Absence of X-chromosome dosage compensation in the primordial germ cells of Drosophila embryos

Ryoma Ota1,2,3*, Makoto Hayashi3,4, Shumpei Morita3,4,5, Hiroki Miura4 & Satoru Kobayashi3,4*

Dosage compensation is a mechanism that equalizes sex chromosome gene expression between the sexes. In Drosophila, individuals with two X chromosomes (XX) become female, whereas males have one X chromosome (XY). In males, dosage compensation of the X chromosome in the soma is achieved by five proteins and two non-coding RNAs, which assemble into the male-specific lethal (MSL) complex to upregulate X-linked genes twofold. By contrast, it remains unclear whether dosage compensation occurs in the germline. To address this issue, we performed transcriptome analysis of male and female primordial germ cells (PGCs). We found that the expression levels of X-linked genes were approximately twofold higher in female PGCs than in male PGCs. Acetylation of lysine residue 16 on histone H4 (H4K16ac), which is catalyzed by the MSL complex, was undetectable in these cells. In male PGCs, hyperactivation of X-linked genes and H4K16ac were induced by overexpression of the essential components of the MSL complex, which were expressed at very low levels in PGCs. Together, these findings indicate that failure of MSL complex formation results in the absence of X-chromosome dosage compensation in male PGCs.

In some reproductive animals, sex chromosomes play key roles in sex determination and differentiation. In such animals, sex-chromosome constitution differs between females and males. For example, in mammals and Drosophila, females and males carry two X chromosomes (XX) and one X chromosome (XY), respectively. This difference in the chromosome constitution causes an imbalance in the number of X-linked genes between the sexes; however, their expression can be equalized between XX females and XY males through a mechanism called dosage compensation.

In the somatic cells of Drosophila males, expression of X-linked genes is upregulated twofold by the dosage compensation mechanism. This upregulation is mainly mediated by the male-specific lethal (MSL) complex. Exclusively in males, this complex binds the X chromosome to acetylate lysine residue 16 in histone H4 (H4K16ac), which in turn hyperactivates X-linked genes. The MSL complex contains the essential components for catalysis, MSL-1 (a scaffold protein), MSL-2 (a ubiquitin ligase), MSL-3 (a chromodomain protein), MOF (a histone acetyltransferase), and MLE (a DNA/RNA helicase), in addition to the non-coding RNAs roX1 and roX2. In the soma, the transcript encoding MSL-2 is repressed translationally in females by Sex-lethal (Sxl), which is a binary switch gene that regulates sex determination and dosage compensation, allowing formation of the MSL complex only in males.

In contrast to the soma, the existence of dosage compensation in the Drosophila germline remains controversial. Rastelli and Kuroda showed that in the germline cells of adult testes, MLE does not localize to the X chromosome, and MSL-3 is not expressed, although H4K16ac is present. Furthermore, Gupta and colleagues performed a microarray analysis that revealed twofold upregulation of X-linked genes in the germline of adult testes. However, recent transcriptome analyses do not support the idea that dosage compensation occurs in the germline of adult testes. The discrepancies may result from differences in sample preparation or the gene sets selected for each analysis. Likewise, varying degrees of contamination of somatic cells in the germline samples

1Department of Biosciences, Faculty of Science and Engineering, Teikyo University, Utsunomiya, Tochigi 320-8551, Japan. 2Division of Integrated Science and Engineering, Graduate School of Science and Engineering, Teikyo University, Utsunomiya, Tochigi 320-8551, Japan. 3Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan. 4Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan. 5Present address: Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02906, USA. *email: ota@nasu.bio.teikyo-u.ac.jp; skob@tara.tsukuba.ac.jp
could yield dissimilar conclusions. Furthermore, meiotic inactivation of X-linked genes in spermatocytes\(^{14}\) raises concerns about the validity of the conclusions.

In this study, we focused on primordial germ cells (PGCs) in embryos, as dosage compensation is linked with sex determination and is an early event in the soma that differs between the sexes\(^{5,6,7,18\text{-}22}\). In Drosophila, PGCs are formed in the posterior pole region of early embryos, and then migrate through the embryo (middle embryogenesis) to reach the embryonic gonads (late embryogenesis). In migrating PGCs, Sxl is expressed in a female-specific manner and induces female fate\(^{47}\), although its downstream targets in these cells are distinct from those in the soma\(^{18\text{-}22}\). By contrast, male PGCs within gonads initiate male fate in response to JAK/STAT signals from the gonadal soma\(^{3,24}\). These observations led us to investigate whether dosage compensation occurs in PGCs during these embryonic stages.

In this study, we found that dosage compensation was undetectable in PGCs during middle to late embryogenesis. Consistent with this, H4K16ac was undetectable in male PGCs. All transcripts encoding the components of the MSL complex, except for MOF, were expressed at very low levels in PGCs. Moreover, overexpression of msl-1, msl-2, msl-3, and roX2 induced hyperactivation of X-linked genes and H4K16ac in male PGCs. Our observations strongly suggest that dosage compensation is absent in male PGCs, due to the failure of MSL expression.

**Materials and methods**

**Flies.** Flies were maintained on standard Drosophila medium at 25 °C. Strains y w, vasa-EGFP\(^{25}\), and nos-Gal4-VP16 (nos-Gal4)\(^{26}\) are maintained in our lab; UAS-RedStinger (UAS-RFP, stock No. 8545)\(^{27}\), M[3xP3-RFP attP]ZH-2A (24484)\(^{28}\), and M[3xP3-RFP attP]ZH-58A (24484)\(^{28}\) obtained from the Bloomington Drosophila Stock Center.

**Production of transgenic flies.** To produce flies carrying UAS-msl-1 and UAS-msl-2, total RNA was isolated from 4 to 8 h AEL (after egg laying) embryos using ISOGENE (NIPPON GENE, 311-0250), and cDNA was synthesized using SuperScript III (Thermo Fisher Scientific, 18080993). The open reading frames (ORFs) of msl-1 and msl-2 were amplified from the cDNA using primer pairs msl-1 Fw/mls-1 Rv and msl-2 Fw/mls-2 Rv, respectively (Table S1). The msl-1 and msl-2 ORFs were cloned into KpnI-digested pUASP-K10-attB\(^{29}\) using the In-Fusion HD Cloning Kit (Takara, Z9648N). DNA spanning from the 5’ terminus of the GAGA-binding site to the 3’ terminus of K10, which contains the msl-1 ORF, was amplified using primers pUASP-K10-attB Fw/ pUASP-K10-attB Rv (Table S1), and the amplicon was cloned into KpnI-digested pUASP-K10-attB containing the msl-2 ORF. The resultant vector was injected into M[3xP3-RFP attP]ZH-58A embryos to produce flies carrying UAS-msl-1 and UAS-msl-2 on the second chromosome. \(w^+\) transformants were collected and used to establish homozygous stocks.

To produce flies carrying UAS-msl-3 and UAS-roX2, the msl-3 ORF and full-length roX2 (RD-type isoform) were amplified from cDNA obtained from 4 to 8 h AEL embryos using primers msl-3 Fw/mls-3 Rv, and roX2 Fw/roX2 Rv (Table S1), respectively. The msl-3 ORF and roX2 fragment were cloned into KpnI-digested pUASP-K10-attB using the In-Fusion HD Cloning Kit. DNA spanning from the 5’ terminus of K10, which contains the msl-3 ORF, was amplified using primers pUASP-K10-attB Fw/ pUASP-K10-attB Rv (Table S1), and the amplicon was cloned into PsI-digested pUASP-K10-attB containing the roX2 fragment. The resultant vector was injected into M[3xP3-RFP attP]ZH-2A embryos to produce flies carrying UAS-msl-3 and UAS-roX2 on the X chromosome. \(w^+\) transformants were collected and used to establish homozygous stocks. As described above, the roX2 transgene (UAS-roX2) was inserted into the X chromosome, because its expression from an autosomal causes assembly of the MSL complex at the expression site and ectopic upregulation of the surrounding autosomal genes\(^{36}\).

**Transcriptome analysis.** For RNA-seq of female and male PGCs at stages 12–16, 1.9–2.3 \(\times\) 10\(^5\) female and male PGCs (Table S2) were isolated by fluorescence-activated cell sorting (FACS), as described\(^{11,12}\), from 8 to 16 h AEL embryos derived from vasa-EGFP/vasa-EGFP; nos-Gal4/nos-Gal4 females mated with UAS-RFP/Y males (Fig. S1a). EGFP and RFP double-positive cells and EGFP-positive cells were isolated as female and male PGCs, respectively. Total RNA was extracted from isolated PGCs using the RNeasy mini Kit (QIAGEN, 74104). Library creation using the TrueSeq Standard mRNA Library Prep Kit (Illumina, 20020594) and RNA-seq using the HiSeq 2500 platform (Illumina) were carried out at the University of Minnesota Genomics Center (UMGC), and approximately 40 million reads per sample (125-bp paired-end reads) were obtained.

For RNA-seq of PGCs at stages 15–16, one hundred each of female and male PGCs were isolated from 12 to 16 h AEL embryos derived from vasa-EGFP/vasa-EGFP; nos-Gal4/nos-Gal4 females mated with UAS-RFP/Y males (Fig. S1a). For RNA-seq of stages 15–16 msl oe male PGCs overexpressing msl-1, msl-2, msl-3, and roX2, 100 male PGCs were isolated from 12 to 16 h AEL embryos derived from UAS-msl-3 UAS-roX2/UAS-msl-3 UAS-roX2; vasa-EGFP/vasa-EGFP; nos-Gal4/nos-Gal4 females mated with UAS-RFP/Y; UAS-msl-1 UAS-msl-2 UAS-msl-1 UAS-msl-2 males (Fig. S1b). cDNAs were synthesized using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech, 634890). Nextera XT library creation and RNA-seq were carried out at UMGC using the HiSeq 2500 platform, and approximately 20 million reads per sample (50-bp paired-end reads) were obtained.

Raw reads obtained from both stage 12–16 and stage 15–16 samples were processed using Trimomatic-0.36\(^{34}\), and then aligned to the transcript model of Drosophila melanogaster (Flybase; dmel-all-transcript-dataset-0.36.32, and then aligned to the transcript model of Drosophila melanogaster (Flybase; dmel-all-transcript-dataset-0.36.32, and then aligned to the transcript model of Drosophila melanogaster).
**Microarray data analysis.** To compare gene expression profiles of somatic tissues between males and females, we used microarray data of thoraxes dissected from adult males and females. These data have been deposited in the Gene Expression Omnibus (GEO) under accession No. GSE30850. Raw values were normalized across samples using quantile normalization, and fold changes in female vs. male thorax were calculated for each gene.

To select zygotically expressed genes, we used microarray data obtained from PGCs at 11 different embryonic stages. These data have been deposited in GEO under Accession No. GSE83460. Raw values were normalized across samples using quantile normalization. Genes expressed at low levels in PGCs at stage 4 (log2 expression values < 7) and at high levels in PGCs at stage 16 (log2 expression values > 8) were selected as zygotically expressed genes.

**Fixation of embryos.** For immunostaining and in situ hybridization, embryos were collected and dechorionated in a sodium hypochlorite solution. The dechorionated embryos were fixed in 1:1 heptane:fixative (4% paraformaldehyde in PBS (130 mM NaCl, 7 mM Na2HPO4, and 3 mM NaH2PO4)) for 30 min. Vitelline membranes of the fixed embryos were removed by vigorous shaking in 1:1 methanol:heptane. The embryos were then rinsed with methanol and stored in methanol at −20 °C until use.

Testes were dissected from adult flies 3–5 days after eclosion and fixed for 15 min. Fixed testes were rinsed with PBS Tr (PBS containing 0.1% Triton X-100) and stored in PBS Tr at 4 °C until use.

**Immunostaining.** Immunofluorescence staining of embryos was performed as described. The following experiments were conducted at room temperature unless otherwise stated. y w nos-Gal4 embryos and embryos derived from UAS-msl-1 UAS-roX2/UAS-msl-3 UAS-roX2; nos-Gal4/nos-Gal4 females mated with UAS-msl-1 UAS-msl-2/UAS-msl-1 UAS-msl-2 males were fixed as described. Fixed embryos were incubated with 7:3, 5:5, and 3:7 methanol:PBS Tr for 5 min each, and then washed with PBS Tr three times for 15 min each. The embryos were then incubated with blocking solution (PBS containing 2% BSA, 0.1% Tween 20, and 0.1% Triton X-100) for 1 h. After blocking, the embryos were incubated overnight at 4 °C with rabbit anti-H4K16ac (1:200; Merck, 07–329) and chick anti-Vasa (1:500). The testes were washed with PBS Tr three times for 15 min each, and then incubated overnight at 4 °C with Alexa Fluor 488–conjugated goat anti-rabbit (1:500; Thermo Fisher Scientific, A11034) and Alexa Fluor 633–conjugated goat anti-chick (1:500; Thermo Fisher Scientific, A21071). The testes were washed with PBS Tr three times for 15 min each, and mounted in VECTASHIELD Mounting Medium (VECTOR, H-1000). The sex of each embryo was determined based on H4K16ac staining in the soma.

Immunofluorescence staining of testes was performed as described. The following experiments were conducted at room temperature unless otherwise stated. Testes of progeny from nos-Gal4/nos-Gal4 females mated with y w males and UAS-msl-3 UAS-roX2/UAS-msl-3 UAS-roX2; nos-Gal4/nos-Gal4 females mated with UAS-msl-1 UAS-msl-2/UAS-msl-1 UAS-msl-2 males were fixed as described. Fixed testes were washed within PBS Tr three times for 15 min each, and incubated with blocking solution for 1 h. After blocking, the testes were incubated overnight at 4 °C with chick anti-Vasa (1:500). The testes were washed with PBS Tr three times for 15 min each, incubated overnight at 4 °C with Alexa Fluor 488–conjugated goat anti-rabbit (1:500; Thermo Fisher Scientific, A11034) and Alexa Fluor 633–conjugated goat anti-chick (1:500; Thermo Fisher Scientific, A21071). The embryos were washed with PBS Tr three times for 15 min each, and mounted in VECTASHIELD Mounting Medium.

**Double staining for in situ hybridization and immunostaining.** To synthesize RNA probes for in situ hybridization, total RNA was isolated from 4 to 8 h AEL embryos, and cDNA was synthesized using Superscript III. cDNAs corresponding to msl-1, msl-2, mof, mle, roX1, and roX2 were amplified using the following primer pairs: msl-1HFw/msl-1HRv, msl-2HFw/msl-2HRv, mofHFw/msl-3HRv, mofHRv/mleHFw/mleHRv, roX1HFw/roX1HRv, and roX2HFw/roX2HRv, respectively (Table S1). Amplified cDNAs were cloned into pGEM-T Easy (Promega, A1360). Templates for RNA probes were amplified from these plasmids using the T7 and SP6 primers, and digoxigenin (DIG)-labeled RNA probes were synthesized from the fragments using T7 and SP6 RNA polymerase (Merck, 10881767001 and 10810274001), respectively.

Whole-mount in situ hybridization combined with immunostaining was performed as described. The following experiments were conducted at room temperature unless otherwise stated. Fixed embryos derived from nos-Gal4/nos-Gal4 females mated with UAS-RFP/Y males were rinsed with ME (50 mM EGTA (pH 8.0) in 90% methanol) and incubated in 7:3, 5:5, and 3:7 ME:fixative for 5 min each. The embryos were re-fixed with fixative for 20 min, and then washed with PBS Tr (PBS containing 0.1% Tween 20) three times for 5 min each. The embryos were digested with 50 µg/ml Proteinase K in PBS Tr for 3 min, and the digestion was stopped by incubation in fixative for 20 min. The digested embryos were washed with PBS Tr three times for 10 min each, and then incubated in pre-hybridization solution (pre-HS; 50% formamide, 5 × SSC (750 mM NaCl and 75 mM sodium-citrate), 100 µg/ml heparin, 100 µg/ml yeast tRNA, 10 mM DTT, and 0.1% Tween 20) for 60 min at 60 °C. They were then hybridized with 2 ng/µl RNA probe in hybridization solution (pre-HS containing 10% dextran sulfate) overnight at 60 °C. After hybridization, the embryos were washed with a washing solution (50% formamide, 5 × SSC, and 0.1% Tween 20) six times for 30 min each at 60 °C. The embryos were rinsed with TNT [100 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] three times and incubated with TNB [0.5% blocking reagent (PerkinElmer, NEL700001KT), 100 mM Tris–HCl (pH 7.5), and 150 mM NaCl] for 30 min. After blocking, the embryos were incubated with TNB containing a horseradish peroxidase–conjugated anti-DIG antibody (1:250; Merck, 11,633,716,001) for 30 min, and then washed with TNT three times for 15 min each. The embryos were incubated in Amplification diluent containing biotin tyramide solution (1:50; PerkinElmer, NEL700001KT) for 20 min, and then washed with TNT three times for 15 min each. The embryos were incubated
overnight at 4 °C in TNB containing chick anti-Vasa (1:500) and rabbit anti-RFP (1:1000; Thermo Fisher Scientific, R10367) and washed with TNT three times for 15 min each. For detection of RNA probes and antibodies, embryos were incubated overnight at 4 °C in TNB containing fluorescein-conjugated streptavidin (1:1000; PerkinElmer, S69), Alexa Fluor 633–conjugated goat anti-chick (1:500, Thermo Fisher Scientific, A21103), and Alexa Fluor 546–conjugated goat anti-rabbit (1:500, Thermo Fisher Scientific, A11035). The embryos were washed with TNT three times for 15 min each, and then mounted in VECTASHIELD Mounting Medium. The sex of each embryo was determined based on RFP staining, except in the case of stage 5 embryos.

Results and discussion
Dosage compensation is indiscernible in the PGCs. To investigate whether dosage compensation occurs in male PGCs, we separately isolated female and male PGCs and compared the expression of X-linked genes between the two sexes. For this purpose, we used females carrying nos-Gal4 and vasa-EGFP mated with males carrying UAS-RFP on the X chromosome. In embryos derived from these mothers, female and male PGCs were double-positive for EGFP and RFP and single-positive for EGFP, respectively (Fig. S1a), as nos-Gal4–driven UAS-RFP is activated only in female PGCs from stage 9 onward, whereas vasa-EGFP is expressed throughout germline development in both sexes. RNA-seq data were obtained from female and male PGCs at stages 12–16. Our data revealed that female-biased expression was significantly more common in transcripts from X-linked genes than in those from autosomal genes, although sex-biased expression was also observed in autosomes (Fig. S2a–f). Expression of the transcripts from X-linked genes was twofold higher on average in female PGCs than in male PGCs, whereas the female/male expression ratio of transcripts from autosomal genes was ~1 (Fig. 1a). Similar female-biased expression was observed when zygotically expressed genes were selected from X-linked genes (Fig. S3), indicating female-biased zygotic expression from X-linked genes. In addition, we found that expression of X-linked housekeeping genes for glycolysis and ATP synthesis was female-biased in PGCs (Fig. S4). By contrast, in the soma, female-biased expression of X-linked genes was not observed (Fig. 1b). In addition, we found that H4K16ac was undetectable in the PGCs, which were surrounded by H4K16ac-positive somatic cells in males (Fig. 1c,d). These observations led us to conclude that expression of genes on the X chromosome is subjected to dosage compensation in the soma, but not in PGCs. However, we cannot rule out the possibility that expression of a fraction of X-linked genes in male PGCs is compensated by mechanisms independent of H4K16ac.

Zygotic expression of ovo and ovarian tumor (otu) genes on the X chromosome is female-biased. We found that these genes exhibited female-biased expression (Fig. S2d). Furthermore, transcripts from marker genes for male PGCs, such as disc proliferation abnormal (dpa), CG9253, no child left behind (ncb), Ran GTPase activating protein (RanGap), Rs1, CG6693, Kinesin-like protein at 61F (Klp61F), and Minichromosome maintenance 5 (Mcm5), exhibited male-biased expression in PGCs (Fig. S2e and f).

Expression of genes encoding the components of MSL complex in male PGCs during embryogenesis. Given that the MSL complex is required for dosage compensation of the X chromosome through H4K16ac in the soma, we next asked whether genes encoding the components of the MSL complex are expressed in male PGCs during embryogenesis. For this purpose, we performed in situ hybridization of embryos produced from females carrying nos-Gal4 mated with males carrying UAS-RFP on the X chromosome. In the PGCs of stage 5 embryos, msl1 and mof mRNAs were detectable (Fig. 2a and p). Expression of these mRNAs is considered to be maternal in origin, as PGCs are transcriptionally repressed at this stage. msl1 and mof are maternal effect genes that are required for germline development. To investigate whether dosage compensation is activated only in female PGCs from stage 9 onward, whereas vasa-EGFP is expressed throughout germline development in both sexes. RNA-seq data were obtained from female and male PGCs at stages 12–16. Our data revealed that female-biased expression was significantly more common in transcripts from X-linked genes than in those from autosomal genes, although sex-biased expression was also observed in autosomes (Fig. S2a–f). Expression of the transcripts from X-linked genes was twofold higher on average in female PGCs than in male PGCs, whereas the female/male expression ratio of transcripts from autosomal genes was ~1 (Fig. 1a). Similar female-biased expression was observed when zygotically expressed genes were selected from X-linked genes (Fig. S3), indicating female-biased zygotic expression from X-linked genes. In addition, we found that expression of X-linked housekeeping genes for glycolysis and ATP synthesis was female-biased in PGCs (Fig. S4). By contrast, in the soma, female-biased expression of X-linked genes was not observed (Fig. 1b). In addition, we found that H4K16ac was undetectable in the PGCs, which were surrounded by H4K16ac-positive somatic cells in males (Fig. 1c,d). These observations led us to conclude that expression of genes on the X chromosome is subjected to dosage compensation in the soma, but not in PGCs. However, we cannot rule out the possibility that expression of a fraction of X-linked genes in male PGCs is compensated by mechanisms independent of H4K16ac.

Zygotic expression of ovo and ovarian tumor (otu) genes on the X chromosome is female-biased. We found that these genes exhibited female-biased expression (Fig. S2d). Furthermore, transcripts from marker genes for male PGCs, such as disc proliferation abnormal (dpa), CG9253, no child left behind (ncb), Ran GTPase activating protein (RanGap), Rs1, CG6693, Kinesin-like protein at 61F (Klp61F), and Minichromosome maintenance 5 (Mcm5), exhibited male-biased expression in PGCs (Fig. S2e and f).

Expression of genes encoding the components of MSL complex in male PGCs during embryogenesis. Given that the MSL complex is required for dosage compensation of the X chromosome through H4K16ac in the soma, we next asked whether genes encoding the components of the MSL complex are expressed in male PGCs during embryogenesis. For this purpose, we performed in situ hybridization of embryos produced from females carrying nos-Gal4 mated with males carrying UAS-RFP on the X chromosome. In the PGCs of stage 5 embryos, msl1 and mof mRNAs were detectable (Fig. 2a and p). Expression of these mRNAs is considered to be maternal in origin, as PGCs are transcriptionally repressed at this stage. msl1 and mof are maternal effect genes that are required for germline development.

Overexpression of msl-1, msl-2, msl-3, and roX2 induces H4K16ac and hyperactivation of X-linked genes in male PGCs. To determine whether depletion of MSL components causes deficiency of dosage compensation in male PGCs, we overexpressed genes encoding MSL components in male PGCs. MLE protein is maternally supplied and expressed in male PGCs during embryogenesis, whereas MSL-1 and MSL-2 proteins are not. Hence, we forced expression of msl-1, msl-2, msl-3, and roX2 in PGCs. Only roX2 was overexpressed because the roX RNAs are functionally redundant. When msl-1, msl-2, msl-3, and roX2 were overexpressed under the control of nos-Gal4 (mls oe), H4K16ac became detectable in the nuclei of male PGCs (Fig. 3a,b).

We next asked whether overexpression of MSL components would result in hyperactivation of X-linked genes in male PGCs. We performed RNA-seq analysis of female and male PGCs and male PGCs overexpressing msl-1, msl-2, msl-3, and roX2 at stages 15–16. We found that X-linked genes exhibited female-biased expression relative to autosomal genes at stages 15–16 (Fig. S6a–c and S7a–c), and that their expression was twofold higher in female PGCs than in male PGCs (Fig. 4a). These data, obtained from PGCs at stages 15–16, were similar to those obtained from PGCs at stages 12–16 (Fig. 1a). When msl-1, msl-2, msl-3, and roX2 were overexpressed in male PGCs (mls oe male), X-linked genes were significantly hyperactivated relative to male PGCs (Fig. 4a,b, Fig. S6d–f, Fig. S7d–f, and Fig. S8). Autosomal genes were also activated, but the degree of upregulation was statistically negligible (Fig. 4a,b). These results indicate that overexpression of msl-1, msl-2, msl-3, and roX2 in
Figure 1. Dosage compensation is absent in embryonic PGCs. (a) Log2 expression ratio of transcripts from genes on the X (pink), second (light blue), and third chromosomes (lime green) between female and male PGCs (female/male) at embryonic stages 12–16. Each box plot represents the median value and first and third quartile values. Error bars represent minimum and maximum values. Red plus signs represent mean values. Outliers are not depicted. The mean values for the transcripts from genes on the X, second, and third chromosomes were 0.94, −0.08, and −0.10, respectively. Significance was calculated by two-sided Mann–Whitney U test (*: $P < 0.05$, ns: not significant). Transcripts for which TPM were zero in all samples were excluded from this analysis. N: number of the transcripts examined. (b) Log2 expression ratio of transcripts from genes on the X (pink), second (light blue), and third chromosomes (lime green) between female and male thoraxes (female/male) dissected from adults. This result was obtained from microarray data deposited by Meiklejohn et al. (GEO accession No. GSE30850). Each box plot represents values as in (a). The mean values for the transcripts from genes on the X, second, and third chromosomes were −0.01, −0.02, and −0.01, respectively. Significance was calculated by two-sided Mann–Whitney U test (ns: not significant). N: number of the probes examined. (c and d) H4K16ac expression in male (c) and female (d) PGCs at embryonic stage 14 (St. 14). y w embryos were stained for H4K16ac (green) and Vasa [a marker of germline cells (magenta)]. Embryos were sexed based on H4K16ac staining in the soma. Scale bar: 10 μm.
| Gene | Stage | St. 5 | St. 10–11 | St. 12–13 | St. 14 | St. 16 |
|------|-------|-------|-----------|-----------|--------|--------|
| msl-1 |      | ![Image](a.png) | ![Image](b.png) | ![Image](c.png) | ![Image](d.png) | ![Image](e.png) |
| msl-2 |      | ![Image](f.png) | ![Image](g.png) | ![Image](h.png) | ![Image](i.png) | ![Image](j.png) |
| msl-3 |      | ![Image](k.png) | ![Image](l.png) | ![Image](m.png) | ![Image](n.png) | ![Image](o.png) |
| mof   |      | ![Image](p.png) | ![Image](q.png) | ![Image](r.png) | ![Image](s.png) | ![Image](t.png) |
| mle   |      | ![Image](u.png) | ![Image](v.png) | ![Image](w.png) | ![Image](x.png) | ![Image](y.png) |
| roX1  |      | ![Image](z.png) | ![Image](aa.png) | ![Image](bb.png) | ![Image](cc.png) | ![Image](dd.png) |
| roX2  |      | ![Image](ee.png) | ![Image](ff.png) | ![Image](gg.png) | ![Image](hh.png) | ![Image](ii.png) |
transplantation of PGCs. Transplantation of XY PGCs overexpressing msl-1, msl-2, msl-3, and roX2 into female soma may clarify whether twofold upregulation of X-linked genes induces femaleness in XY PGCs.

Biological significance of the absence of dosage compensation in male PGCs. In Drosophila, the sexual identity of the germline is regulated by both cell-autonomous cues, which are produced depending on X-chromosome constitution, and the sex of the surrounding soma.\(^23,46–50\). In the absence of dosage compensation, X-linked genes are expressed at twofold higher levels in female (XX) PGCs than in male (XY) PGCs at stages 12–16 (Fig. 1a). This biased expression of X-linked genes may be one of the determinants of femaleness.

Male PGCs can hyperactivate X-linked genes. Thus, failure of MSL complex formation resulting from low or no expression of msl-1, msl-2, msl-3, and roX RNAs causes the lack of dosage compensation in male PGCs. Future studies focusing on the regulatory mechanisms of msl-1, msl-2, msl-3, and roX expression will represent an important step toward elucidating how dosage compensation is repressed in male PGCs.

Although hyperactivation of X-linked genes was evident in male PGCs overexpressing msl-1, msl-2, msl-3, and roX2 (Fig. 4a,b, Fig. S6d–f, Fig. S7d–f, and Fig. S8), the upregulation ratio was only ~1.5-fold (Fig. 4b). Thus, overexpression of msl-1, msl-2, msl-3, and roX2 in male PGCs is insufficient to induce twofold upregulation of X-linked genes, as observed in male soma (Fig. 1b). One possible explanation for this is that dosage compensation requires factors other than the MSL complex in male PGCs. The functions of JIL-1 kinase and Topoisomerase 2 are also needed for dosage compensation in male soma.\(^46–47\). The possibility that overexpression of these molecules, along with msl-1, msl-2, msl-3, and roX2, can induce twofold upregulation of X-linked genes in male PGCs remains to be tested.
Data availability

All data and materials produced by this study are available from the corresponding authors upon reasonable request.

Received: 26 October 2020; Accepted: 16 February 2021
Published online: 01 March 2021

References

1. Bachtrog, D. et al. Sex determination: why so many ways of doing it? PLoS Biol. 12, e1001899 (2014).
2. Disteche, C. M. Dosage compensation of the sex chromosomes. Annu. Rev. Genet. 46, 537–560 (2012).
3. Kuroda, M. I., Hilfiker, A. & Lucchesi, J. C. Dosage compensation in Drosophila—a model for the coordinate regulation of transcription. Genetics 204, 435–450 (2016).
4. Turner, B. M., Birley, A. J. & Lavender, J. Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei cell. Cell 69, 375–384 (1992).
5. Bone, J. R. et al. Acetylated histone H4 on the male X chromosome is associated with dosage compensation in Drosophila. Genes Dev. 8, 96–104 (1994).
6. Penalva, L. O. F. & Sánchez, L. RNA binding protein sex-lethal (Sxl) and control of Drosophila sex determination and dosage compensation. Microbiol. Mol. Biol. Rev. 67, 343–359 (2003).
7. Kelley, R. L. et al. Expression of msll-2 causes assembly of dosage compensation regulators on the X chromosomes and female lethality in Drosophila. Cell 81, 867–878 (1995).
8. Kelley, R. L., Wang, J., Bell, L. & Kuroda, M. I. Sex lethal controls dosage compensation in Drosophila by a non-splicing mechanism. Nature 387, 195–199 (1997).
9. Rastelli, L. & Kuroda, M. I. An analysis of maleless and histone H4 acetylation in Drosophila melanogaster spermatogenesis. Mech. Dev. 71, 107–117 (1998).
10. Gupta, V. et al. Global analysis of X-chromosome dosage compensation. J. Biol. 5, 1–22 (2006).
11. Meiklejohn, C. D., Landeen, E. L., Cook, J. M., Kingan, S. B. & Presgraves, D. C. Sex chromosome-specific regulation in the Drosophila male germline but little evidence for chromosomal dosage compensation or meiotic inactivation. PLoS Biol. 9, e1001126 (2011).
12. Argyridou, E. & Parsch, J. Regulation of the X chromosome in the Germline and Soma of Drosophila melanogaster males. Genes (Basel) 9, 242 (2018).

Figure 4. Overexpression of msll-1, msll-2, msll-3, and roX2 induces hyperactivation of X-linked genes in male PGCs. (a) Log2 expression ratio of transcripts from genes on the X (pink), second (light blue), and third chromosomes (lime green) between female and male PGCs (female/male) at embryonic stages 15–16. Each box plot represents values as in Fig. 1a. Mean values for the X, second, and third chromosomes were 1.03, 0.23, and −0.21, respectively. Blue dotted lines indicate log2 expression ratios of 0 and 1. Significance was calculated by two-sided Mann-Whitney U test (*, P < 0.05; ns, not significant). Transcripts for which TPM was zero in all samples were excluded from this analysis. N: number of the transcripts examined. (b) Log2 expression ratio of transcripts from genes on the X (pink), second (light blue), and third chromosomes (lime green) between msloe male PGCs and male PGCs (msloe male/male) at embryonic stages 15–16. Each box plot represents values as in Fig. 1a. Mean values for the X, second, and third chromosomes were 0.51, 0.06, and 0.04, respectively. Blue dotted lines indicate log2 expression ratios of 0 and 1. Significance was calculated by two-sided Mann-Whitney U test (*, P < 0.05; ns, not significant). Transcripts for which TPM was zero in all samples were excluded from this analysis. N: number of transcripts examined. Expression ratio of the autosomal genes in msloe male/male was higher than in female/male in (a) (P values, calculated by two-sided Mann–Whitney U test for the second and third chromosomes, were <0.05), but the effect sizes calculated by Cliff’s Delta for the second and third chromosomes were statistically negligible (0.07 and 0.08, respectively)51. Expression ratio of X-linked genes in msloe male/male was significantly lower than in female/male in (a) (P values, calculated by two-sided Mann–Whitney U test, were <0.05), and the effect size was non-negligible (0.21).
Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (KAKENHI the Bloomington Drosophila Stock Center for providing us with fly stocks. This work was supported in part by

Acknowledgements

13. Shi, Z. et al. Single-cyst transcriptome analysis of Drosophila male germline stem cell lineage. *Development* **147**, 184259 (2020).
14. Vihrovanovski, M. D., Lopes, H. F., Kerr, T. L. & Long, M. Stage-specific expression profiling of Drosophila spermatogenesis suggests that meiotic sex chromosome inactivation drives genomic relocation of testis-expressed genes. *PLoS Genet* **5**, e1000731 (2009).
15. Cline, T. W. A male-specific lethal mutation in *Drosophila melanogaster* that transforms sex. *Dev. Biol.* **72**, 266–275 (1979).
16. Lucchesi, J. C. & Skrippy, T. The link between dosage compensation and sex differentiation in Drosophila melanogaster. *Chromosoma* **82**, 217–227 (1981).
17. Hashiyama, K., Hayashi, Y. & Kobayashi, S. *Drosophila Sex lethal* gene initiates female development in germline progenitors. *Science* **333**, 885–888 (2011).
18. Marsh, J. L. & Wieschaus, E. Is sex determination in germ line and soma controlled by separate genetic mechanisms?. *Nature* **272**, 249–251 (1978).
19. Schüpbach, T. Autosomal mutations that interfere with sex determination in somatic cells of *Drosophila* have no direct effect on the germline. *Dev. Biol.* **90**, 117–127 (1982).
20. Oliver, B. Genetic control of germline sexual dimorphism in *Drosophila*. *Int. Rev. Cytol.* **219**, 1–60 (2002).
21. Ota, R. et al. Transcripts immunoprecipitated with Sxl protein in primordial germ cells of *Drosophila* embryos. *Dev. Growth Differ.* **59**, 713–723 (2017).
22. Primus, S., Pozzamenter, C., Baxter, K. & Van Doren, M. Tudor-domain containing protein 5-like promotes male sexual identity in the *Drosophila* germline and is repressed in females by Sex lethal. *PLoS Genet.* **15**, e1007617 (2019).
23. Waever, M. et al.Somatic control of germline sexual development is mediated by the JAK/STAT pathway. *Nature* **436**, 563–567 (2005).
24. Casper, A. & Van Doren, M. The control of sexual identity in the *Drosophila* germline. *Development* **133**, 2783–2791 (2006).
25. Sanó, H., Nakamura, A. & Kobayashi, S. Identification of a transcriptional regulatory region for germline-specific expression of *vasa* gene in Drosophila melanogaster. *Mech. Dev.* **112**, 129–139 (2002).
26. Van Doren, M., Williamson, A. L. & Lehmann, R. Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr. Biol.* **8**, 243–246 (1998).
27. Barolo, S., Castro, B. & Posakony, J. W. New *Drosophila* transgenic reporters: insulated P-element vectors expressing fast-maturing RFP. *Biotechniques* **36**, 436–442 (2004).
28. Bischof, J., Maeda, R. K., Hediger, M., Xarch, F. & Basler, K. An optimized transgenesis system for *Drosophila* using germ-line-specific cp31 integrases. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 3312–3317 (2007).
29. Koch, R., Ledermann, R., Urywyler, O., Keller, M. & Suter, B. Systematic functional analysis of bicaudal-D serine phosphorylation and intragenic suppression of a female sterile allele of *bicD*. *PLoS ONE* **4**, e4552 (2009).
30. Park, Y., Kelley, R. L., Oh, H., Kuroda, M. I. & Mellor, V. H. Extent of chromatin spreading determined by roX RNA recruitment to MSL proteins. *Science* **298**, 1620–1623 (2002).
31. Shigenobu, S., Kitade, Y., Noda, C. & Kobayashi, S. Molecular characterization of embryonic gonads by gene expression profiling in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13728–13733 (2006).
32. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
33. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* **34**, 525–527 (2016).
34. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2009).
35. Morita, S., Ota, R., Hayashi, M. & Kobayashi, S. Repression of G1/S transition by transient inhibition of mrl-1044 expression in *Drosophila* primordial germ cells. *iScience* **3**, 100950 (2020).
36. Ota, R. & Kobayashi, S. Myc plays an important role in *Drosophila* P-M hybrid dysgenesis to eliminate germline cells with genetic damage. *Commun. Biol.* **3**, 185 (2020).
37. Hayashi, M. et al. Conserved role of Ovo in germline development in mouse and *Drosophila*. *Sci. Rep.* **7**, 40056 (2017).
38. Casper, A. L. & Van Doren, R. The establishment of sexual identity in the *Drosophila* germline. *Development* **136**, 3881–3880 (2009).
39. King, R. C., Bahns, M., Horowitz, R. & Larramendi, P. A mutation that affects female and male germ cells differentially in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). *Int. J. Insect Morphol. Embryol.* **7**, 359–375 (1978).
40. Puil, D., Oliver, B. & Mahowald, A. P. The role of the ovarian tumor locus in *Drosophila melanogaster* germline sex determination. *Development* **119**, 123–134 (1993).
41. Hanyu-Nakamura, K., Sonobe-Nojima, H., Tanigawa, A., Lasko, P. & Nakamura, A. *Drosophila* Pgc protein inhibits P-TEFb recruitment to chromatin in primordial germ cells. *Nature* **451**, 730–733 (2008).
42. Seydoux, G. & Dunn, M. A. Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of Caenorhabditis elegans and *Drosophila melanogaster*. *Development* **124**, 2191–2201 (1997).
43. Rastelli, L., Richman, R. & Kuroda, M. I. The dosage compensation regulators MLE, MSL-1 and MSL-2 are interdependent since early embryogenesis in *Drosophila*. *Mech. Dev.* **53**, 223–233 (1995).
44. Mellor, V. H. & Rattner, B. P. The roX genes encode redundant male-specific lethal transcripts required for targeting of the MSL complex. *EMBO J.* **21**, 1084–1091 (2002).
45. Jin, Y., Wang, Y., Johansen, J. & Johansen, K. M. JIL-1, a chromosomal kinase implicated in regulation of chromatin structure, associates with the male specific lethal (MSL) dosage compensation complex. *J. Cell Biol.* **149**, 1005–1010 (2000).
46. Leraas, S. et al. JIL-1 kinase, a member of the male-specific lethal (MSL) complex, is necessary for proper dosage compensation of eye pigmentation in *Drosophila*. *Genetics* **143**, 213–215 (2005).
47. Cugusi, S. et al. Topoisomerase II plays a role in dosage compensation in *Drosophila*. *Transcription* **4**, 238–250 (2013).
48. Van Deusen, E. B. Sex determination in germ line chimeras of *Drosophila melanogaster*. *Development* **37**, 173–185 (1977).
49. Steinmann-Zwicky, M., Schmid, H. & Nöthiger, R. Cell-autonomous and inductive signals can determine the sex of the germ line of *Drosophila* by regulating the sex *Sxl* gene. *Cell* **57**, 157–166 (1989).
50. Murray, S. M., Yang, S. Y. & Van Doren, M. Germ cell sex determination: a collaboration between soma and germline. *Curr. Opin. Cell Biol.* **22**, 722–729 (2010).
51. Romano, J., Kromrey, J. D., Coraggio, J. & Skowronnek, J. Appropriate statistics for ordinal level data: Should we really be using t-test and Cohen'sd for evaluating group differences on the NSSE and other surveys. *Annual Meeting of the Florida Association of Institutional Research 1–3 (2006).*

Acknowledgements

We thank the University of Minnesota Genomic Center for Illumina next-generation sequencing. We also thank the Bloomington Drosophila Stock Center for providing us with fly stocks. This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (KAKENHI Grant Numbers: 24247011, 25114002, 18H05552, 18K14739, and 20H03287) and a Grant from the TARA project (Grant Number: 202012).
Author contributions
R.O. and S.K. designed the experiments. R.O., M.H., S.M., and H.M. performed the experiments. R.O. and S.K. wrote the paper. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-84402-7.

Correspondence and requests for materials should be addressed to R.O. or S.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021