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Coordinated Regulation of Heterochromatic Genes in *Drosophila melanogaster* Males

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ABSTRACT

Dosage compensation modifies the chromatin of X-linked genes to assure equivalent expression in sexes with unequal X chromosome dosage. In Drosophila dosage compensation is achieved by increasing expression from the male X chromosome. The ribonucleoprotein dosage compensation complex (DCC) binds hundreds of sites along the X chromosome and modifies chromatin to facilitate transcription. Loss of *roX* RNA, an essential component of the DCC, reduces expression from X-linked genes. Surprisingly, loss of *roX* RNA also reduces expression from genes situated in proximal heterochromatin and on the small, heterochromatic fourth chromosome. Mutation of some, but not all, of the genes encoding DCC proteins produces a similar effect. Reduction of *roX* function suppresses position effect variegation (PEV), revealing functional alteration in heterochromatin. The effects of *roX* mutations on heterochromatic gene expression and PEV are limited to males. A sex-limited role for the *roX* RNAs in autosomal gene expression was unexpected. We propose that this reflects a difference in the heterochromatin of males and females, which serves to accommodate the heterochromatic Y chromosome present in the male nucleus. *roX* transcripts may thus participate in two distinct regulatory systems that have evolved in response to highly differentiated sex chromosomes: compensation of X-linked gene dosage and modulation of heterochromatin.

MANY male animals carry a euchromatic, gene-rich X chromosome and a largely heterochromatic, gene-poor Y chromosome (Charlesworth 1991). Highly differentiated sex chromosomes pose several problems for the organisms that carry them, the most obvious being that males have a potentially lethal imbalance in X-linked gene dosage. The process of dosage compensation equalizes X-linked gene expression between XY males and XX females, thereby maintaining a constant ratio of X-linked to autosomal gene products (Gupta *et al.* 2006; Nguyen and Disteche 2006). While strategies to accomplish this differ between species, a unifying theme is coordinated regulation of a whole chromosome by selective recruitment of chromatin-modifying proteins (Lucchesi *et al.* 2005). Intriguing links between seemingly unrelated systems that coordinately regulate large groups of genes have been observed. A recent study has shown that the *Caenorhabditis elegans* dosage compensation protein DPY-28 also limits meiotic crossover, another chromosomewide process (Tsai *et al.* 2008). In an interesting parallel, we show here that some members of the *Drosophila melanogaster* dosage compensation complex (DCC) also regulate heterochromatin function by modulating the expression of heterochromatic genes in males.

The Drosophila DCC, also known as the male-specific lethal or MSL complex, mediates dosage compensation by increasing expression from the single X chromosome of males. Two noncoding RNAs, *roX1* and *roX2* (***R**NA **o**n the **X** 1* and *2*), are essential components of this complex but are functionally redundant for dosage compensation (Meller and Rattner 2002). The *roX* transcripts assemble with the MSL proteins, encoded by *maleless* (*mle*), the *male-specific lethals 1*, *-2*, and *-3* (*msl1*, *-2*, and *-3*) and *males absent on first* (*mof*). A cotranscriptional targeting mechanism localizes the complex within the body of genes (Kind and Akhtar 2007; Larschan *et al.* 2007). The complex directs acetylation of histone H4 on lysine 16 (H4Ac16), a modification associated with increased expression (Akhtar and Becker 2000). Mutation of a single *roX* gene is without phenotype, but simultaneous mutation of both *roX* genes dramatically lowers male survival (Deng *et al.* 2005). Females appear unaffected by loss of the *roX* transcripts. In *roX1 roX2* males the MSL proteins bind at ectopic autosomal

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sites and the chromocenter. Microarray expression analysis of *roX1 roX2* (null for *roX* function) and *roX2* (control) male larvae confirmed that *roX* RNA is required for full expression of X-linked genes in males (Deng and Meller 2006).

The highly preferential binding of the Drosophila DCC to the X chromosome promoted the idea that these molecules contribute solely to X-linked gene expression, but this notion has been challenged by the finding that some of the MSL proteins act outside of the complex as general transcriptional regulators. MLE is enriched on the male X chromosome but also found at sites of active transcription in males and females (Kotlikova *et al.* 2006). MOF is an integral member of the DCC and is enriched on the male X chromosome. In addition, MOF is more modestly enriched at the 5′ end of transcribed genes throughout the genomes of both sexes (Kind *et al.* 2008). In spite of the general role of these two factors, mutations in *mle* and *mof* are lethal only to males.

We now show that *roX* RNA influences the expression of heterochromatic genes, including those on the small fourth chromosome and in heterochromatic regions of the second and third chromosomes, in male larvae. The MSL1 and MSL3 proteins are necessary for full expression of these genes, but MSL2, a protein of central importance for X chromosome dosage compensation, is unnecessary. This demonstrates that the intact DCC is not involved in regulation of heterochromatic genes. Consistent with the idea that the *roX* RNAs affect heterochromatin function in males, we find that a *roX1 roX2* chromosome is a potent modifier of position effect variegation (PEV) in males, but not in females. We propose a new role for these molecules in sex-specific regulation of heterochromatin. We further speculate that this serves to accommodate the differences in heterochromatin content in males and females that result from the presence of a large, heterochromatic Y chromosome in the male nucleus.

MATERIALS AND METHODS

Drosophila strains: Flies were maintained at 25° on standard cornmeal–agar fly food in a humidified incubator. The $roX1^{ex6}$, $roX1^{SMC17A}$, $roX1^{mb710}$, roX^{ex7B}, and $roX1^{ex33}$ mutations have been described (Meller *et al.* 1997; Deng *et al.* 2005). Elimination of *roX2* is accomplished by combining a lethal deletion removing *roX2* and essential flanking genes, Df(1)52, with a cosmid insertion carrying essential deleted genes but lacking *roX2* [$w^+4\Delta4.3$] (Meller and Rattner 2002). For convenience this combination is referred to as *roX2*. A deleted cosmid lacking w^+ was used in studies of PEV [$w^-4\Delta4.3$]. Mutations in *msl1*, *msl2*, *msl3*, and *pof* have been previously described (Lindsley and Zimm 1992; Johansson *et al.* 2007). Variegating transgene insertions used in this study have been described (Sun *et al.* 2000; Maggert and Golic 2002; Yan *et al.* 2002).

Males mutated for *msl1* and *msl2* generally are not healthy enough to be selected by gonad morphology. To select $msl1^1$ males, *yw*; $msl1^1$ females were mated to y^+w; $msl1^1/CyOy^+$ males. $msl1^1$ males are *y* and have brown mouth hooks. To select $msl2^1$ males, *yw*; $msl2^1$ females were mated to y^+w [w^+PD27]; $msl2^1$ males. The [w^+PD27] insertion carries *msl2* and rescues $msl2^1$ males (Kelley *et al.* 1995). All male offspring from this cross are *yw*; $msl2^1$ and all female offspring are y^+.

Histology: Immunhistochemical detection on polytene chromosomes of wild- type and $roX1^{ex6}roX2$ males was done as previously described (Kelley *et al.* 1999). POF antibody, a generous gift from J. Larsson, was used as previously described (Larsson *et al.* 2001).

Quantitative RT–PCR: Total RNA was made from three groups of at least 50 larvae of each genotype. One microgram of total RNA was reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). Quantitative PCR was performed as previously described (Deng *et al.* 2005). A total of 34 genes were selected from four different gene groups (2 and 3 euchromatic, 2 and 3 heterochromatic, fourth chromosomal, and X chromosomal). The selected genes were expressed at moderate levels, displayed uniform absorbance in arrays of the same genotype, and reflected the average change in expression for their gene group in *roX1 roX2* males. An exception is the X-linked *Lsp-1α* gene, which is known to escape dosage compensation. *Bigmax* and *Dmn* are autosomal genes that proved reliable for normalization of expression (data not shown). The primers used in this study are presented in supporting information, Table S1.

Gene expression microarrays: Total RNA was prepared from groups of at least 50 third instar larvae using the TRIzol method (Invitrogen) and purified using the RNeasy kit (QIAGEN). Three independent RNA preparations for each genotype served as templates for probe synthesis (see protocol at www.Affymetrix.com). Probes were hybridized to Affymetrix Drosophila Genome 2.0 chips (Santa Clara, CA). Background corrected intensity values were quantile normalized (Irizarry *et al.* 2003). In brief, all probe intensities from mutant and control arrays were assembled into a single ranking. Probes from individual chips were assigned the value of the corresponding quantile, thus preserving the rank order within a chip and standardizing intensity distribution across all chips. Intensities were summarized into one expression value per sample and probe set using the robust multi-array average (RMA) algorithm. The Affymetrix MAS5.0 Present/Absent calls were used to filter out probe sets not present in at least two out of three replicates of each genotype.

Genes and probe sets (Berkeley Drosophila Genome Project annotation release 5.8) were sorted to enrich for heterochromatic genes on the basis of the boundaries between heterochromatic and euchromatic regions (Smith *et al.* 2007; Hoskins *et al.* 2007). The coordinates of these boundaries are: 2R;1-1285689, 2L;22000975-23011544, 3R;1-378656, 3L;22955576-24543557, X;22030326-22422827. The coordinates for heterochromatin that is not contiguous with assembled arm sequences are 2LHet;1-368872, 2RHet;1-3288761, 3LHet;1-2555491, 3RHet;1-2517507, XHet;1-204112, YHet; 1-347038. Only probe sets assigned to a chromosome were used. Genes and probe sets assigned to heterochromatic regions were obtained from FlyBase GBrowse. The corresponding gene and probe set information was obtained from the Affymetrix Drosophila_2 annotation file (Drosophila_2.na25) released on March 17, 2008 (Liu *et al.* 2007).

Statistical methods and descriptions: The $\log_2$ fold change of each gene was computed as the $\log_2$ mean RMA expression of mutant samples minus the $\log_2$ mean RMA expression of control samples. The significance of differences between groups was assessed by the Wilcoxon test. Analyses were performed in the R software environment (www.r-project.org)

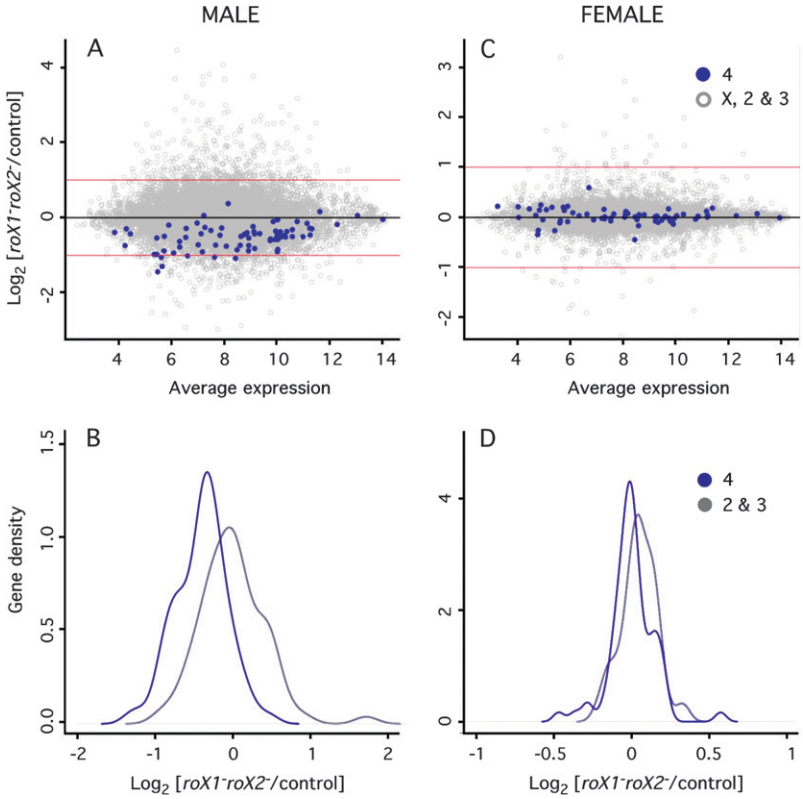

Figure 1.—Expression of the fourth chromosome is reduced in *roX1 roX2* males. (A) In *roX1 roX2* males the expression of fourth-linked genes (blue) decreases in comparison with the rest of the genome (gray). Points represent the $\log_2$ of the ratio of gene expression in $roX1^{SMC17A}$ *roX2* males to control males (*roX2*) plotted against expression level ($\log_2$ absorbance). Numbers and types of genes plotted are 9880 nonfourth-linked genes and 74 fourth-linked genes. (B) The density distribution of $\log_2$ expression (mutant/control) for fourth-linked genes (blue) and second and third chromosome genes (gray) in males. The distribution of fourth-linked genes differs significantly from the remaining autosomal genes (adjusted *P*-value $< 6.6 \times 10^{-16}$; Wilcoxon test). (C) In *roX1 roX2* females the expression of fourth-linked genes (blue) is unchanged. The rest of the genome is shown in gray. Data is presented as the $\log_2$ of the ratio of gene expression in $roX1^{SMC17A}roX2$ females to control females ($roX1^{SMC17A}roX2$; [w^+Hs83-*roX1*$^+$]) plotted against expression level ($\log_2$ absorbance). Genes contributed to this analysis are 8433 nonfourth-linked and 69 fourth-linked genes. (D) The density distribution of $\log_2$ expression (mutant/control) for fourth-linked genes (blue) and second and third chromosome genes (gray) in female larvae. The distribution of fourth-linked genes is not significantly different from that of the second and third chromosomes (adjusted *P*-value 0.92). Only genes present in at least two out of three replicates were included. See File S1 for details of microarray hybridization and analysis.

using Bioconductor (www.bioconductor.org) (Gautier *et al.* 2004; Smyth 2005). The raw data can be downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, GSE3990; GSE12054; GSE12076).

qRT–PCR data were analyzed by the efficiency corrected comparative quantification method (Pfaffl 2001). Ct values for three biological replicates (each containing two technical replicates) per genotype were averaged into one Ct value per gene. The relative quantities (mutant:control) were tested for normality using the Shapiro–Wilk test. As the MSL1 and MSL3 data sets were not normally distributed, the nonparametric Mann–Whitney *U* test was used to determine significance. Descriptive statistics are in File S1.

RESULTS

Fourth-linked genes are underexpressed in *roX1 roX2* male larvae: A microarray study was conducted to compare genomewide expression in *roX1 roX2* males (null for *roX* function) and *roX2* (control) males. *roX1 roX2* males rarely survive past the third larval instar, but males mutated for a single *roX* gene are developmentally normal with full survival. In support of this, the localization of the MSL proteins on the polytenized X chromosome of *roX2* and wild-type males appears identical on chromosome preparations (Deng and Meller 2006). An overall reduction in X-linked gene expression was observed in the *roX1 roX2* male larvae (Deng and Meller 2006). However, to our surprise, expression of genes situated on the small fourth chromosome was also reduced by ~50% in *roX1 roX2* males (Figure 1A). qRT–PCR confirmed the reduction in fourth–linked gene expression (Table S2). A plot of the $\log_2$ of the expression ratio (mutant: control) of euchromatic genes on the second and third chromosomes has a distribution centered near zero, but the fourth chromosome is shifted left, reflecting this overall decrease (Figure 1B). The change in expression of fourth-linked genes is significantly different from that of any other chromosome (Wilcoxon test, *P*-value $< 10^{-16}$).

To determine if reduced expression is due to loss of the *roX* transcripts, the expression of fourth-linked genes was examined in *roX1 roX2* males carrying a *roX1* transgene, [w^+Hs83-*roX1*$^+$], which rescues both male survival and X localization of the MSL proteins. This transgene fully restores expression of five X-linked genes (Figure 2A). Expression of 10 of 11 fourth chromosome genes is also largely restored. We conclude that the absence of *roX* RNA reduces expression of fourth–linked genes.

The *roX* RNAs appear completely redundant for dosage compensation of the X chromosome (Meller and Rattner 2002). To determine if the *roX* genes are also redundant for regulation of the fourth chromosome, expression of individual genes was compared in *roX1 roX2* males (value set to 1, Figure 2B), *roX2*

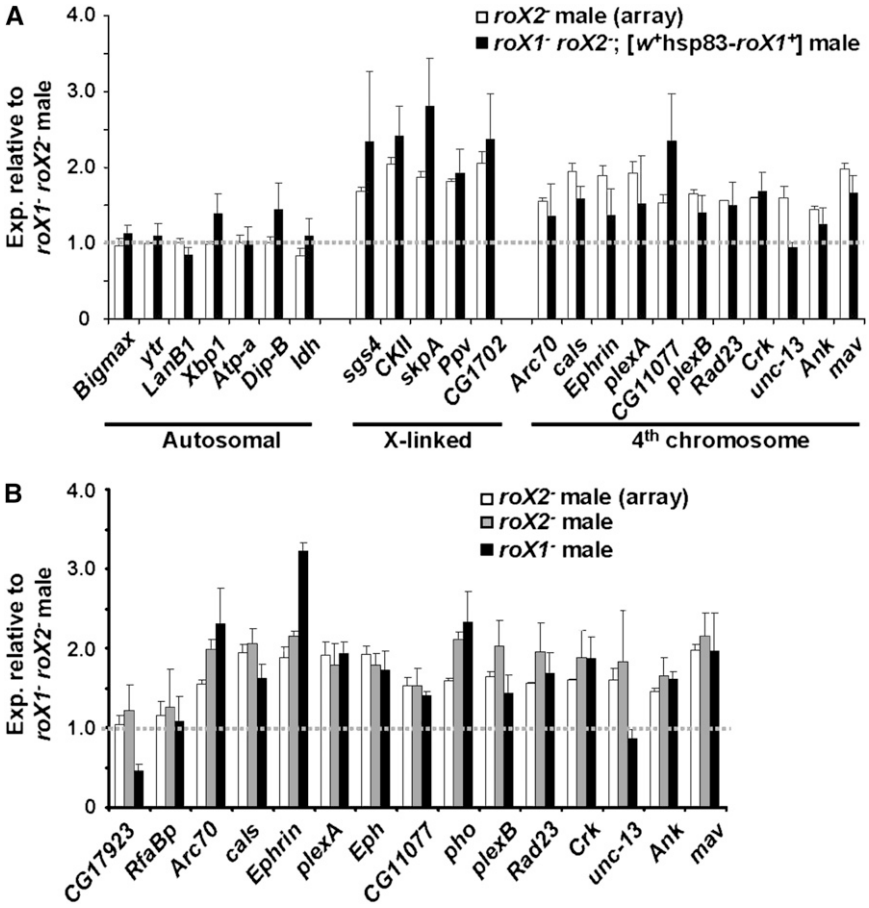

FIGURE 2.—*roX1* and *roX2* are necessary but redundant for full expression of fourth-linked genes. (A) Expression of fourth-linked genes is restored by a *roX1* transgene. Quantitative RT–PCR was used to compare the expression of individual genes in male $roX1^{SMC17A}roX2$ larvae (set to 1) and $roX1^{SMC17A}roX2$; [w^+hsp83-$roX1^+$] larvae (solid bars). The expression in *roX2* larvae as determined by microarray (Figure 1A) is shown for comparison (open bars). (B) *roX1* and *roX2* are redundant for expression of fourth-linked genes in males. Expression of individual genes was measured by qRT–PCR in male $roX1^{SMC17A}roX2$ larvae (set to 1), *roX2* larvae (shaded bars) and $roX1^{SMC17A}$ larvae (solid bars). The expression of each gene in male *roX2* larvae by microarray analysis is presented for comparison (open bars).

males (shaded bars) and *roX1* males (solid bars). Almost all of the fourth-linked genes display similar expression in single mutants but have considerably lower expression in the *roX1 roX2* double mutant. Only two tested genes, *CG17923* and *unc-13*, behave differently in *roX2* and *roX1* male larvae. We speculate that these genes are influenced by genetic factors on the *roX1* mutant chromosome used in these studies. This is consistent with the inability of a *roX1* transgene to restore *unc-13* expression (Figure 2A). We conclude that the *roX* genes are redundant for expression of fourth-linked genes.

The idea that an autosome is differentially regulated in males and females seemed highly unlikely. However, *roX1* is abundant in early embryos of both sexes, and thus might contribute to the expression of fourth-linked genes in both sexes (MELLER 2003). To determine if *roX* also influences fourth-linked gene expression in females, microarrays were hybridized to probes generated from *roX1 roX2* and *roX1 roX2*; [w^+Hs83-$roX1^+$] female larvae. Females are not developmentally disrupted by elimination of *roX1* and *roX2*, which may account for the narrow range of expression ratios (compare Figure 1, A and C). The presence of the constitutively expressed *roX1* transgene had no influence on fourth-linked genes in *roX1 roX2* females (Figure 1, C and D). The *roX* RNA requirement for full expression of fourth-linked genes, therefore, appears limited to males. Several fourth-linked genes were measured in wild-type male and female larvae to determine whether their expression is normally higher in males. With the exception of the male-preferential *CG17923*, the fourth-linked genes examined are expressed at similar levels in males and females (Figure S1). This conclusion is supported by published microarray studies of *D. melanogaster* male and female larvae and adults (PARISI *et al.* 2003; LIU *et al.* 2005).

Ectopic localization of the MSL proteins does not repress fourth-linked genes: There are several potential mechanisms by which loss of the *roX* transcripts might reduce expression from the fourth chromosome in males. The fourth chromosome shows particularly strong ectopic binding of the MSL proteins in *roX1 roX2* males (MELLER and RATTNER 2002; DENG *et al.* 2005). It is possible that the abnormal binding of these proteins to the fourth chromosome represses expression. To test this idea, we examined expression of fourth-linked genes in female larvae that display an identical pattern of MSL localization. MSL2 is normally present only in males. When MSL2 is expressed in females from the [w^+Hs83-M2] transgene, intact MSL complexes form and bind to both X chromosomes, resulting in high female mortality (KELLEY *et al.* 1995).

Females are rescued by mutation of both *roX* genes. These females have normal survival but display ectopic MSL localization indistinguishable from that observed in *roX1 roX2* males (Deng *et al.* 2005). We reasoned that if mislocalized MSL proteins repress expression, this repression will be evident in *roX1 roX2*; [w^+Hs83-M2] females, but not in *roX1 roX2* females. Expression of a panel of genes was measured in female larvae of these genotypes by qRT–PCR. Autosomal genes on the second and third chromosomes remain unchanged, but expression from the fourth chromosome actually increases when MSL2 is expressed in *roX1 roX2* females (Figure S2). Although unexpected, this increase is consistent with the accumulation of low levels of H4Ac16 at sites of mislocalized MSL proteins (Deng and Meller 2006). We conclude that ectopic binding of the MSL proteins to the fourth chromosome does not cause repression of fourth-linked genes in *roX1 roX2* males.

The possibility that mislocalized MSL proteins disrupt centromere function, leading to frequent loss of the fourth chromosome, was also considered. Examination of mitotic neuroblast preparations revealed that the number of visible fourth chromosomes was identical in nuclei from *roX1 roX2* and control males (Figure S3). Elevated loss of the fourth chromosome in somatic tissues is thus unlikely to be the source of reduced fourth-linked gene expression.

***roX* does not interact genetically with *painting of fourth*:** Several lines of evidence suggest a close relationship between the fourth and the X chromosomes of *D. melanogaster* (Larsson and Meller 2006). Most suggestive is the chromosomewide targeting mechanism revealed by *painting of fourth* (POF), which binds along the banded portion of the fourth chromosome in both sexes. In the related *D. ananassae* and *D. malerkotliana*, POF binds to the fourth chromosome of both sexes but colocalizes with MSL3 on the male X chromosome, suggesting a role in X chromosome compensation in these species (Larsson *et al.* 2004). While POF is nonessential in *D. melanogaster* with two fourth chromosomes, it is necessary for the survival of flies with a single fourth chromosome, suggesting that POF functions in dosage compensation of the *D. melanogaster* fourth chromosome (Johansson *et al.* 2007). POF is a putative RNA-binding protein. This raised the suspicion that, like the MSL proteins, POF might require *roX* RNA for correct localization. We examined the localization of POF in *roX1 roX2* and control males, but no differences were discernible by immunostaining of polytene chromosomes (Figure S4). While the *roX* RNAs are easily detected over the polytenized X chromosome of males, they are not detected binding to the fourth chromosomes (data not shown). The *roX* RNAs are therefore unnecessary for POF binding and do not colocalize with POF on the fourth chromosome in the larval salivary gland.

No evidence of genetic interactions between *roX1 roX2* chromosomes and *pof* mutations could be detected. *roX1 roX2* chromosomes carrying partial loss-of-function *roX1* mutations that allow the recovery of escaper males were used for these analyses. Mutation of one copy of *pof* does not reduce the recovery of males carrying the severely affected $roX1^{mb710}roX2$ chromosome (Table S3). Elimination of POF in males carrying the partial loss-of-function $roX1^{ex7B}roX2$ chromosome resulted in a modest decrease in survival consistent with differences in genetic background (22% $roX1^{ex7B}roX2$ escapers and 18% $roX1^{ex7B}roX2$; $pof^{\Delta 119}$ escapers; see Table S4). Unlike *pof* mutants, flies carrying a single fourth chromosome and the partial loss-of-function $roX1^{ex7B}roX2$ chromosome were recovered (Table S5). This suggests that the *roX* transcripts do not participate in compensation of fourth-linked genes. While POF is proposed to function in dosage compensation of the fourth chromosome in both sexes, *roX* RNA has a male-limited effect on expression of fourth-linked genes. These studies indicate that the *roX* and *pof* genes do not participate in the same process.

Autosomal genes in heterochromatic regions require *roX* for full expression: The fourth chromosome has additional unusual features, including enrichment for heterochromatin (reviewed in Riddle and Elgin 2006). Genes embedded in heterochromatin are presumed to have specialized regulatory features that enable expression in spite of their repressive heterochromatic environment (Yasuhara and Wakimoto 2006). If the proximity of fourth-linked genes to heterochromatin makes them dependent upon *roX* RNA, expression of genes in pericentric heterochromatin on the second and third chromosomes may also depend on *roX* RNA. To enrich for genes in or near heterochromatin, microarray probe sets were sorted using the heterochromatin/euchromatin boundaries from the Drosophila genome annotation (see materials and methods). Expression of heterochromatin-enriched genes on the second and third chromosomes decreased by 17% in *roX1 roX2* males, but remained unchanged in *roX1 roX2* females (Figure 3). This suggests that proximity to heterochromatin could account for the dependence of fourth-linked genes on *roX* RNA.

MSL1 and MSL3, but not MSL2, contribute to expression of heterochromatic genes: It is possible that the failure of dosage compensation affects fourth-linked and heterochromatic genes indirectly. This might occur by underexpression of critical X-linked factors, or by the global redistribution of chromatin proteins upon disruption of the male X chromosome. To determine if other mutations preventing dosage compensation also reduce expression of fourth-linked and heterochromatic genes, expression was measured in animals lacking different components of the DCC. MSL1 and MSL2 are of central importance to dosage compensa-

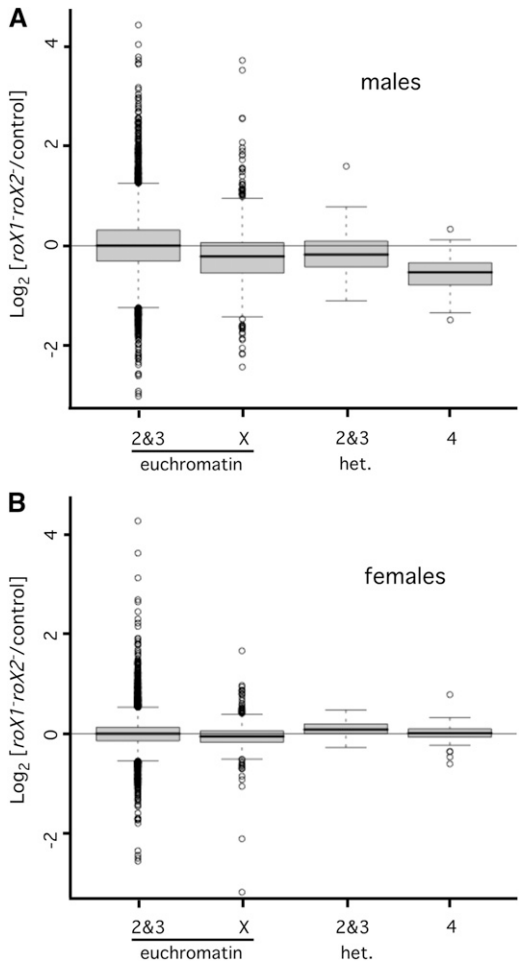

Figure 3.—Genes situated in proximal heterochromatin require *roX* RNA for full expression in males. (A) Genes in proximal heterochromatin have reduced expression in *roX1*^{SMC17A}*roX2* male larvae. Box plots were generated using the $\log_2$ expression ratios (mutant/control) presented in Figure 1A. The mean expression of genes in proximal heterochromatin on the second and third chromosomes decreases by 0.17 in *roX1*^{SMC17A}*roX2* males (adjusted *P*-value of 0.003). The mean expression of X-linked genes decreases by 0.24, and expression of fourth-linked genes decreases by 0.58. Changes of the X and fourth chromosome have an adjusted *P*-value of $<6.6 \times 10^{-16}$. Only genes present in at least 2 out of 3 arrays contributed to this analysis (8347 in second and third euchromatin; 1533 in X euchromatin, 73 in second and third heterochromatin, and 74 on the fourth chromosome). (B) Fourth-linked and heterochromatic genes do not require *roX* RNA for full expression in females. Box plots were generated using the $\log_2$ expression ratios (mutant/control) presented in Figure 1C. The mean change in expression of X-linked genes in *roX1*^{SMC17A}*roX2* females is −0.04. Second and third chromosome heterochromatic genes and fourth-linked genes have a slight average increase (0.06 and 0.01, respectively) that is not statistically significant. Only genes present in at least 2 out of 3 arrays contributed to this analysis (7097 in second and third euchromatin, 1336 in X euchromatin, 57 in second and third heterochromatin, and 69 on the fourth chromosome). Enrichment for heterochromatic genes is described in File S1.

tion (Copps *et al.* 1998; Li *et al.* 2005). All chromatin binding by the remaining DCC proteins is absent in males lacking either MSL1 or MSL2 (Baker *et al.* 1994; Palmer *et al.* 1994). Reduced X chromosome expression has been demonstrated in male S2 cells following RNAi knock down of *msl2* (Hamada *et al.* 2005). Examination of data from this study detected no change in fourth-linked or heterochromatic genes (Figure S5). While this is suggestive, it is possible that RNAi knock down is incomplete, or that MSL2 acts transiently to establish a male-specific configuration of heterochromatin. To address these concerns, microarrays were hybridized to probes from *msl2*^{1} male larvae and *msl2*^{1}/+ controls. MSL2 is not maternally deposited, therefore *msl2*^{1} larvae lack this protein entirely (Rastelli *et al.* 1995). Expression from the X chromosome was reduced by 21% in male *msl2*^{1} larvae (Bonferroni corrected *P*-value $< 2.2 \times 10^{-16}$; Figure 4A). While this is less than the 50% reduction expected for dosage compensation failure, it compares well with the 22% decrease upon MSL2 knock down in S2 cells (Hamada *et al.* 2005). By contrast, expression of heterochromatic and fourth-linked genes appears unchanged or slightly increased in *msl2*^{1} males (0.07- and 0.11-fold, respectively). We conclude that the intact DCC is not necessary for full expression of fourth-linked and heterochromatic genes.

To determine the role of other key MSL proteins, we examined the expression of a panel of autosomal and fourth-linked genes in *msl1*^{1} and *msl1*^{1}/+ male larvae. Expression of fourth-linked genes decreased by 38% in comparison with euchromatic genes on the second and third chromosomes, and genes situated in heterochromatic regions were reduced by 33% (Figure 4B). These changes are significant at the 0.003 and 0.002 level when evaluated using a two-tailed Mann–Whitney *U* test (see materials and methods for details). Expression was also measured in *msl3*^{2} males and their *msl3*^{2}/+ brothers (Figure 4C). Expression from fourth-linked genes decreased by 24%, and expression from heterochromatic genes decreased by 36% in *msl3*^{2} males. These changes are statistically significant at the 0.032 and 0.036 level. Taken together, these findings indicate that some, but not all, of the MSL proteins are necessary for full expression of fourth-linked and heterochromatic genes in male flies.

***roX* RNA contributes to normal heterochromatin function in males:** Transgenes inserted in heterochromatin display a variegated silencing (PEV) (Wallrath and Elgin 1995). Modulation of this silencing is a sensitive reporter for local chromatin architecture and has been used to identify mutations that affect heterochromatin function. As loss of *roX* influences the expression of genes situated in heterochromatic regions, we suspected that *roX1 roX2* chromosomes might also affect PEV. Adult male *roX1*^{ex33A}*roX2* and *roX2* (control) flies that carry transgenes displaying PEV were generated. *roX1*^{ex33A} is a partial loss-of-function

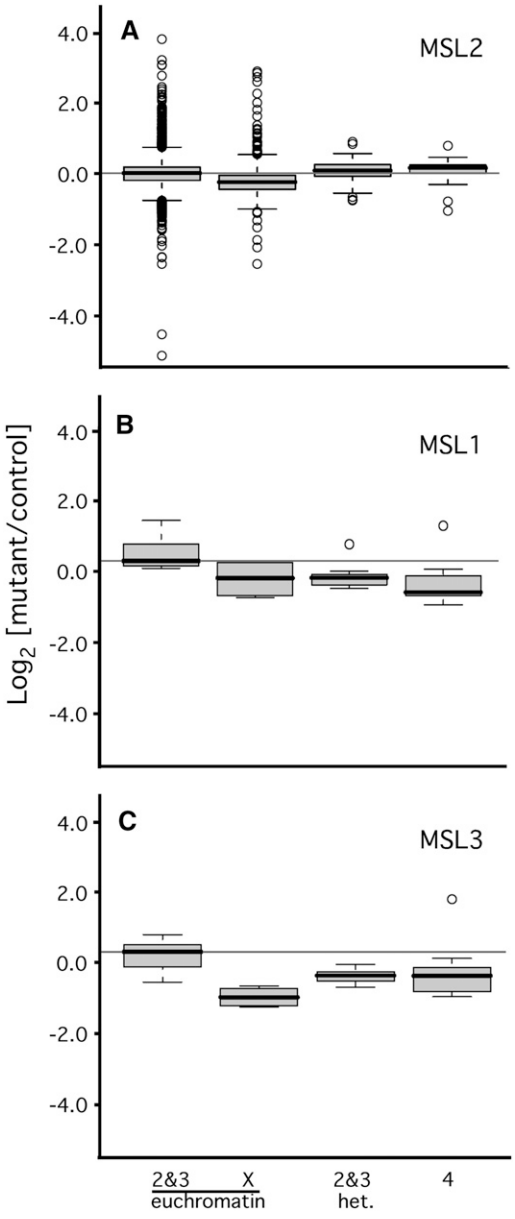

Figure 4.—MSL1 and MSL3, but not MSL2, are required for full expression of autosomal genes. (A) Fourth-linked and heterochromatic genes are not misregulated in *msl2*^{1} male larvae. Box plots represent the $\log_2$ (mutant:control) expression of the indicated groups of genes. Expression was measured by hybridizing microarrays with probes generated from *msl2*^{1} and *msl2*^{1}/+ (control) male larvae. Enrichment for heterochromatic genes is described in materials and methods. (B) Fourth-linked and heterochromatic genes require MSL1 for full expression. Expression of a panel of genes was measured in *msl1*^{1} males and their heterozygous brothers (controls). Seven euchromatic genes on the second and third chromosomes; 4 euchromatic X-linked genes, 12 fourth-linked genes, and 10 heterochromatic genes on the second and third chromosome were assayed (see Table S1). Expression of heterochromatic and fourth-linked genes differs from euchromatic genes on the second and third chromosomes at the 0.003 and 0.002 confidence level, respectively. (C) Fourth-linked and heterochromatic genes require MSL3 for full expression. Expression of a panel of genes was measured in *msl3*^{2} males and their heterozygous brothers (controls). Seven euchromatic genes on the second and third chromosomes, 4 euchromatic X-linked genes, 13 fourth-linked genes, and 10 heterochromatic genes were measured. Expression of heterochromatic and fourth-linked genes differs significantly from euchromatic genes on the second and third chromosomes at the 0.032 and 0.036 confidence levels. *Dmn* was used to normalize amplification. Box plots were generated using Bioconductor R. See materials and methods for details of statistical analyses.

mutation that supports ~50% survival of males lacking *roX2* (Deng *et al.* 2005). PEV was detected by expression of the *white* (*w*) eye color marker. Increased silencing, or enhancement of PEV, results in fewer red facets in the eye, and decreased silencing, or suppression of PEV, results in more red pigmentation. The expression of *w* from two different insertions on the Y chromosome is similar in *roX1*^{ex33A}*roX2* and control *roX2* males (Figure 5C). In contrast, expression from three insertions on the fourth chromosome is dramatically increased in *roX1*^{ex33A}*roX2* males, but not in females (Figure 5, A and B). The modifying effect of *roX1 roX2* on PEV is therefore limited to males. As was observed for other *roX* phenotypes, suppression of PEV requires simultaneous mutation of both *roX* genes (data not shown). Expression from insertions on the second chromosome is modestly increased in *roX1*^{ex33A}*roX2* males (Figure 5D). The *roX1*^{ex33A}*roX2* chromosome is therefore a potent suppressor of PEV, but suppression depends on the sex of the fly as well as the position of the variegating transgene.

DISCUSSION

Our findings suggest that *roX* RNA participates in two distinct biological processes that coordinate regulation of large regions; X chromosome dosage compensation and normal heterochromatin function. One of the most striking and unexpected features of our study is the male specificity of *roX* involvement at heterochromatin. In light of this, it is interesting that mutations in heterochromatin proteins do present sex-biased phenotypes. Depletion of HP1, a major component of heterochromatin, causes higher male lethality and considerably more gene misregulation in males (Liu *et al.* 2005). The same study identified differences in HP1 distribution in males and females. Our findings suggest that these differences may arise from the fact that heterochromatin itself is different in males and females. The genetic control of heterochromatin has been the subject of many screens for modifiers of PEV. Neither *msl* nor *roX* genes have been identified by this method. Simultaneous mutation of both *roX* genes is required to suppress PEV, making their identification through random mutagenesis highly unlikely (data not shown). In addition, loss of any MSL protein is lethal in

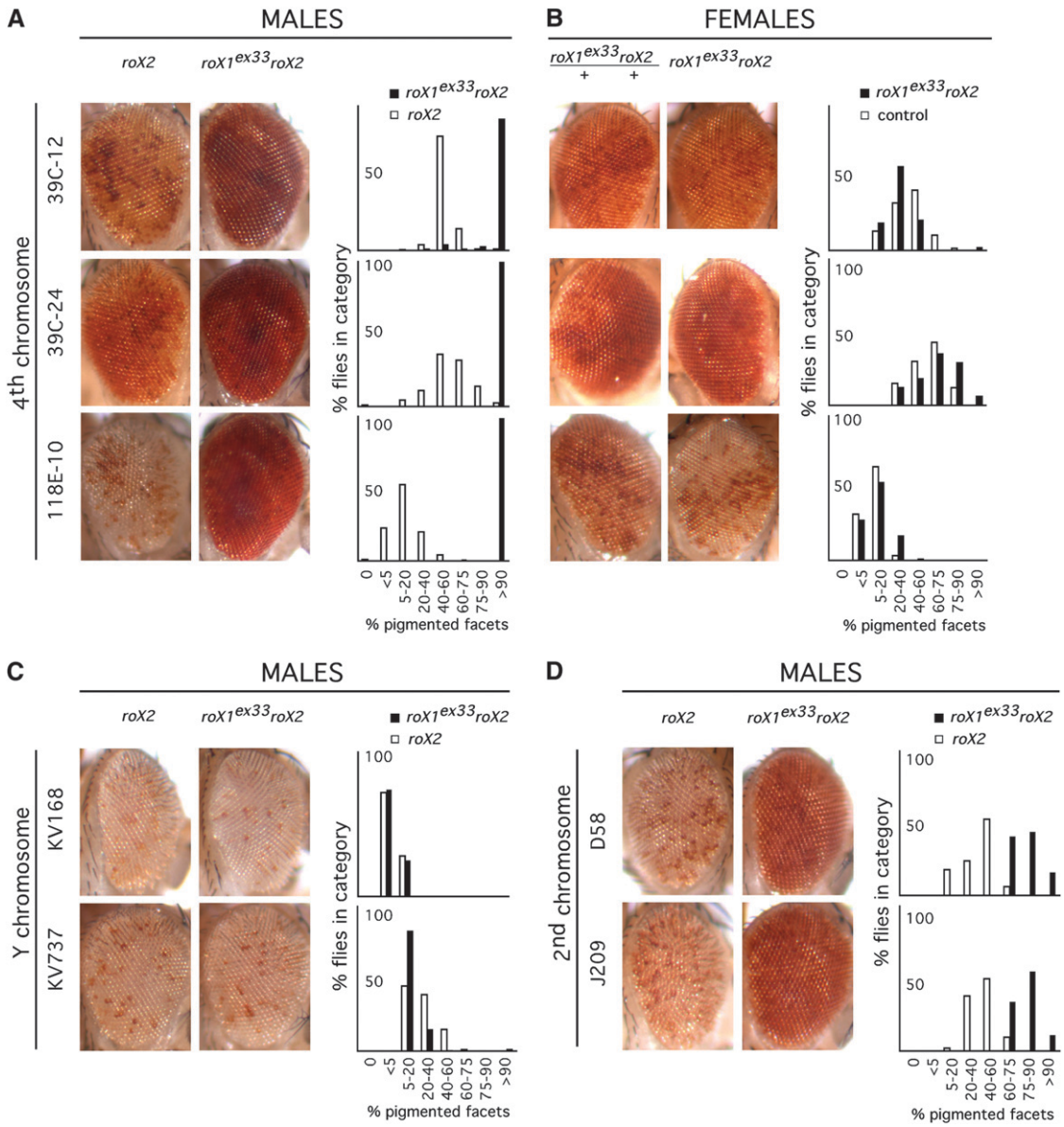

FIGURE 5.—A *roX1 roX2* chromosome modifies position effect variegation (PEV) in males. (A) $roX1^{ex33A}roX2$ suppresses variegation of a w^+ marker inserted on the fourth chromosome of males. Control males carry a wild-type *roX1* gene. (B) $roX1^{ex33A}roX2$ females and control females with variegating fourth-linked insertions. Control females are $roX1^{ex33A}roX2/{+}{+}$. (C) $roX1^{ex33A}roX2$ does not modify PEV of insertions on the Y chromosome. (D) $roX1^{ex33A}roX2$ suppresses variegation of insertions in proximal heterochromatin of the second chromosome in males. Representative eyes are presented beside a histogram illustrating the range of pigmentation in $roX1^{ex33A}roX2$ (black) and control (white). Details of fly genotypes are presented in MATERIALS AND METHODS.

males, the sex in which modification of PEV would be expected. It is thus unsurprising that the *roX* and *msl* genes have not appeared in screens for modifiers of PEV.

The observation that *roX* RNA is required for normal heterochromatin function in males is particularly intriguing in light of previous studies suggesting links between dosage compensation and heterochromatin. HP1 is modestly enriched on the male X chromosome and mutation of HP1 or Su(var)3-7, an HP1 binding partner, disrupts the structure of the polytenized male X chromosome (DE WIT *et al.* 2005; SPIERER *et al.* 2005, 2008). The JIL-1 kinase is genetically linked to dosage compensation and also enriched on the male X chromosome (LERACH *et al.* 2005). JIL-1 mutations suppress PEV in pericentromeric regions and permit proximal heterochromatin to spread into euchromatic regions (EBERT *et al.* 2004; LERACH *et al.* 2006; ZHANG *et al.* 2006). While JIL-1 is a plausible link between dosage compensation and heterochromatin, the effect of JIL-1 mutation on heterochromatin and PEV is observed in both sexes.

Although the molecular basis of *roX* regulation of autosomal genes is currently speculative, we have

eliminated the most plausible sources of an indirect effect. MSL1 and MSL2 directly interact and both are thought central to chromatin recognition by the DCC. However, MSL1 is necessary for full expression of heterochromatic genes in males but MSL2 is not. This is inconsistent with the idea that heterochromatic genes are misregulated by a redistribution of chromatin proteins following the failure of dosage compensation. Our studies reinforce the notion that the intact DCC, containing MSL2, is dedicated to recognition of the X chromosome. It is interesting that a short sequence motif recognized by the DCC is enriched on the X chromosome but depleted from the fourth chromosome (Alekseyenko *et al.* 2008). This suggests selective pressure to prevent inappropriate binding of the DCC to the fourth chromosome and supports our conclusion that the intact DCC does not regulate fourth-linked genes.

It is tempting to speculate that *roX* RNA, MSL1, and MSL3 associate, as they do in the MSL complex. Although efforts to detect *roX1* and MSL proteins on the fourth chromosome of polytene preparations have not been successful, it is possible that these molecules have a transient role in heterochromatic regions. While members of the DCC are interdependent in larvae, in early embryos maternally deposited MSL1 and MSL3 are present and stable prior to the zygotic expression of MSL2 at 3 h after egg laying. Similarly, *roX* transcripts are unstable in larvae lacking any MSL protein, but *roX1* produced in early embryos is stable for several hours, even in the absence of MSL2 (Meller *et al.* 1997; Meller 2003; Rattner and Meller 2004). *roX1* is first transcribed >1 hr before dosage compensation is initiated, but just before heterochromatin becomes visible (Vlassova *et al.* 1991; Lu *et al.* 1998). MSL1, MSL3, and *roX1* are therefore present during the initial formation of heterochromatin, making it plausible that they serve a transient role at this time. Expression of MSL2 in males at 3 hr triggers formation of the intact DCC and sequestration of MSL proteins and *roX1* RNA to the X chromosome. The window between 1.2 and 3 hr may thus be a critical time during which *roX* influences heterochromatin structure. This idea is currently under investigation.

The response of variegating insertions to loss of *roX* RNA depends on the position of the insertion, raising the possibility of chromosome-specific factors that modulate sensitivity. Y-linked insertions are unaffected by loss of *roX* RNA, consistent with adaptation of the Y chromosome for expression in male germ cells lacking MSL1 and MSL3 (Rastelli and Kuroda 1998). In contrast, suppression of PEV by loss of *roX* is strongest for insertions on the fourth chromosome. The fourth chromosome has several unusual features. It is composed of interspersed euchromatin and heterochromatin and is thus enriched for the boundaries between these chromatin states (Sun *et al.* 2000; Yasuhara and Wakimoto 2008). This organization may influence the sensitivity of fourth-linked genes to loss of the *roX* transcripts. The DNA sequence elements that underlie heterochromatin formation on the fourth chromosome also appear unusual (Riddle *et al.* 2008). All heterochromatic regions are marked by H3K9me, which creates a binding site for HP1. While Su(var)3-9 is responsible for the majority of H3K9me deposition throughout the rest of the genome, the dSETDB1 methyltransferase localizes to the fourth chromosome and is responsible for H3K9me accumulation there (Seum *et al.* 2007; Tzeng *et al.* 2007). Knock down of ISWI in S2 cells leads to a modest decrease in expression of fourth-linked genes (Bonaldi *et al.* 2008). This is interesting as the dosage-compensated male X chromosome is particularly sensitive to loss of ISWI (Corona *et al.* 2002). Finally, PEV of insertions on the distal fourth chromosome are modified by the dose of the fourth chromosome, while insertions on the second chromosome are not (Haynes *et al.* 2007). This reinforces the idea that factors required for fourth chromosome heterochromatin differ from other heterochromatic regions. At present no evidence suggests that these biochemical features are male limited, but they do support the notion that the structure and regulation of the fourth chromosome is unusual. These differences may render the fourth chromosome particularly sensitive to loss of the *roX* RNAs.

The observation that heterochromatic genes with similar expression in males and females are differentially regulated raises the question of why this difference exists. A clue may lie in the Y chromosome. The Y chromosome represents 12% of the male genome and is entirely heterochromatic. It has far-reaching affects on other heterochromatic regions because it absorbs a large portion of the proteins that assemble into heterochromatin. Loss of the Y chromosome frees these proteins and enables them to bind elsewhere, thus promoting heterochromatin formation and enhancing PEV throughout the nucleus (Weiler and Wakimoto 1995). As a result, loss of the Y chromosome silences transgenes in proximal heterochromatin and on the fourth chromosome. Loss of *roX* RNA has the opposite effect, increasing expression from these transgenes. Indeed, the partial loss of function $roX1^{ex33A}roX2$ chromosome largely restores expression from a variegating fourth chromosome insertion in males lacking a Y chromosome (data not shown). *roX* and the Y chromosome thus exert opposing influences on heterochromatic silencing.

Dosage compensation is essential in animals with highly differentiated X and Y chromosomes. During the evolution of sex chromosome pairs, the Y chromosome irreversibly loses coding potential and accumulates repetitive sequences, which, in turn, promotes the formation of heterochromatin (Rice 1996). Although the precise origin of the *D. melanogaster* Y chromosome is

debatable, it is similar to mammalian Y chromosomes in being gene poor and heterochromatin rich (Carvalho 2002). In spite of the evidence that the Drosophila Y chromosome exerts a far-reaching influence throughout the nucleus, a mechanism that counteracts the effects of the Y chromosome has never been identified. The *roX*-dependent modulation of heterochromatin that we have observed is male limited and it influences PEV in a manner opposite to that of the Y chromosome. It thus displays two key features expected for a system that accommodates Y heterochromatin. This model places the *roX* RNAs in two different domainwide regulatory systems: dosage compensation of the X chromosome and modulation of heterochromatin in males. It is intriguing that both processes serve to accommodate different problems resulting from sex chromosome differentiation.

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Coordinated Regulation of Heterochromatic Genes in *Drosophila melanogaster* Males

Xinxian Deng, S. Kiran Koya, Ying Kong and Victoria H. Meller

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FILE S1

MSL1

Descriptive statistics for relative quantities (Ct mutant : Ct control)

			Range			
Gene Group	**No of genes**	**Mean**	**Min**	**Max**	**Std. Deviation**	**Std. Error**
2&3 Euchromatic	7	1.37148	1.02	2.208	0.473859	0.179102
X chromosomal	4	0.888	0.634	1.12	0.268492	0.134246
2&3 Heterochromatic	10	0.9161	0.738	1.5	0.219706	0.069477
4th chromosomal	12	0.85038	0.566	2.03	0.394979	0.114021

MSL1 gene group comparisons

			Mann-Whitney U Test		
2&3 Euchromatic vs	**Mean Diff**	**Std. Error**	**Z score**	**Exact Sig. [2*(1-tailed Sig.)][a]**	**Asymp. Sig. (2 tailed)**
X chromosomal	0.483476	0.224306	-1.515	0.164	0.13
2&3 Heterochromatic	0.455376	0.17636	-2.928	0.002	0.003
4th chromosomal	0.521101	0.170201	-3.043	0.001	0.002

a. not corrected for ties

MSL3

Descriptive statistics for relative quantities (Ct mutant:Ct control)

			Range			
	No of genes	**Mean**	**Min**	**Max**	**Std. Deviation**	**Std. Error**
2&3 Euchromatic	7	1.17098	0.717	1.63	0.340002	0.128509
X chromosomal	4	0.56162	0.468	0.667	0.099245	0.049622
2&3 Heterochromatic	10	0.8063	0.658	0.969	0.104504	0.033047
4th chromosomal	13	0.93238	0.557	3.01	0.647348	0.179542

MSL3 gene group comparisons

			Mann-Whitney U Test		
2&3 Euchromatic vs	**Mean Diff**	**Std. Error**	**Z score**	**Exact Sig. [2*(1-tailed Sig.)][a]**	**Asymp. Sig. (2 tailed)**
X chromosomal	0.609355	0.276784	-2.646	0.006	0.008
2&3 Heterochromatic	0.36468	0.21762	-2.147	0.033	0.032
4th chromosomal	0.238595	0.207022	-2.1	0.037	0.036

a. not corrected for ties

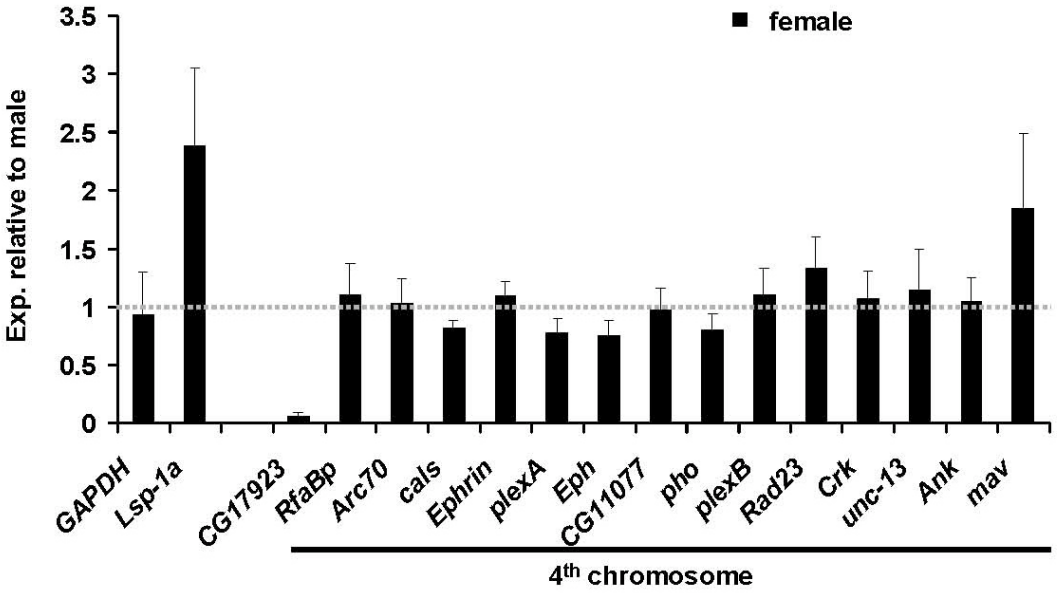

FIGURE S1.—Expression of the 4th chromosome is similar in males and females. Expression of individual genes in wild type male and female larvae was measured by qRT PCR. RNA levels are set to 1 for males (gray line). *GAPDH* is autosomal and the X-linked *Lsp-1α* escapes compensation. Error bars indicate the standard deviation of at least three independent measurements.

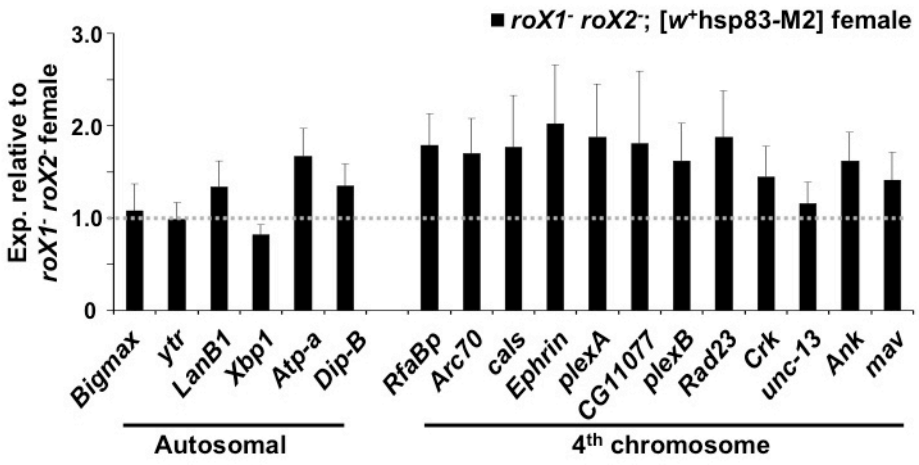

FIGURE S2.—Ectopic binding of MSL proteins to the 4th chromosome does not repress expression. Expression of individual genes was measured by qRT PCR in *roX1*^{SMC17A} *roX2* (set to 1) and *roX1*^{SMC17A} *roX2*; [*w*$^{+}$Hs83-M2] females. Misexpression of MSL2 in *roX1*^{SMC17A} *roX2*; [*w*$^{+}$Hs83-M2] females produces a pattern of ectopic MSL binding indistinguishable from that observed in *roX1*^{SMC17A} *roX2* males. Error bars indicate standard deviation of at least three independent measurements.

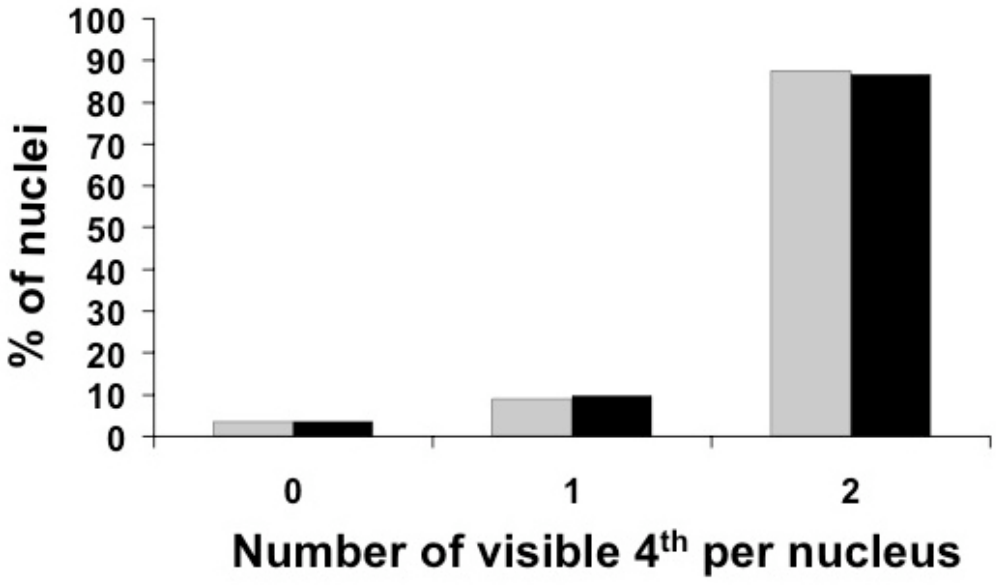

FIGURE S3.—The 4th chromosome is not lost at a higher rate in *roX1 roX2* males. The number of visible 4th chromosomes in mitotic neuroblast preparations from control males (gray bars) and *roX1 roX2* males (black bars) was determined. 540 nuclei were counted from control males and 361 from *roX1*^{SMC17A} *roX2* males.

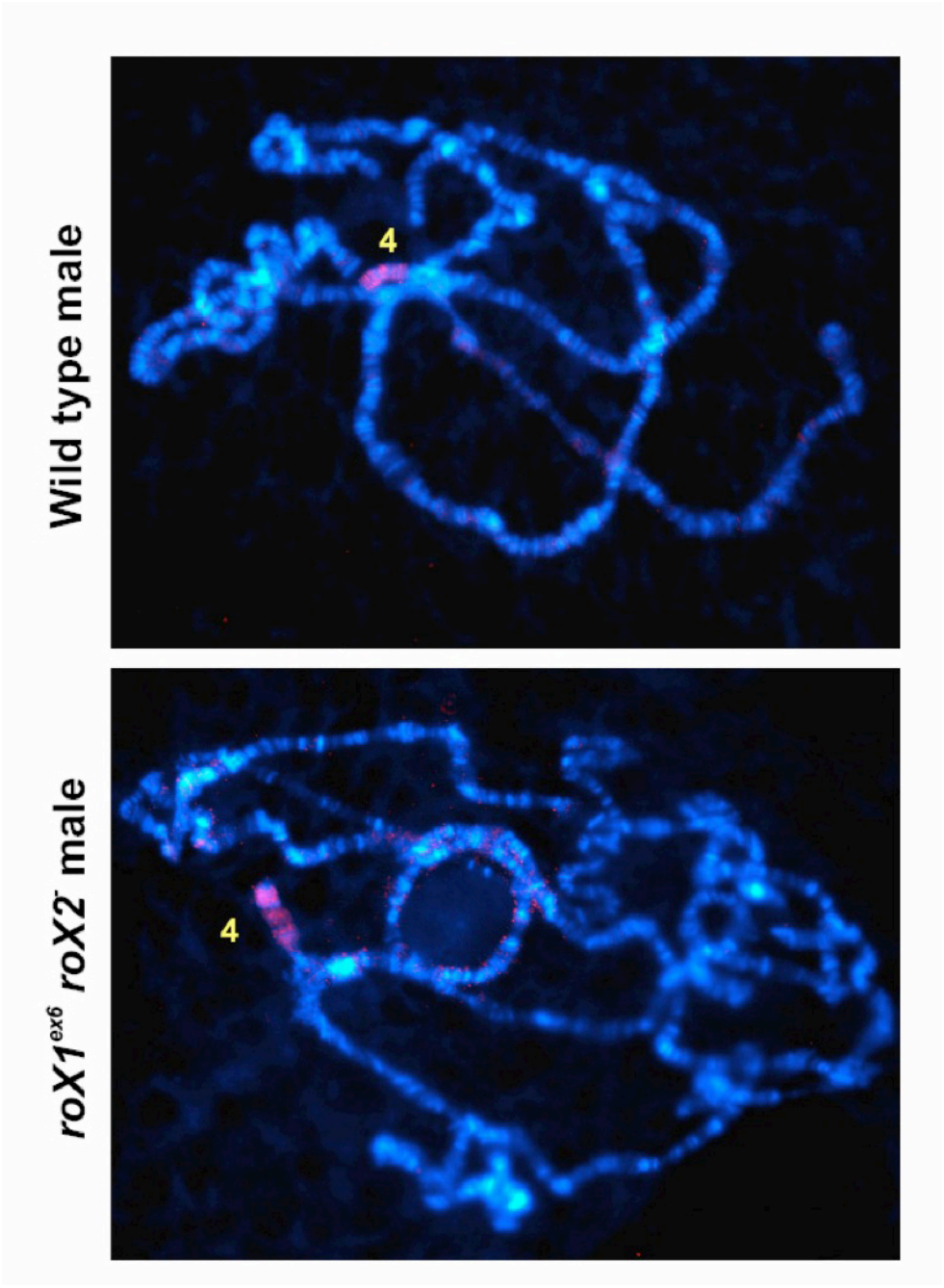

Figure S4.—POF is not redistributed in *roX1 roX2* males. Polytene chromosome preparations from control (top) and $roX1^{ex6}roX2$ males (bottom) were immunostained for POF. $roX1^{ex6}$ is a severe loss of function mutation. POF localization appears identical in $roX1^{ex6}roX2$ and $roX1^{SMC17A}roX2$ male larvae, but the salivary glands of $roX1^{ex6}roX2$ males produce better polytene chromosomes, and this genotype was therefore used. DNA is detected by Hoechst 33258 (blue; 40 ms exposure). POF is detected by Texas Red (red; 40 ms exposure). 4: the 4th chromosome.

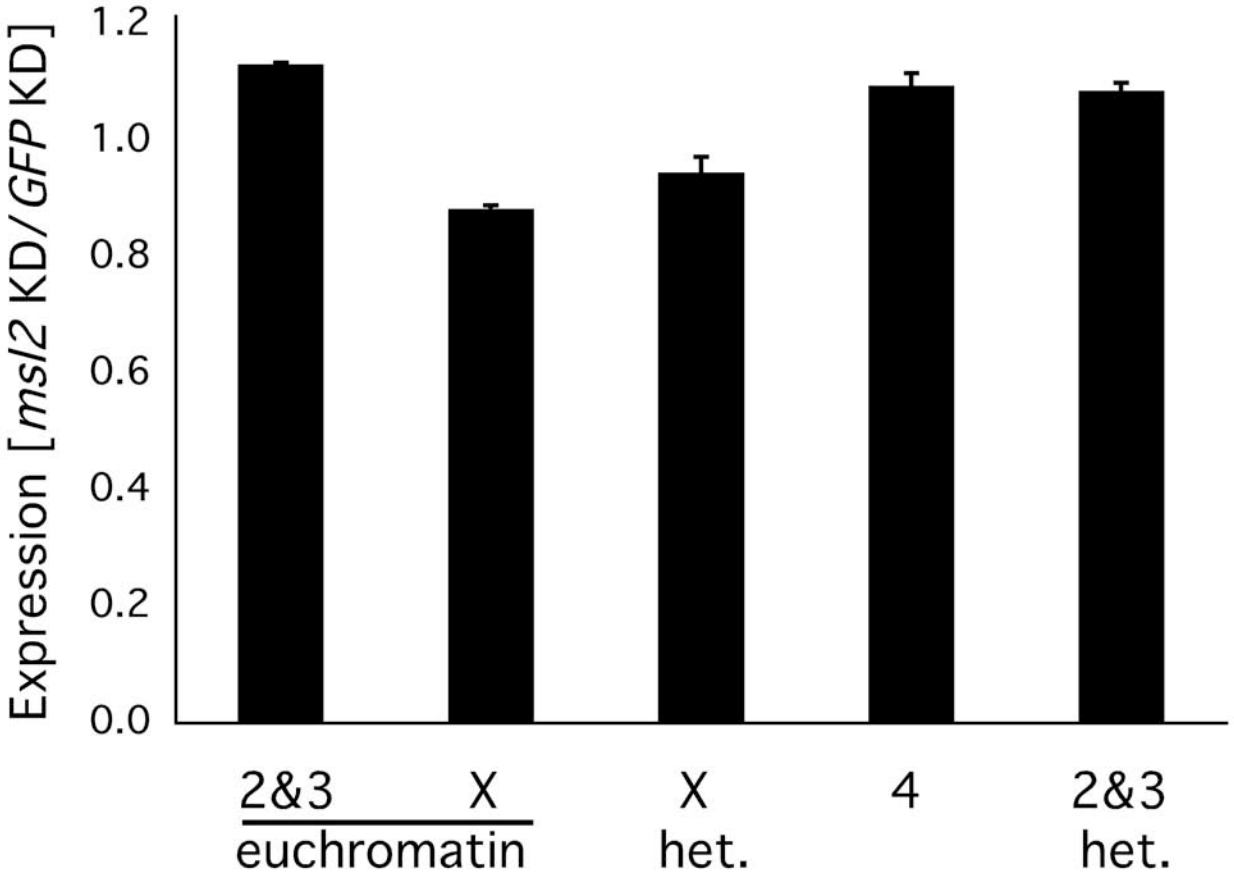

FIGURE S5.—Knock down of MSL2 does not reduce expression of 4th-linked genes. Data from a published microarray study documenting the effect of MSL2 knock down in male S2 cells was examined for evidence of an effect on 4th-linked gene expression (Hamada et al., 2005). Enrichment for heterochromatic genes is described in Supplemental Methods.

TABLE S1

Primers used for quantitative real time PCR

Gene		Primer sequence	MSL1	MSL3
Second and third chromosome euchromatic				
Dmn	F	GACAAGTTGAGCCGCCTTAC	normalizer	normalizer
	R	CTTGGTGCTTAGATGACGCA		
ytr	F	ATTTTGGACCAGCACCACTC	+	+
	R	CAAAATCCCTGCAATTTCGT		
LanB1	F	TCAACGAGCACCTGATTCAC	+	-
	R	GCAAATGGATGTTTCCCAAT		
Idh	F	TAGGACCCCAAAACCCATAA	-	+
	R	GGG AGA GCA TAC CAC ACT TG		
Xbp1	F	GGGAGAGCAACTTTGACGAG	+	+
	R	GCCGGCCAAACTTAACAATA		
Dip-B	F	AGGATCACGCCAGAAGACTG	+	+
	R	AGTCACTGGGACGGAGAATG		
Atp-a	F	ACCCACACTGCTACACTCCC	+	+
	R	TCCTGGTTGCTCTTGTTGTG		
GAPDH	F	GCCCTGAACGGCAAGCT	+	+
	R	GTAAGATCCACAACGGAGACATTG		
Bigmax	F	CTCGGCGCACAATTCAGA	+	+
	R	CCCTTCTCCGCTCCTTGTAA		
X chromosome euchromatic				
CKII	F	CCTGGTTCTGTGGACTTCGT	+	+
	R	GTAGTCCTCATCCACCTCGC		
skpA	F	CTAAAAGTCGACCAGGGCAC	+	+
	R	CCAGATAGTTCGCTGCCAAT		
PpV	F	TTGACCACCCATGAACTCAA	+	+
	R	GTGTTTGCTATGCTTGGGGT		
CG1702	F	GACATCTTTGCAGCCTGTGA	+	+
	R	GCCCTGATCTTGGGGTACTT		
4th chromosome				
Arc70	F	ATCGTACAACAACGAGCCCT	+	+
	R	CAGCGTGAAAGAAACGTCAA		
cals	F	AGTTTGTCAGCCCTCACCTT	+	+
	R	CTCCTATGCATTGCGACAGA		
Ephrin	F	TTGCAATTCTTGGCATTCAC	+	+
	R	CATAGAGGTCGCGGTGATTT		
plexA	F	AAAGCAGCGATTGGCTTTTA	+	+
	R	GGCGCAGCTCTTATTCTGAC		

CG17923	F	CCCCCTCCAGACTTAAAGGA	+	+
	R	CCATTCTTGTCCGTAGCCAT		
RfaBp	F	ACTGTCGCTGTCTTCCGATT	-	+
	R	GATTTTGCCTTTTTGTTCGC		
Eph	F	CTACCGTTTACCAGCTCCGA	+	+
	R	TTGCCAGCAATCCAACATTA		
CG11077	F	GCCCTCGTATGGAACTGCTA	+	+
	R	CCGTATTACTTATGCGGGGA		
pho	F	TCACGCAAAAGCAAAGAGAA	+	+
	R	ATTCAGCGTTTGAACAACCA		
plexB	F	AACGGAACCACAAAAGATCG	+	+
	R	ATGTTACCGAGCGAACCAAC		
Rad23	F	GCGGATAACGAAGACTTGGA	+	+
	R	TAGCCGTTCTATTGCGTCCT		
Crk	F	AACATTAATGGGCAATGGGA	+	+
	R	CATCGACAAATTCAACGTGC		
Ank	F	TGCAGAGTTTGGCACTCATC	+	+
	R	TCGCCATCTTTTTCAATTCC		
Mav	F	GATAAAATCGACGAGGCCAA	+	+
	R	TTTTCCTAGATCCTGGCCCT		
Second and third chromosome heterochromatic				
lt	F	CGAAGCTTCAACTGGCAATC	+	+
	R	AGATAATGGTGCAAGCCCAC		
CG17540	F	TCGAAAGTGCAATTAAAGCTG	+	+
	R	TAGTTGTAAAACCCCGCTCG		
CG40439	F	TCTCGAGCATTGGGAGTTCT	+	+
	R	TGCCTTCCAAAGCTGCTATC		
CG17665	F	GCAGAAGCAATAGCCGAATC	+	+
	R	AAACGCCTCCCAAGTGTATG		
CG17683	F	AGGACTTTTTCAGCAAGGCA	+	+
	R	TGCTGAGCCTCACTAAGCAA		
Gprk1	F	TCTAAAAGGCTTGGGTGCAT	+	+
	R	TGTAAACCTGGTGCCAATCA		
CG12547	F	TTTTCCCGCAAAGATTGTTC	+	+
	R	CCCCGTATCAGCAATAGCAT		
Rad21	F	CCCAGAACCACCTTCGTAGA	+	+
	R	GGACTGTGAAGGCATTGGTT		
MED21	F	GGAAGTAGTGCAAAAAGGCG	+	+
	R	TGAGCAATGCATTCCAAAGA		
CG41099	F	GTGCGACATGGAATTGACAC	+	+
	R	CAATTGCGCGATGTAAAAGA		

TABLE S2

Quantitative reverse transcription-PCR (qRT-PCR) validation of microarray analysis

Gene		position	Microarray Average		Fold Change	
			Mutant	Control	Δ Array (*p*-value)	Δ QPCR (std. dev.)
Bigmax	1628490	97F1	508	491	1.04	-
Unchanged 4th chromosome genes						
CG17923	1635946	101F1	231	239	0.97 (0.69)	0.86 (0.23)
RfaBp	1637843	102D3-4	5887	6668	0.88 (0.25)	0.87 (0.28)
Down regulated 4th chromosome genes						
Arc70	1628767	102C3	862	1331	0.65 (3.5E-5)	0.50 (0.03)
cals	1628842	102D4	874	1700	0.51 (5.1E-5)	0.49 (0.05)
Ephrin	1634035	102C2	294	522	0.53 (1.1E-4)	0.46 (0.01)
plexA	1640271	102D1	662	1264	0.52 (1.7E-4)	0.57 (0.09)
Eph	1641458	102C2	435	836	0.52 (0.0009)	0.55 (0.04)
CG11077	1636950	102D1	562	857	0.66 (0.001)	0.66 (0.10)
pho	1626947	102D6	1120	1777	0.63 (0.001)	0.47 (0.02)

The expression of selected genes was examined by qRT-PCR. cDNA templates were generated from RNA samples used for microarray analysis. Unchanged genes have a *t*-test *p*-value of >0.2 in microarray analysis. *Bigmax* was used as the normalizing gene.

TABLE S3

Reducing POF does not affect the recovery of *roX1 roX2* males

		daughters		sons	
mother	father	$\frac{+}{+}$	$\frac{pof}{+}$	$\frac{+}{+}$	$\frac{pof}{+}$
y roX1^{mb710} roX2	*pof*	-	1375	-	78 (5.7%)
y roX1^{mb710} roX2	+	687	-	47 (6.8%)	-

Females homozygous for *roX1^{mb710}roX2* were mated to males homozygous for a *pof* null (*pof^{D119}*) or to wild type males. The survival of *roX1^{mb710}roX2* sons (parentheses) is based on the recovery of females from the same cross.

TABLE S4

Elimination of POF does not prevent recovery of *roX1^{ex7B}roX2* males

		daughters			sons		
		+	*pof^{D119}*	*pof^{D119}*	+	*pof^{D119}*	*pof^{D119}*
mother	father	+	CyO [*w^{+}roX1^{+}*]	*pof^{D119}*	+	CyO [*w^{+}roX1^{+}*]	*pof^{D119}*
roX1^{ex7B} roX2; pof^{D119} Binsincy	*pof^{D119}* CyO [*w^{+}roX1^{+}*]	-	321	479	-	354	65 (18.4%)
roX1^{ex7B} roX2 *roX1^{ex7B} roX2*	+	389	-	-	85 (21.9%)	-	-

Females homozygous for the partial loss of function chromosome *roX1^{ex7B}roX2* and heterozygous for *pof^{D119}* were mated to *pof^{D119}* males. Sons inheriting CyO[*w^{+}roX1^{+}*] will have one copy of *pof^{+}* and a rescuing *roX1* transgene. Their survival is expected to be near 100%. As anticipated, similar numbers of daughters and sons carrying the CyO[*w^{+}roX1^{+}*] chromosome were recovered. The survival of *roX1^{ex7B} roX2; pof^{D119}* males is based on the recovery of their *roX1^{ex7B} roX2; pof^{D119}*/CyO [*w^{+}roX1^{+}*] brothers. Elimination of *roX2* is achieved by combining the lethal Df(1)52 with an insertion of the cosmid [*w^{+}*4D4.3] on the 3rd chromosome. This cosmid carries all essential genes removed by Df(1)52 but lacks *roX2* (Meller and Rattner, 2002). Fathers in both matings are homozygous for [*w^{+}*4D4.3].

TABLE S5

***roX1 roX2* males with a single 4th chromosome are recovered**

		daughters			sons		
mother	father	4/0	4/4	4/4	4/0	4/4	4/4
roX1^{ex7B} *roX2* ; *sv* / *roX1*^{ex7B} *roX2* *sv*	+	-	389	-	-	85	-
roX1^{ex7B} *roX2* ; *sv* / *roX1*^{ex7B} *roX2* *sv*	C(4)*ey*^{R}*ci*^{1}	156	-	2268	16	-	20

Females homozygous for the partial loss of function chromosome *roX1*^{ex7B}*roX2* and the recessive 4th chromosome marker *sv* are mated to either wild type males or to males with a compound 4th chromosome. Haplo 4 offspring are *sv*. Elimination of *roX2* is achieved by combining the lethal Df(1)52 with an insertion of the cosmid [*w*$^{+}$4D4.3] on the 2nd chromosome. This cosmid carries all essential genes removed by Df(1)52 but lacks *roX2* (Meller and Rattner, 2002). All mothers are homozygous for [*w*$^{+}$4D4.3].