex or influence the activity of *Sxl*. Might there still be a role for zygotically expressed products of these, or other, MSL complex genes in *Sxl* activation? The question is important because data presented in figure 2B of Gladstein *et al.* (2010) were interpreted as showing that zygotically contributed *mle^{1}*, *msl1^{L60}*, *msl2^{1}, msl3^{1}, males absent on the first^{2}* (*mof^{2}*), and *RNA on the X^{ex6}* (*rox^{ex6}*) alleles all enhanced the lethality of *sc$^{3\text{-}1}$ sisA^{1}/++* females. Any such zygotic lethal interactions should have been apparent in the results shown here in Table 2 as a reduction in the number of *msl/+* female offspring compared to their balancer-bearing sisters. In no case was such a deficit observed (Table 2, crosses D, F, H, J, L, and N). Reasoning that reducing the dose of two zygotic components of the MSL complex should have a stronger impact on viability than the loss of one, I asked whether a *msl1^{L60} msl2* double mutant chromosome enhanced the lethality of females carrying the *sc* and *sisA* mutations. Once again, I found no evidence of a lethal interaction as both the double *msl1 msl2* mutant and balancer chromosome-bearing *sc^{71} sisA^{1} /++* females were equally viable (Table 3A).

The *rox-1* RNA component of the MSL complex is not provided maternally and the transcript is expressed zygotically in early embryos (Lim and Kelley 2012). Gladstein *et al.* (2010) reported that the *rox-1^{ex6}* allele exhibited a strong zygotic female-lethal synergism with *sc* and *sisA* mutations. Cross C of Table 3 shows the results of a cross in which females carrying marked *rox-1^{ex6}* and *rox-1^{+}* X chromosomes were crossed to *sc$^{3\text{-}1}$ sisA^{1}*/Y males. I scored male and female progeny nonrecombinant for the *w ct* interval and found no evidence for a lethal interaction between the *rox-1^{ex6}* mutation and the XSEs. Indeed, the viability of *sc$^{3\text{-}1}$ sisA^{1}/++* females inheriting the wild-type *rox-1^{+}* allele (57%) was more compromised than those bearing the *rox-1^{ex6}* mutation (80% survival).

msl3^{1}, alone among the *msl* lines I tested, exhibited lethal interactions with XSE mutants (Table 1, Table 2, and Table S1). Gladstein *et al.* (2010) reported that this lethal interaction also had a zygotic component. The results of cross N of Table 2 indicate, however, that the lethal interaction with *msl3^{1}* flies is exclusively maternal as both *msl3^{1}/+* and *TM3/+* female progeny were equally viable. Because balancer chromosomes are not necessarily neutral in their effects on primary sex determination (Cline 1988), I wished to determine if there was any evidence of a zygotic interaction between *msl3^{1}* and *sc* and *sisA* under conditions where the *TM3* balancer normally carried in the *msl3^{1}* stock was absent. Accordingly, I crossed males transheterozygous for *msl3^{1}* and the dominant eye marker *Rap^{1}* with females carrying the *sc^{96}* and *sisA^{1}* alleles balanced with *FM7*. Heterozygous *sc^{96} sisA^{1}/++* progeny were equivalently viable whether they carried *msl3^{1}* or *Rap^{1}*, confirming there is no zygotic component of the lethal interaction between the *msl3^{1}* chromosome and the XSEs (Table 3, cross B).

The data presented here do not support the hypothesis that the MSL complex functions in the early regulation of *Sxl*. Despite extensive tests, I was unable to identify any of the genetic interactions that would be predicted to occur between *mle*, *msl1*, or *msl2* and either *Sxl* or the two key zygotic regulators of *Sxl*, *scute*, and *sisA*. Nor was I able to find support for the claimed zygotic role of *rox-1* in early *Sxl* activation. In this context, the strong female-lethal maternal effect

Table 4 *msl3* is not sufficient for the maternal-effect female-lethal interaction with XSE mutations

Maternal 3rd Chr. genotype	Viability (%) of females of indicated X Chr. genotype compared to sibling males (no. reference)			
	sisA^{1}/ +		*sc*^{M6}/ +	
msl3^{1}	42	(117)	9.2	(174)
msl3$^{MAK\text{-}1}$/*msl3*^{1}	110	(152)	103	(241)
Df(3L)Exel6110/msl3^{1}	102	(101)	102	(477)
Df(3L)BSC224/msl3^{1}	92	(104)	99	(135)

Experimental mothers derived from crosses: *msl3*^{1} *red* ♀♀ X ♂♂ *msl3*^{1} *red/TM3, Sb*^{1} *Ser*^{1} or ♂♂ *msl3*$^{MAK\text{-}1}$*/TM3, Sb*^{1} *Ser*^{1} or ♂♂ *w*^{1118}; *Df(3L)Exel6110/TM6, Tb*^{1}, or ♂♂ *w*^{1118}; *Df(3L)BSC224/TM6c, Sb*^{1} *cu*^{1}. Paternal genotypes: *y cm*^{1} *ct*^{6} *sisA*^{1}*/Y* or *sc*^{M6} *w/Y*.

observed with *msl3*^{1} females suggests one of two explanations: Either *msl3* regulates *Sxl* independent of the MSL complex or the *msl3*^{1} stock contains an unrecognized mutation(s) responsible for the maternal effect interaction.

An unidentified mutation is responsible for the interactions seen with an msl3 mutant

To determine which of these alternatives is true, I examined other *msl3*^{1} isolates and additional *msl3* alleles to see if they retained the interaction with the X-signal components. I obtained four additional *msl3*^{1} isolates from J. Birchler (University of Missouri), V. Meller (Wayne State University), and M. Kuroda (Harvard University), some labeled by their historical synonyms, *mle(3)132* and *msl3*^{P}. Each of the *msl3*^{1} stocks, regardless of origin, exhibited maternal-effect lethal interactions with *Sxl*, *scute*, and *sisA* (data not shown). I also received the *msl3*$^{MAK\text{-}1}$ allele from J. Birchler but found that the stock had acquired a mutation, rendering the second chromosome homozygous lethal, so I was unable to test directly if *msl3*$^{MAK\text{-}1}$ mutants exhibited the maternal-effect lethal interaction. Instead, I carried out complementation tests with *msl3*^{1}, *msl3*$^{MAK\text{-}1}$, and two chromosomal deficiencies, *Df(3L)Exel6110* and *Df(3L)BSC224*, which are deleted for all *msl3* coding sequences (Table 4). I found that *msl3*$^{MAK\text{-}1}$ and the two deficiencies fully complemented the female-lethal maternal effect of the *msl3*^{1} chromosome as evidenced by the absence of any lethal interaction with *sisA*^{1} or *sc*^{M6} (Table 4). The complementation tests thus demonstrate the female-specific maternal effects previously attributed to *msl3* are likely due, at least in part, to one or more additional mutations in *msl3*^{1} fly stocks.

An important question is whether the maternal effect lethal interaction requires the loss of *msl3* function, as might be the case if both a deficit in *msl3* and another gene were required, or if the lethal effect is entirely independent of *msl3*. To address this point, I asked if *msl3*^{1} females that carried an *msl3*$^{+}$ transgene that fully complements *msl3* male lethality also exhibited the maternal effect. Initially I tested a *y w*; *P[w*$^{+}$; *msl3*$^{+}$*-TAP]*; *msl3*^{1} stock (Alekseyenko *et al.* 2006) and found that the stock did not exhibit the female-lethal maternal effect (data not shown). This result could have occurred either if an *msl3* mutation is necessary, but not sufficient for the maternal defect, or if the responsible mutation(s) had been lost from the strain. I therefore generated two independent *w*^{1118}; *msl3*^{1}*/TM3* stocks that retained the maternal-effect female-lethal interaction and introduced the *P[w*$^{+}$; *msl3*$^{+}$*-TAP]* transgene into each, taking care to ensure that the suspect *msl3*^{1} chromosome from the *y w* transgenic stock was excluded. Sibling *w*^{1118}; *msl3*^{1} mothers, differing only in whether or not they carried a copy of *P[w*$^{+}$; *msl3*$^{+}$*-TAP]*, were tested to determine if there was a female-lethal interaction with the *sc*^{M6} allele (Table 5). I found that the presence of the fully functional *msl3*$^{+}$ transgene had no effect on the female-lethal interaction as *sc*^{M6}/+ female viability was equally compromised regardless of whether the maternal genotype was functionally *msl3*$^{+}$ or *msl3*$^{-}$ (Table 5). Note that crosses A and B in Table 5 incorporated a second chromosome duplication of *sc*$^{+}$ as a control to ensure that the female-lethal interactions observed remained dependent on the *sc*^{M6} allele present in the progeny females. Taken together, the complementation experiments with *msl3* mutants (Table 4) and *msl3*$^{+}$ transgenes (Table 5) establish that *msl3* mutations are neither necessary nor sufficient for the observed maternal-effect interactions with the XSE genes.

Collectively, the data presented here argue that neither the MSL complex nor its component parts participate in the early embryonic activation of *Sxl*. This undercuts the notion that females use the MSL complex to amplify the X chromosome signal (Gladstein *et al.* 2010) and eliminates an important challenge to standard models for sex determination and dosage compensation. My experiments also suggest the existence of at least one novel maternal regulator of primary sex determination. The molecular identification of this locus, which has escaped detection in a variety of screens, is a high priority, as it is likely to offer new insights into the mechanism of X-chromosome counting.

Comparisons of differing results

The conclusions I reach in this paper are in direct opposition to those made earlier by Gladstein *et al.* (2010) and Horabin (2012), and the data also appear to be in conflict. How then can one explain the differences between the results in this report and those published previously? With respect to *msl3* the answer is clear. Because Gladstein *et al.* (2010) neither mapped the responsible lesion nor performed a complementation test, they reached the incorrect conclusion about the involvement of *msl3* in sex determination. Remarkably, Uenoyama *et al.* (1982) offers evidence that the original isolate of *msl3*^{1} contained a maternal-effect mutation exhibiting a female-lethal interaction with *Sxl*^{f1}. For convenience, data from tables 1 and 2 of Uenoyama *et al.* (1982) are reproduced here with explanatory notes and current nomenclature as Table S2. Crosses 1a and 1b of Uenoyama *et al.* (1982), comparing sibling *msl3*^{1} *red* and *msl3*^{1} *red/TM3* mothers, showed a modest female-lethal maternal-effect interaction with *Sxl*^{f1}. Crosses 2a and 2b, however, reveal that when the experimental mothers carried a recombinant *msl3*^{1} *red* chromosome

Table 5 The *msl3¹* mutation is not necessary for the female-lethal maternal-effect interactions with the XSE *scute*

	♀♀ X ♂♂ sc^{M6} *w/Y; Dp(1;2)Hw^{bap} (sc⁺)/+*				
Crosses A and B	Maternal genotype	2nd Chr. genotype	Female viability (%) compared to brothers	No. female progeny	No. male progeny (reference)
Cross A	*w; msl3¹*	*+/Dp(sc⁺)*	98	65	66
	"	+/+	4	3	79
Cross B	*w: P{msl3⁺-TAP}/+; msl3¹*	*P{msl3⁺-TAP}/Dp(sc⁺)*	98	63	64
	"	*P{msl3⁺-TAP}/+*	7	4	60
	"	*+/Dp(sc⁺)*	127	71	56
	"	+/+	8	6	72
Crosses C and D			♀♀ X ♂♂ sc^{M6} *w/ Y*		
	Maternal genotype	2nd Chr. genotype	Female viability (%) compared to brothers	No. female progeny	No. male progeny (reference)
Cross C	*w; msl3¹*	+/+	2	6	284
Cross D	*w: P{msl3⁺-TAP}/+; msl3¹*	+/+	0.9	1	113
	"	*P{msl3⁺-TAP}/+*	<1.1	0	94

Females in crosses A–D were derived from independently isolated w^{1118}; *msl3¹* lines. It is not known if the *msl3¹* chromosomes retain the *red* allele. Experimental mothers derived from crosses between w^{1118}; *msl3¹* females and w^{1118}; *P{w⁺,msl3⁺-TAP}/+; msl3¹* males and differ only in the presence or absence of the *msl3⁺* transgene. Male genotypes are as shown.

that also included the distal marker *ebony* (*e*), the maternal-effect lethal interaction was lost (see Table S2). In retrospect, it is not surprising that the *msl3¹* stock would carry additional mutations. The *msl3¹* chromosome was recovered from a wild population in Japan in the 1970s (Uchida *et al.* 1981) and may well have experienced mobilization of *P*-elements while introducing markers from laboratory strains. In fact, Uchida *et al.* (1981) reported the original chromosome also bore a nonsex-specific maternal-effect lethal that mapped between *red* and *ebony*. The relationship between that mutation and the one responsible for the interactions with *Sxl* and the XSEs is not known.

It is less obvious what might explain the differences between what is reported here and what was published earlier for *mle*, *msl1*, and *msl2*. I suggest the resolution is that the differences are more illusory than real because Gladstein *et al.* (2010) drew their conclusions from equivocal genetic experiments. Consider figures 1B and 2B of Gladstein *et al.* (2010), which addressed whether maternal or zygotic *msl* mutations enhanced the female-lethal effects of the $sc^{3\text{-}1}$ $sisA^1$ X chromosome. Instead of comparing the viability of the offspring of heterozygous and homozygous *msl* mothers, or between sibling *sc sis/++; msl/+* and *sc sisA/++; +/+* females, Gladstein *et al.* (2010) measured viability with reference to the unrelated wild-type Oregon R strain. They concluded that each *msl* had maternal and zygotic effects on female viability because the lethal effects observed with the *msl* mutants were greater than seen with the Oregon R controls. The problem with this approach is that it relies on quantitative comparisons between flies of unrelated genetic backgrounds. This was compounded by the choice of Oregon R, a strain particularly ill-suited to serve as a reference because it is among those least sensitive to the female-lethal effects of reduced XSE gene dose (Cline 1988). In effect, the choice of control strain may have predetermined that stronger female-lethal effects would be observed with the *msl* mutant strains than with the control, which would have given the appearance of female-lethal interactions where none existed.

The only cases where there appear to be actual conflicts between my findings and those published earlier are with respect to the abilities of maternal $msl1^{L60}$, $msl2^1$, and perhaps mle^1, to create synthetic female-lethal interactions with a *Sxl* null allele (figure 1B in Gladstein *et al.* 2010 *vs.* Table 1 and Table S1). I cannot explain the discordant data because *Sxl/+* heterozygotes should be fully viable in the absence of interacting mutations, but note that Gladstein *et al.* (2010) examined only a single mutant line for each locus and did not determine if the effects were dominant or recessive or if they were maternal. The latter points are important because if the maternal effects were equal between mothers homozygous and heterozygous for these null, or near null, *msl* alleles, that result would suggest the lethally interacting loci were unlikely to be the *msl* genes.

Maternal effects of the msl2 gene?

The question of whether there are maternally contributed *msl* gene products is of crucial importance for assessing a possible role of the MSL complex in the early embryo, whether related to sex determination or early dosage compensation (Gladstein *et al.* 2010; Lott *et al.* 2011; Horabin 2012). There is strong evidence that maternally provided *mle* and *msl1* products function in the embryo as assembly of the MSL complex is delayed, and the lethal period shortened, in the male progeny of mothers mutant for the two loci (Belote and Lucchesi 1980; Franke *et al.* 1996). The most crucial questions concern *msl2* as the protein is essential for the formation and function of the MSL complex. Numerous experimental measures suggest that there is no maternal contribution (Bashaw and Baker 1995; Kelley *et al.* 1995; Rastelli *et al.* 1995; Franke *et al.* 1996; McDowell *et al.* 1996; Lott *et al.* 2011, 2014). RNA-seq data are potentially helpful, but findings from precisely staged embryos that lack *msl2* mRNA (Lott *et al.* 2011) have been criticized for lack of statistical

power (Horabin 2012), whereas bulk samples from 0- to 2-hr-old embryos that contain *msl2* mRNA (FlyBase, cited by Gladstein *et al.* 2010 and Horabin 2012) could be explained by even moderate contamination with older embryos. It is thus worth reexamining the genetic evidence cited (Gladstein *et al.* 2010; Horabin 2012) in support of a maternal effect of *msl2*. Apart from the incomplete experiment in figure 1A of Gladstein *et al.* (2010), which does not formally address a maternal effect, that evidence comes from crosses performed by Uenoyama *et al.* (1982). As reprinted in Table S2, two effects were observed in crosses between $msl2^{27}$ mothers and Sxl^{f1}/Y; $msl2^{27}/SM1$ fathers: a small female-lethal effect and a low frequency of apparent sex transformations in homozygous $msl2^{27}$ female progeny (Uenoyama *et al.* 1982). With respect to viability, there was no evidence for a maternal effect as there was no difference between the female progeny of homozygous and heterozygous $msl2^{27}$ mothers (88 *vs.* 86% viability). With respect to sex transformation, 8% of female $msl2^{27}$ progeny of homozygous mothers exhibited at least one sex-transformed structure compared to 4% of the $msl2^{27}$ progeny of heterozygous mothers (Table S2, crosses IIIa, IIIb; Uenoyama *et al.* 1982). Given that the basis of the sex transformations remains mysterious, that they occur only in 4–8% of progeny, and are affected by the zygotic genotype, the 1982 sex transformation data do not make a convincing case for an *msl2* maternal effect. In light of the strong evidence that there is no detectable maternal *msl2* protein, or mRNA, and the absence of genetic evidence for an *msl2* function in embryos, the simplest interpretation is that *msl2* mRNA and protein first appear, and the MSL complex first assembles, after *Sxl* activity has been set in cycle 14 (Franke *et al.* 1996; Lott *et al.* 2011, 2014).

Conclusions

The data presented here indicate that the MSL complex does not participate in the early regulation of *Sxl* in female embryos. Most *msl* mutants did not exhibit the genetic interactions predicted to occur if the complex directly, or indirectly, affects the early steps in sex determination. In the sole case where an *msl* mutant did interact as predicted, the *msl3* mutation was shown to be unnecessary for the effects, which appear instead, to be the result of at least one unidentified maternal-effect locus. My experimental findings nullify the rationale behind an important alternative model of primary sex determination. By extension, they also undercut the proposal that the MSL complex regulates the process by which some X-encoded genes are dosage compensation prior to the large-scale activation of the zygotic genome (Lott *et al.* 2011).

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Primary Sex Determination in *Drosophila melanogaster* Does Not Rely on the Male-Specific Lethal Complex

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TABLE S1. There are no female-lethal interactions between maternal *mle*, *msl1*, or *msl2* *mutations* and zygotic sex determination mutations.

	Viability (%) of females of indicated X chromosome genotype relative to sibling males (# reference).					
maternal MSL genotype	Sxl^{f1}/+		$sisA^{1}$/+		sc^{M6}/+	
mle^{1}			110	(212)	97	(173)
mle^{1}/mle^{9}	99	(205)	107	(183)	96	(119)
$msl1^{1}$			103	(119)	101	(131)
$msl1^{1}/ msl1^{KmB}$			95	(118)	102	(194)
$msl1^{1}/msl1^{\gamma 216}$					104	(113)
$msl1^{L60}$	104	(168)			123	(109)
$msl1^{L60}$ $msl2/msl1^{KmB}$					97	(150)
$msl1^{L60}$ $msl2/msl1^{1}$					110	(182)
$msl2^{1}$					111	(98)
$msl2^{1}/msl2^{227}$					104	(98)
+ $msl2^{1}/msl1^{L60}$ $msl2$					110	(70)
$msl2^{P17}/ msl2^{227}$			97	(115)	100	(95)
$msl2^{P22}/ msl2^{KmA}$					97	(175)

Paternal genotypes: *y w* cm^{1} Sxl^{f1} ct^{6}/Y; *y* cm^{1} ct^{6} $sisA^{1}/Y$; sc^{M6} *w/Dp(1;Y)*y^{2}*61l (sc+) or* sc^{M6} *w/Y (msl-*1^{L60} cross). Female parents from stocks: *w;* pr^{1} mle^{1}*/In(2LR)Gla;* mle^{9} cn^{1} bw^{1}*/CyO;* $msl1^{1}$ cn^{1} bw^{1}*/CyO;* $msl1^{KmB}$*/CyO;* $msl1^{\gamma 216}$ cn^{1} bw^{1}*/CyO;* $msl1^{L60}$*/In(2LR)Gla; y w;* $msl1^{L60}$ $msl2^{unidentified}$*/In(2LR)Gla;* $msl2^{1}$*/CyO;* $msl2^{227}$ bw^{1}*/CyO; In(2L)*$msl2^{P17}$*/CyO; In(2L)*$msl2^{P22}$*/CyO,* $msl2^{KmA}$*/CyO.*

TABLE S2. The data of Uenoyama et al. (1982) do not provide evidence for a maternal-effect female-lethal interaction between the *msl*3 and *msl2* mutations and Sxl^{f1}.

Cross		No. female progeny		No. male progeny (reference)	Female viability compared to reference males		Female progeny w/ sex transformations
		msl/msl	*msl* /+	msl/+			
Ia	$msl3^1$ *red* ♀♀ *X* ♂♂ Sxl^{f1}/*Y;* $msl3^1$ *red/TM3*	198	121	215	$msl3^1$ $msl3^1$/+ combined	92% 56% 74%	42% 15% -
Ib	$msl3^1$ *red /TM3* ♀♀ *X* ♂♂ Sxl^{f1}*/Y;* $msl3^1$ *red/TM3*	106	252	231	$msl3^1$ $msl3^1$/+ combined	92% 109% 100%	8% 4% -
IIa	$msl3^1$ *red e* ♀♀ X ♂♂ Sxl^{f1}/Y; $msl3^1$ *red*/TM3	269	221	252	$msl3^1$ $msl3^1$/+ combined	107% 88% 97%	14% 7% -
IIb	$msl3^1$ *red e /TM3* ♀♀ *X* ♂♂ Sxl^{f1}*/Y;* $msl3^1$ *red/TM3*	129	240	223	$msl3^1$ $msl3^1$/+ combined	116% 108% 111%	0% 0%
IIIa	$msl2^{27}$ ♀♀ *X* ♂♂ Sxl^{f1}*/Y;* $msl2^{27}$*/SM1*	195	239	247	$msl2^{27}$ $msl2^{27}$/+ combined	82% 97% 88%	8% 0.4%
IIIb	$msl2^{27}$*/SM1* ♀♀ *X* ♂♂ Sxl^{f1}*/Y;* $msl2^{27}$*/SM1*	241	560	603	$msl2^{27}$ $msl2^{27}$/+ combined	80% 93% 86%	4% 1%

All data are extracted from Tables 1 and 2 of Uenoyama et al. (1982). The original tables also include data on two *mle* alleles. *msl3*^{1} was reported by its synonym *mle(3)-132*. *msl2*^{27} appears to be a different allele than *msl2*$^{227.}$