



UNIVERSITÀ
DEGLI STUDI DI TRIESTE

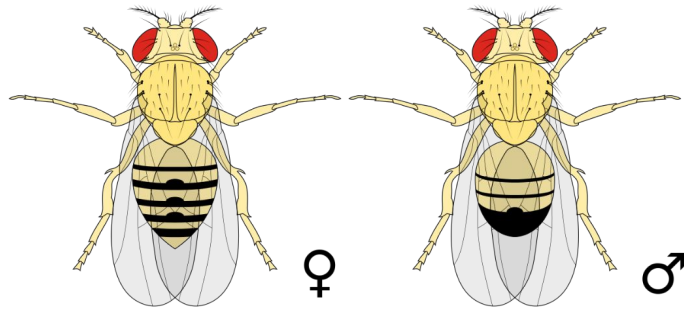


DIPARTIMENTO DI
SCIENZE DELLA VITA

Dosage Compensation in *Drosophila*

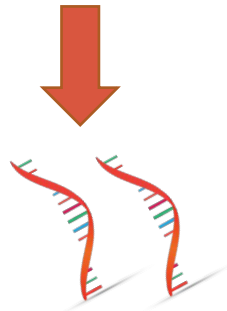
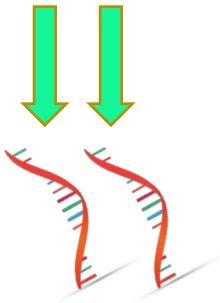
Simone Bellini
Séverine Nozownik
Roberta Palmitessa

Dosage Compensation was discovered in *Drosophila*



XX

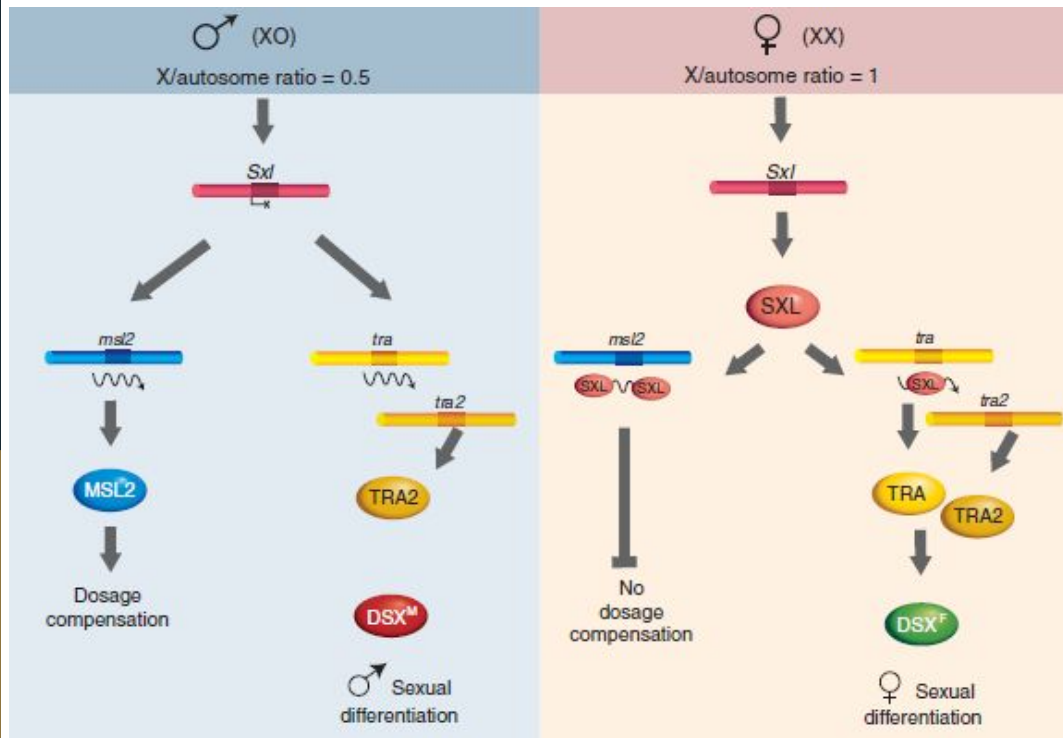
XY



- **X** chromosome carries many **genes** involved in **housekeeping functions/developmental** pathways.
- **Loss-of-function** mutations of *msl1*, *msl2*, *msl3* and *mle* (**MSL complex**) are **lethal** in males.
- **MSL complex** is **present** in males and **absent** in females.

Females have twice the number of these genes, yet the products level is the same in both sexes. The first step in dosage compensation is to establish this sex specificity.

Regulators of Dosage Compensation



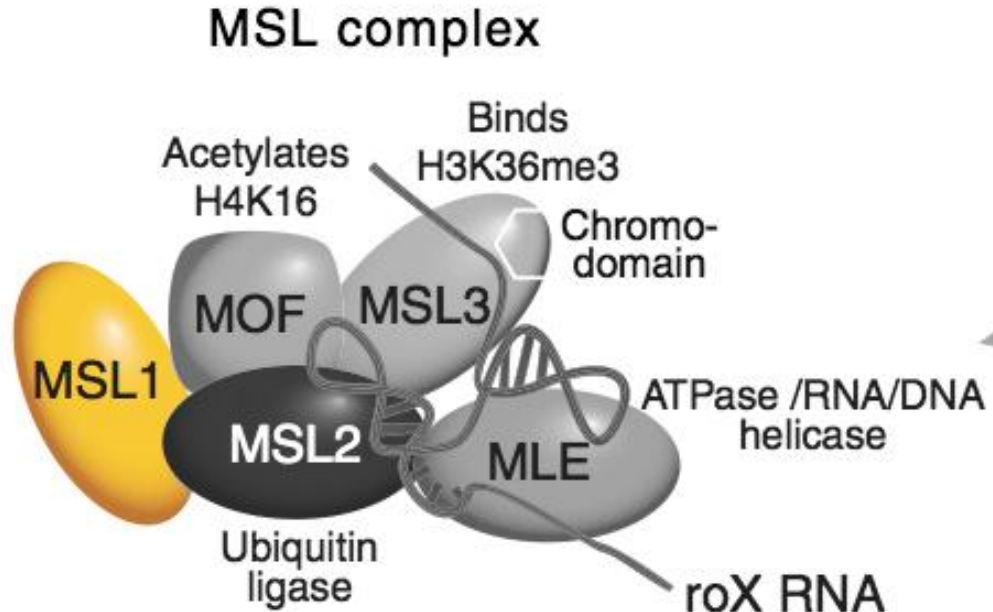
- **X:autosome ratio controls both sex and dosage compensation.**
- ***Sxl* encodes a female-specific RNA binding protein regulating sex determination and dosage compensation pathway.**
- ***Sxl* is positively regulated by transcription factors encoded by the X.**

The key target of SXL is *msl2* mRNA, repressing its translation in female.

Assembly of the complex responsible for compensation

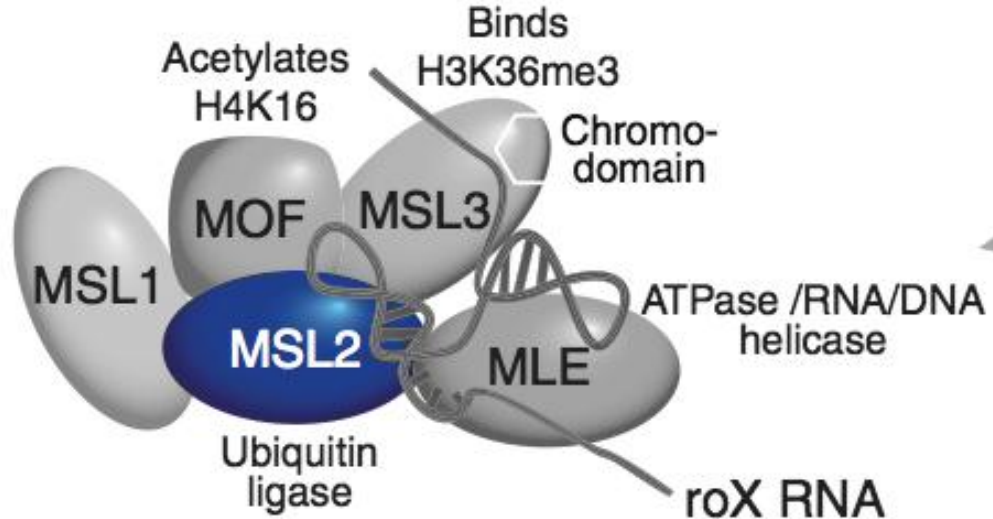
MSL1 forms a scaffold for interaction with **MSL3** and **MOF**.

Association of **MSL1** and **MSL2** is essential to chromatin.



Assembly of the complex responsible for compensation

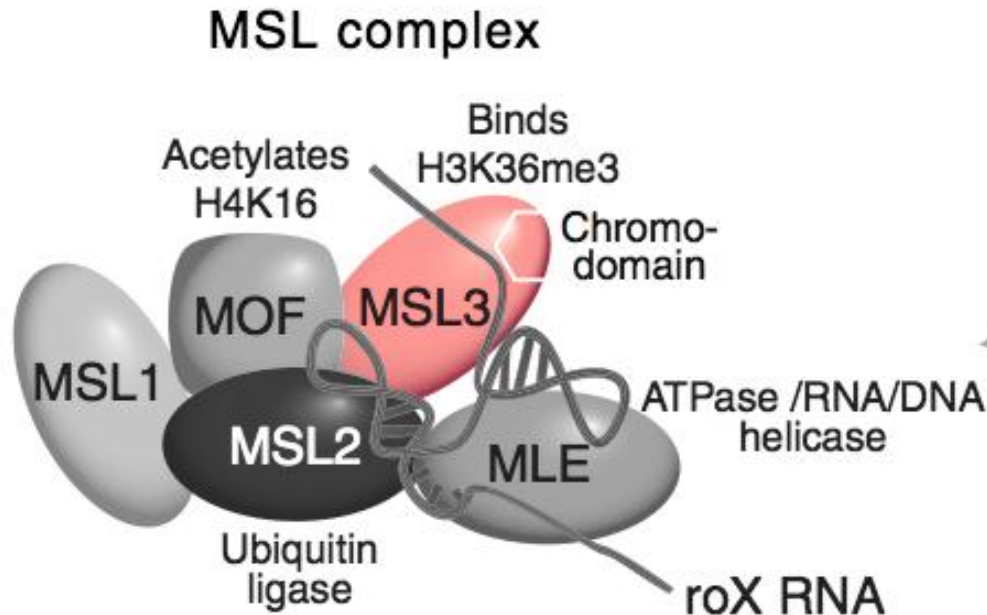
MSL complex



In the **absence** of interaction with **MSL2**, **MSL1** is destabilized.

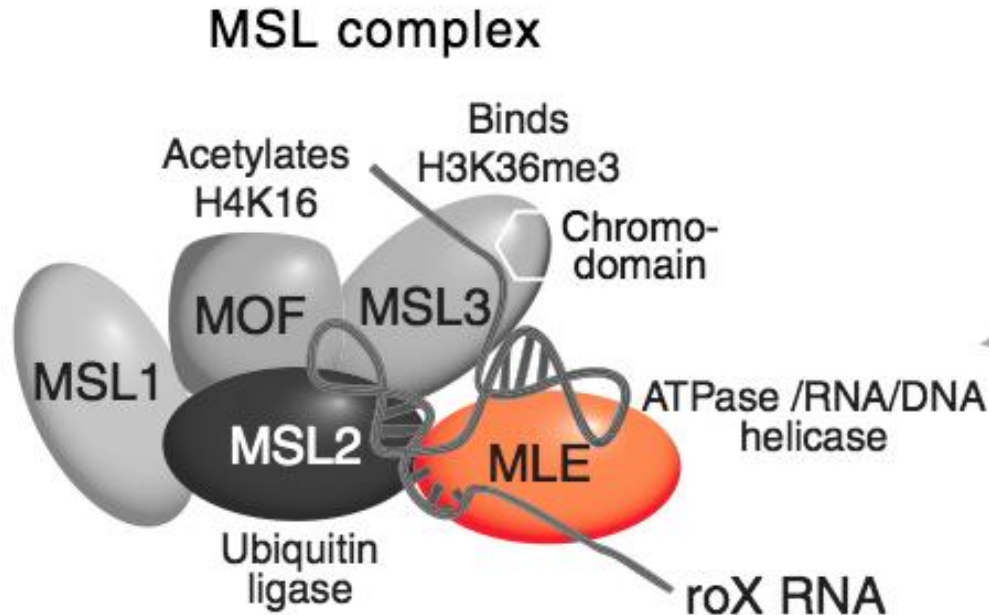
MSL2 ubiquitinates **MOF**, **MSL1**, **MSL3** and itself.

Assembly of the complex responsible for compensation



Interaction between the MSL3 chromodomain and active chromatin marks may help the MSL complex to locate target genes.

Assembly of the complex responsible for compensation



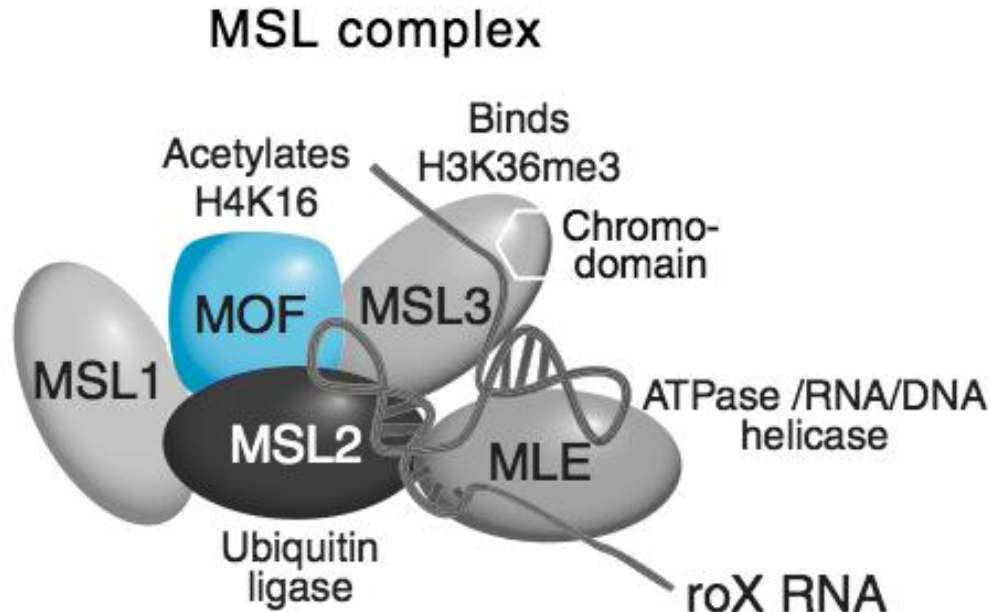
MLE shows RNA/DNA helicase, ATPase and single-stranded RNA/DNA binding activities.

MLE performs its function by interacting with the roX RNAs.

Assembly of the complex responsible for compensation

MOF can acetylate H4K16.

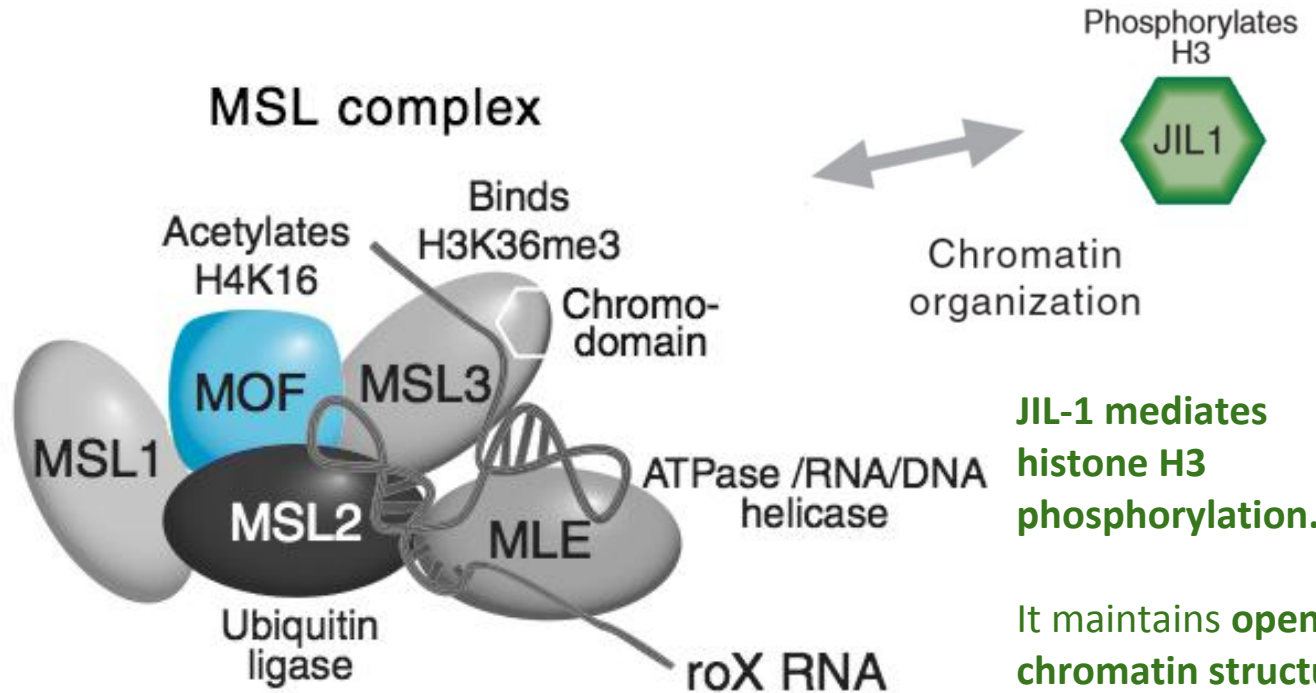
The principal role of the rest of the complex may be to localize MOF to its targets on the X chromosome.



Assembly of the complex responsible for compensation

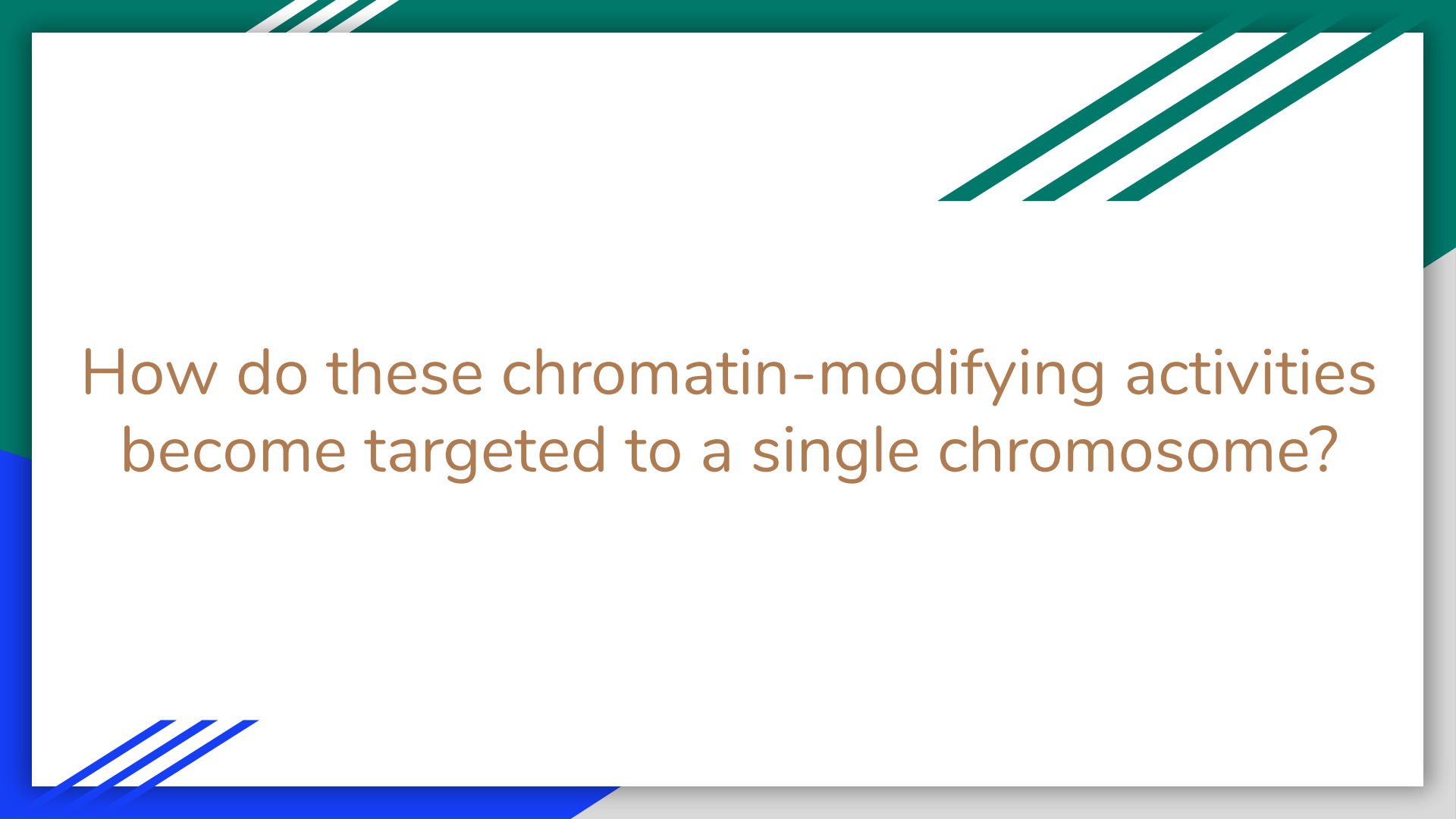
MOF can acetylate H4K16.

The principal **role** of the rest of the **complex** may be to **localize MOF** to its targets on the X chromosome.



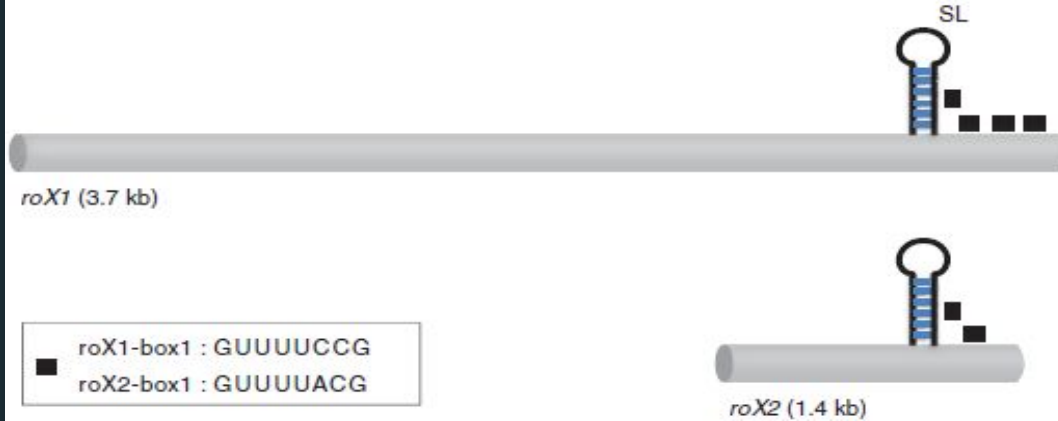
JIL-1 mediates histone H3 phosphorylation.

It maintains **open chromatin structure** in transcriptionally active regions.



How do these chromatin-modifying activities become targeted to a single chromosome?

roX function and role in Dosage Compensation



RNA on X 1 and 2 are **dissimilar** in size and sequence

↓
yet **function redundantly** to target MSL complex to the male X chr

X-chromosome **mutant**
for both **roX1** and **roX2**

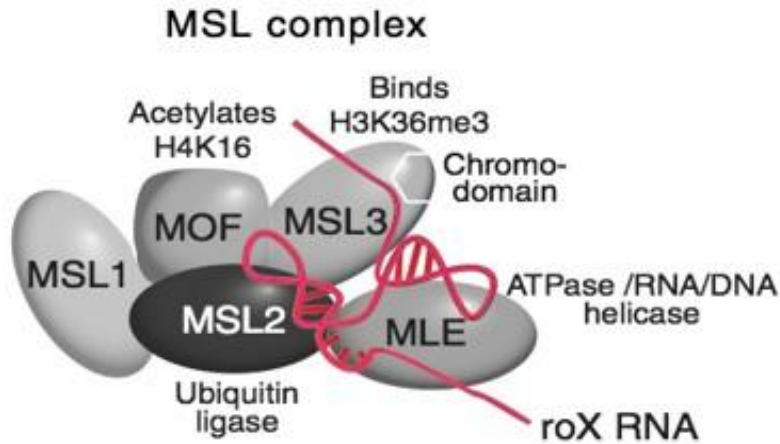


- ❑ most **double mutants die**
- ❑ **single mutants** have no known phenotype



They are capable of stimulating the H4K16 acetylation activity of the MSL complex

roX RNAs facilitate assembly and targeting of MSL complex on X chromosome



They are recovered after CHIP of MSL proteins → **physical association** of the RNAs with the complex

Minimal protein core **complex lacking roX RNAs** can still specifically **H4K16ac** and overexpression of MSL proteins can **partially overcome** the **lack of roX RNAs**

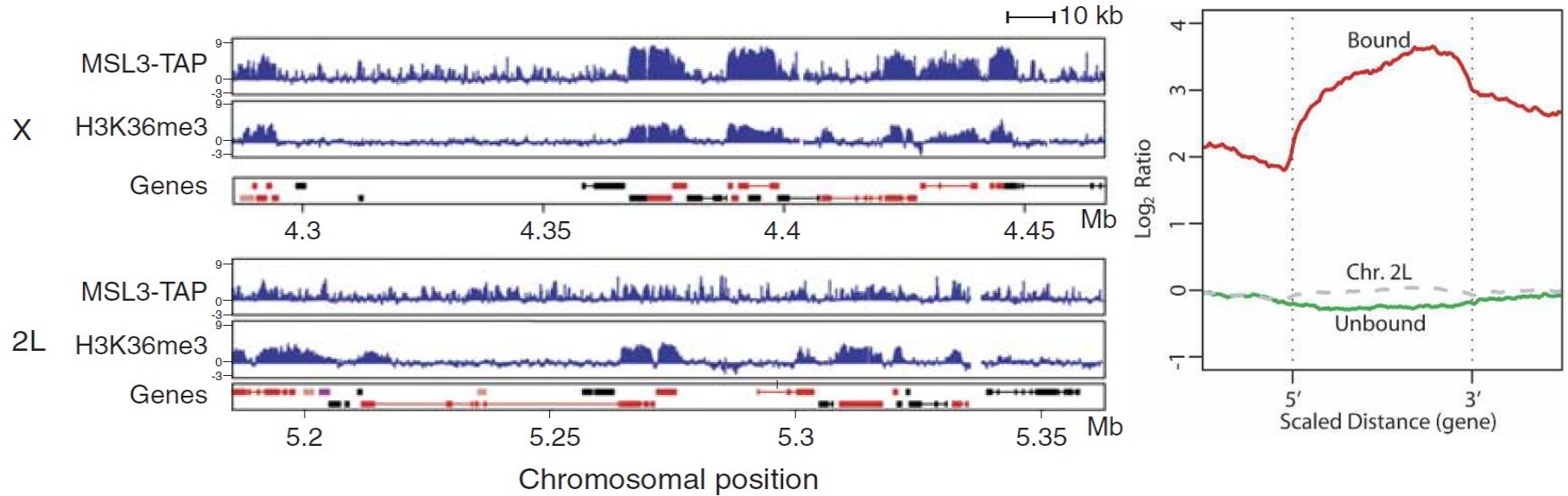


Proteins possess the essential functions of Dosage Compensation, but require the RNAs to stimulate assembly and spreading



But where does MSL complex
localize along X chromosome?

High resolution analysis of MSL binding on the X-chromosome



→ High resolution CHIP-chip analysis, SL2 cells

Red boxes → expressed genes

Black boxes → non expressed genes



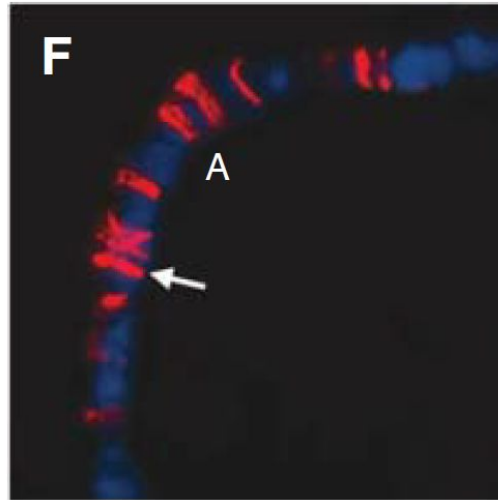
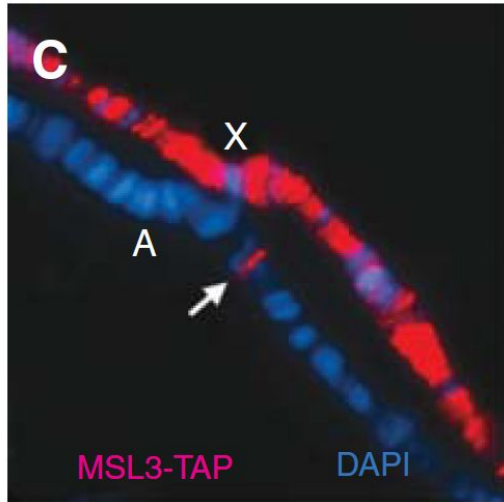
MSL colocalizes with H3K36me3 on middle and 3'ends of transcribed genes: it might act downstream at the level of *elongation*

Targeting model for MSL complex along X chromosome

- set of initiation sites dispersing the complex in *cis*
- full set of targets along the chromosome

WT

roX1⁻ roX2⁻



Arrow → autosomal roX transgene



roX ncRNAs mostly act in *cis*, and X-chromosome has additional targeting signals beyond the two *roX*

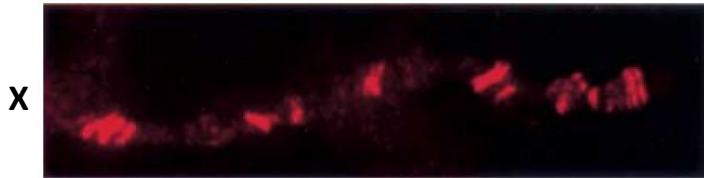
Targeting signals of the MSL complex

MSL-binding sites



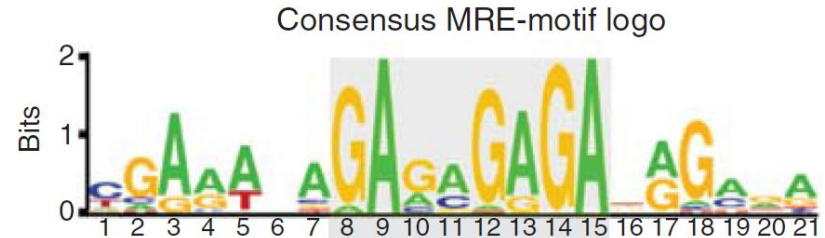
Complete complex → numerous sites

High-affinity sites



Mutant or incomplete complex → fewer sites: CESs and HASs

Common sequence motif (21-bp GA-rich): MRE



MRE mutations abolish MSL recruitment → key role in MSL recognition of the X chr

CLAMP (chromatin-linked adaptor for MSL proteins): without it, MSL complex is depleted along X chr → key role in recruiting the complex to initial binding sites



These entry sites enable sequence-specific binding of the MSL complex to the X-chromosome

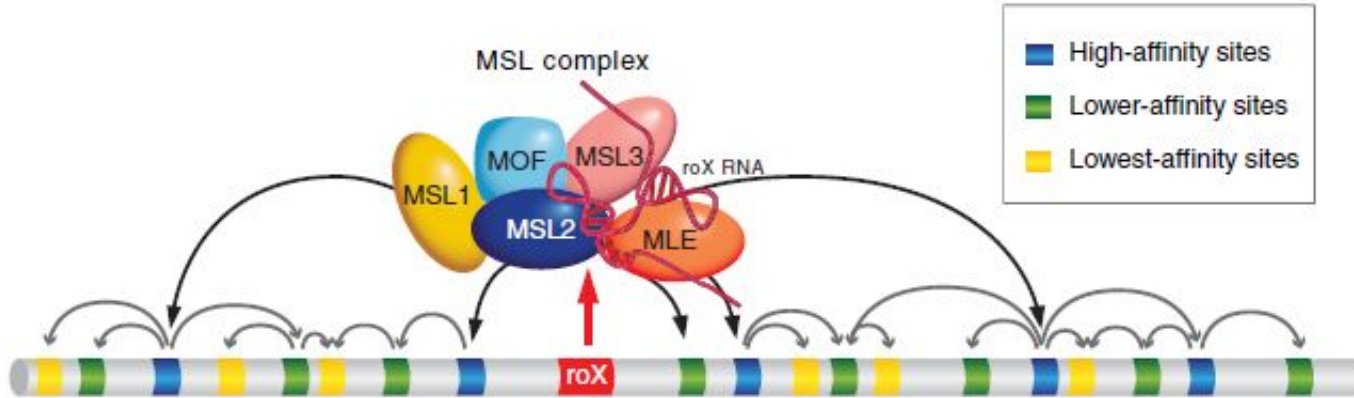
Transition from initiation sites to target genes

1. Recruitment of MSL to CESs
2. Spreading to sites of lower affinity: movement to **active genes**

→ What is the **mechanism for recognizing active genes?**

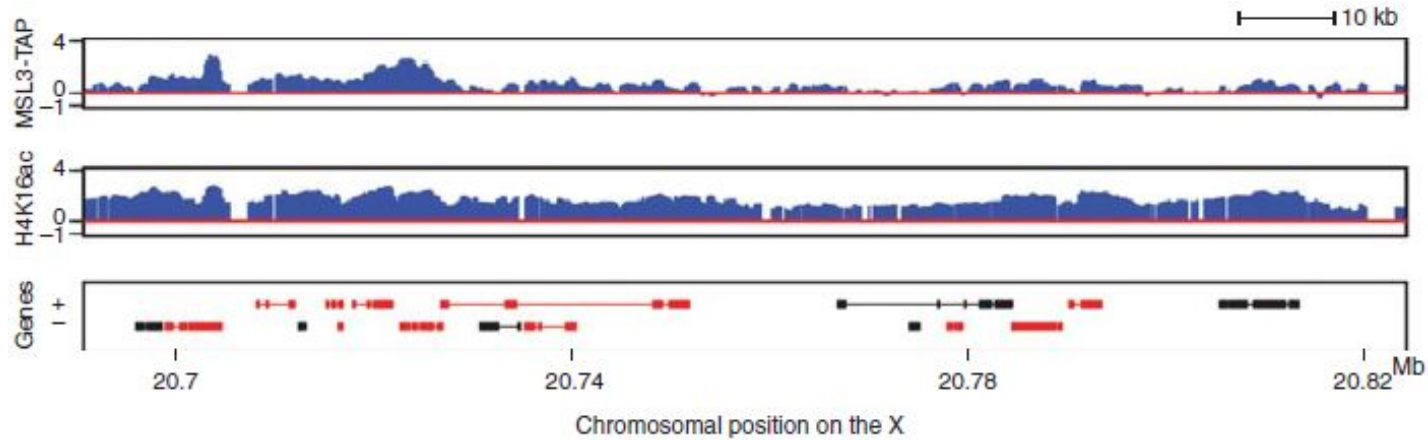
MSL complex **distribution** → strongly coincident with **H3K36me3** pattern

Absence of SET2 (HKMET) → MSL binding to target genes is decreased



➔ Spreading facilitated by MSL3 binding to H3K36me3 + roX RNA contribution

Chromatin modifications associated with Dosage compensation



H4K16ac:
MSL-dependent
mark

↓
**bias toward the
middle and the
3'end**

MSL binding → H4K16ac → weaken repressive internucleosomal structures

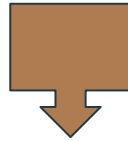


The presence of H4K16ac renders the chromatin of dosage compensated genes more accessible to factors or complexes

Mechanism of compensation

Transcriptional enhancement of X-linked genes responsible for dosage compensation occurs at **elongation step**:

- ❖ **H4K16ac → 3' ends bias (and not promoter)**
- ❖ **MSL colocalizes with H3K36me3 on middle and 3' ends of transcribed genes**
- ❖ **Strong / Weak promoters coexist on the X chromosome in males**
→ **All are two fold enhanced by dosage compensation mechanism**



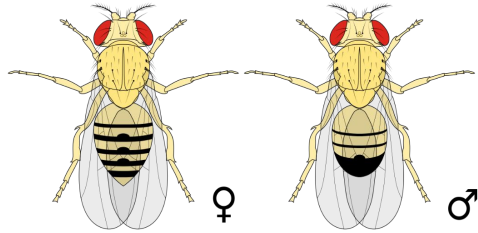
This mechanism seems based on enhancing the elongation rate, but it's not sufficient to explain dosage compensation.

Other processes must occur as :

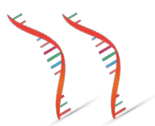
- **Increase in the frequency of recruitment of Pol or release from pausing**
- **Improvement of RNA Pol II processivity**

Dosage compensation in *Drosophila*

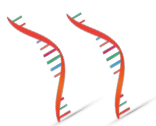
TAKE HOME MESSAGE



XX



XY

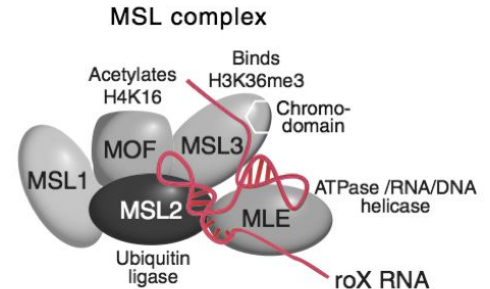


Regulation by a number of different mechanisms :

- **X-specific non-coding RNAs : roX1 and roX2**
- **Chromosome-wide targeting by MSL complex**
- **Site-specific histone acetylation : H4K16ac**

RNA-on-X 1 and 2 in *Drosophila melanogaster* fulfill separate functions in dosage compensation

Maria Kim, Marie-Line Faucillion, Jan Larsson



Introduction: roX1 and roX2

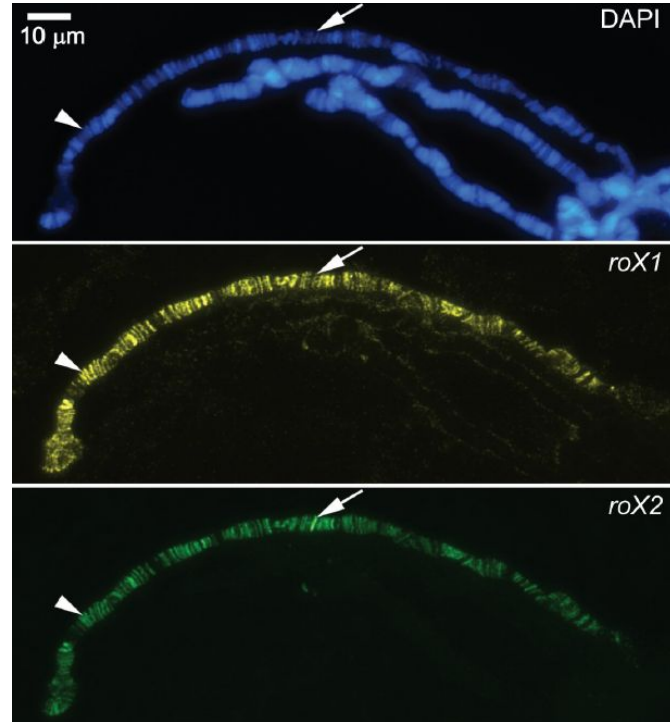
- Two different roX RNAs, **one per MSL complex**.
- roX1 transcribed in male and female blastoderm, then expression fades and **roX2 appears only in males**.
- roX redundancy allows mutations of roX1/2 alone, but **double mutations are lethal** for most males.



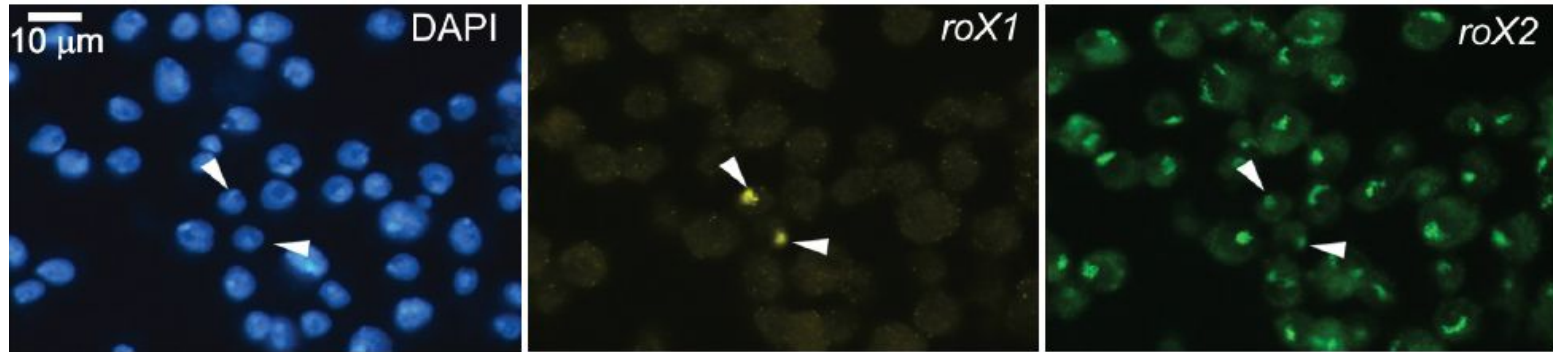
Expression of roX1 and roX2 is differentially regulated throughout cell cycle

- Polytene chromosomes immunostaining reveals **roX1/roX2** localization.

roX1/roX2 signals correlate closely both in intensity and patterns on X-chromosome.



Expression of roX1 and roX2 is differentially regulated throughout cell cycle



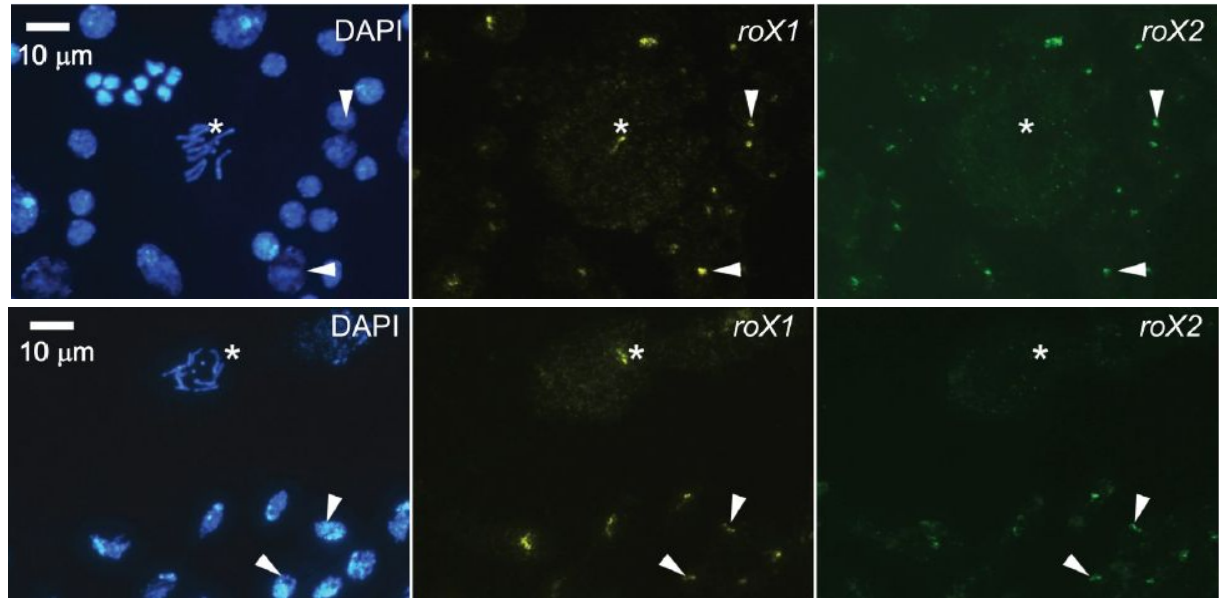
- In Schneider 2 cells ***roX2*** is expressed more strongly than ***roX1***, visible only in a small fraction of the cells.

Only a small fraction of S2 cells express both *roX* RNAs and all those expressing *roX1* also express *roX2*

Expression of roX1 and roX2 is differentially regulated throughout cell cycle

- **Neuroblasts** of male larvae and embryos subjected to **RNA *in situ* hybridization analysis**

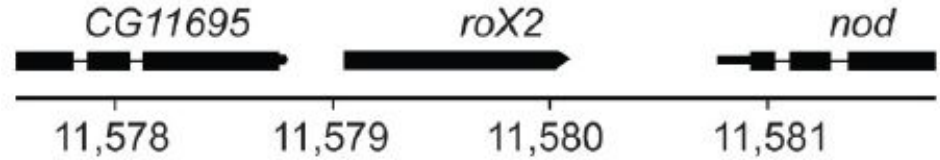
- **Only *roX1* signals were detected** on the distal part of the metaphase X-chromosome



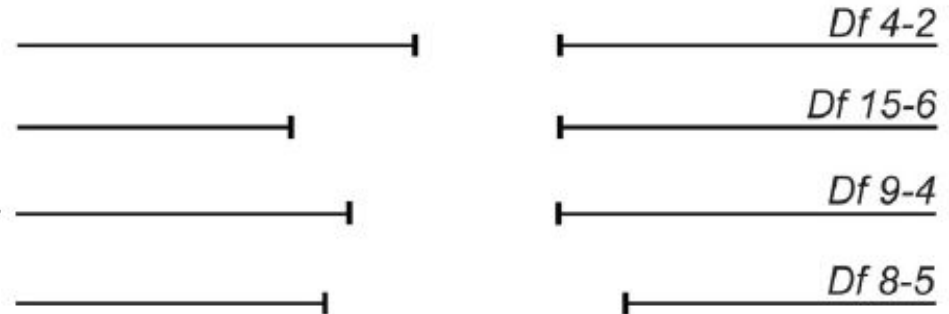
Expression of *roX* RNAs is differentially regulated and *roX1* RNA is the most bound to the X-chromosome during mitosis

Generation of new roX2 mutant alleles

• **Deletion mutant of *roX2* was created without affecting adjacent genes.**



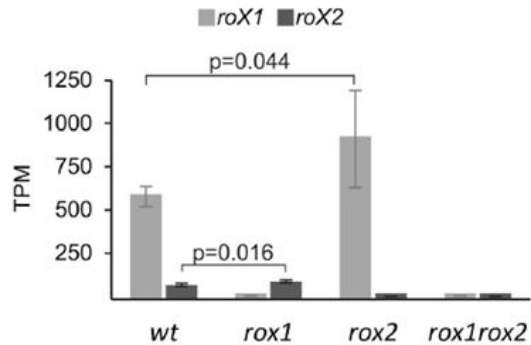
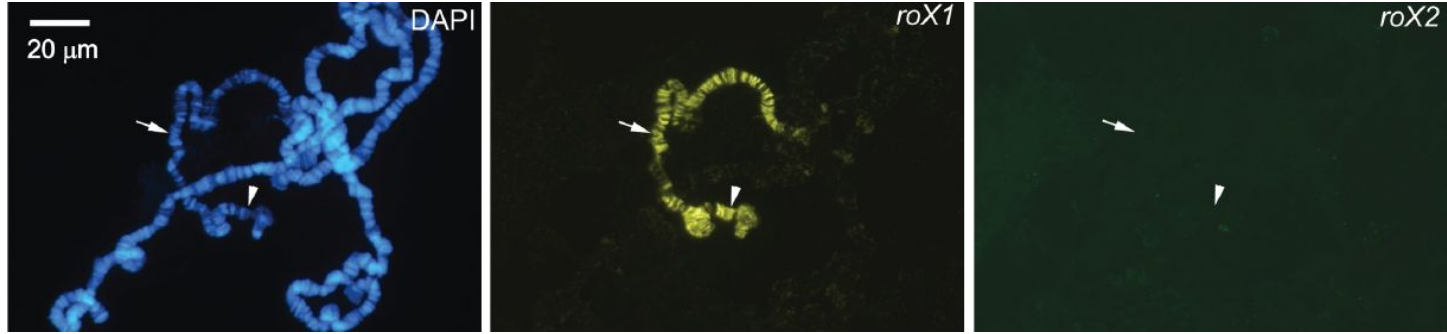
• **CRISPR-Cas9 technique was used to induce two double-strand breaks simultaneously in the *roX2* locus.**



Four *roX2* deletion mutant strains were obtained.

Df 9-4 was chosen for experiments and recombined with *roX1* mutant to obtain double mutant.

Generation of new roX2 mutant alleles



RNA *in situ* hybridization confirmed the absence of roX2 RNA in salivary glands.

High Throughput sequencing data and transcriptome analysis of *roX1*, *roX2* and *roX1roX2* mutant flies

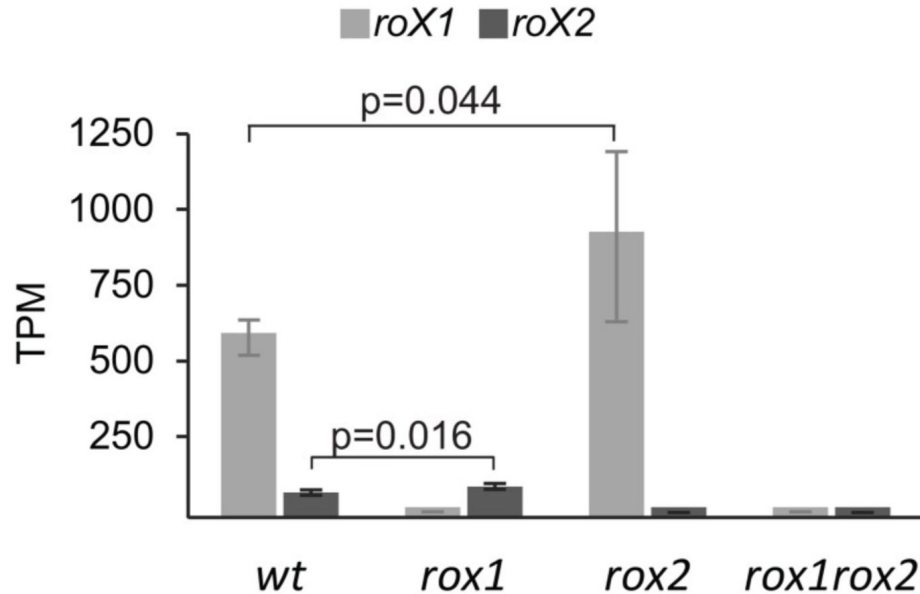
- **Obtention of 1st instar male larvae** : 80–100 virgin females, *y1 w1118* (used as *wild type*), *y1 w1118 roX1ex6* (*roX1* mutant), *y1 cho2 v1 roX29-4* (*roX2* mutant), and *y1 w1118 roX1ex6 v1 roX29-4/FM7i, P[w+mC ActGFP]JMR3* (*roX1 roX2* mutant), were crossed with 50–80 *FM7i, P[w+mC ActGFP]JMR3/Y* males. Non-GFP 1st instar larvae were collected (20 per sample)
- **Total RNA extraction** : Tri Reagent (Ambion)
- **Libraries** : TruSeq RNA Sample Prep Kit v2 (Illumina). In total, three wildtype, *roX2* mutant and *roX1 roX2* mutant bio-logical replicates were prepared and four *roX1* mutant replicates.
- **Sequencing** : HiSeq2500 instrument at SciLife lab (Uppsala)

High Throughput sequencing data and transcriptome analysis of roX1, roX2 and roX1roX2 mutant flies

- **Mapping to *Drosophila Melanogaster* genome** : version 6.09 using STAR v2.5.1b
- **Read counting** : samples used for the analysis had 29.3–56.2 M reads mapping quality values of 22.9–52.1 and mean mapping coverage of 201–497
- **Differential expression analysis** : DESeq2 package on R
 - Filters for exclusion : Threshold (Minimum 20 reads per genes) /Most variable genes/ White gene and neighbors
 - **Included genes** : 2356, 2659, 2571, 3164, 105, 10750 and 2042 genes on chromosomes 2L, 2R, 3L, 3R, 4, all autosomes except chromosome 4, and X, respectively
- **Average differential expression between replicates : log2-transformed and mean-centred**

What are the specific roles of the *roX* RNA species in dosage compensation ?

Average expression of roX1 and roX2 RNAs

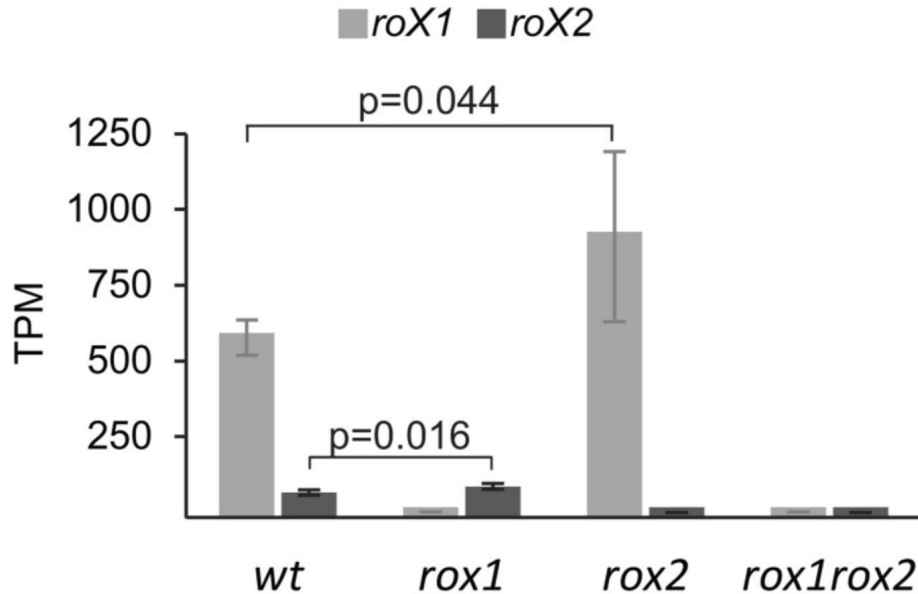


RNA sequenced from **1st instar male** larvae :

- **minimized indirect effects of dosage compensation** in *rox1rox2* mutant

TPM = Transcripts per kilobase per million

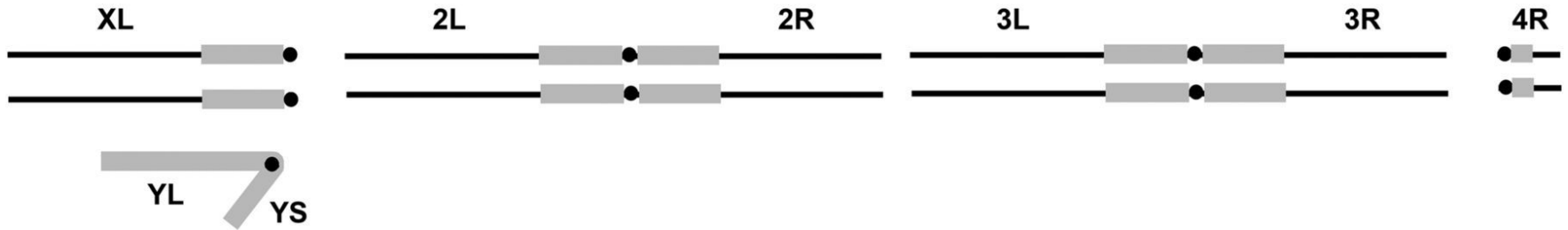
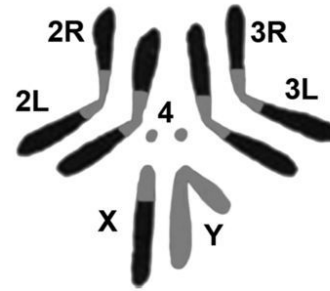
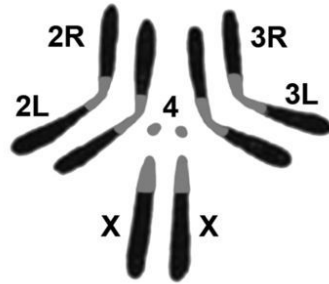
Average expression of roX1 and roX2 RNAs



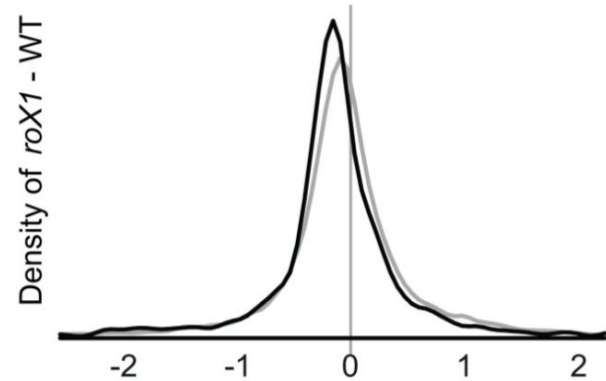
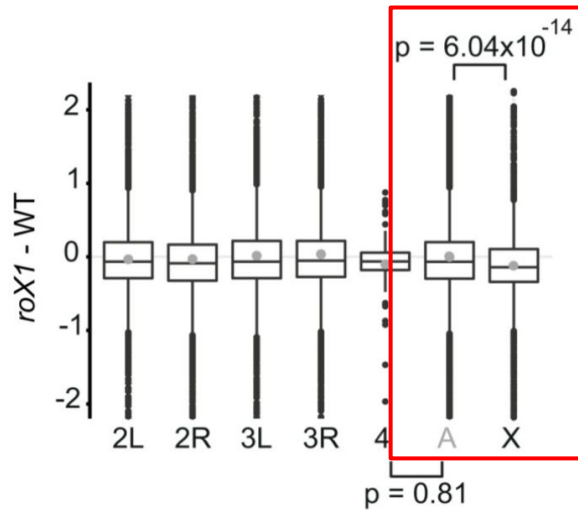
- **roX1 : 89% reductions** in roX RNA levels
- **rox2 : 45% increase** in roX1 RNA abundance
- Single mutants differ considerably in levels of roX RNA

Efficiency of dosage compensation significantly compromised in the *rox1* mutant and in the *rox1rox2* mutant

Drosophila chromosomes



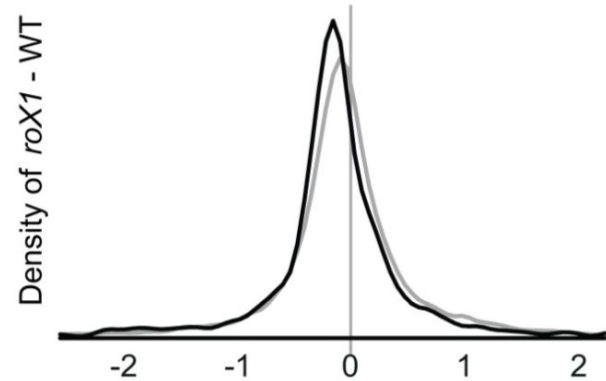
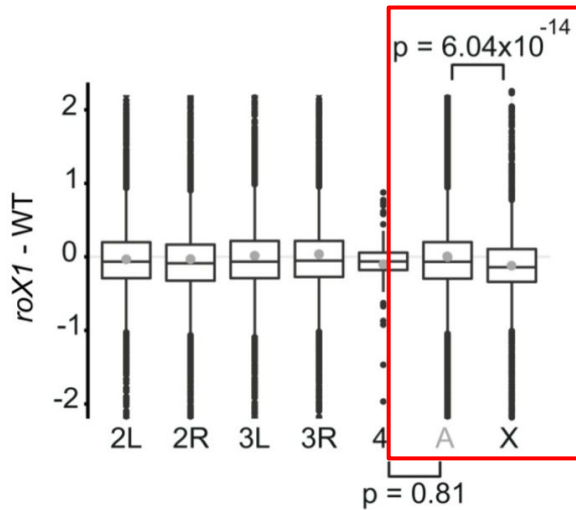
Chromosome-specific effects in roX mutants



➔ **8.6% reduction** in average expression of X chr genes relative to genes on the major autosomes arms

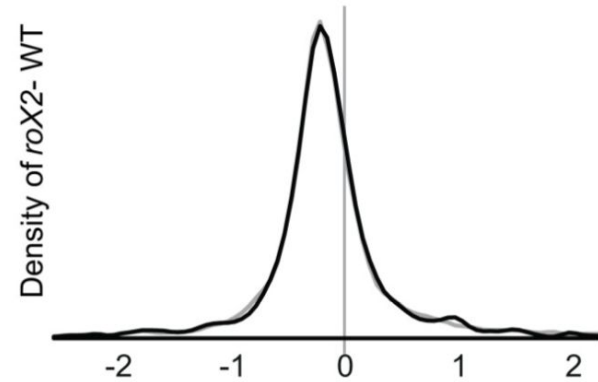
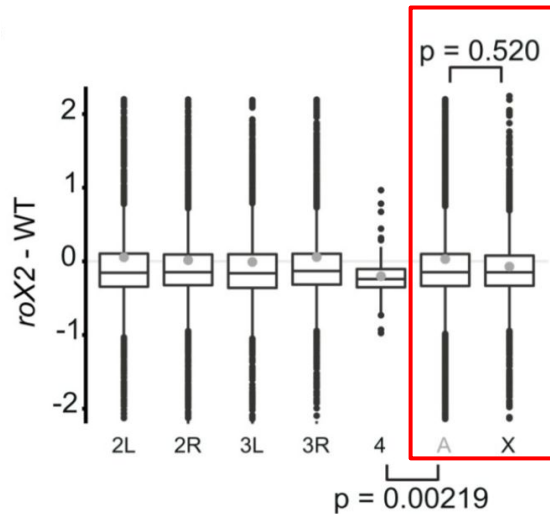
➔ Density distribution for X and autosomal expression ratios have **slight differences**

Chromosome-specific effects in *roX* mutants



Global X-chromosome transcription is slightly affected in the *roX1* mutant

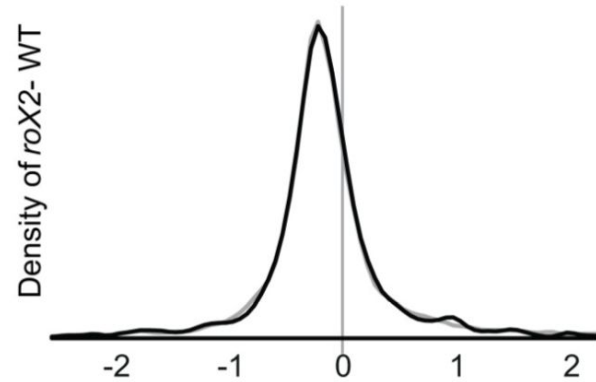
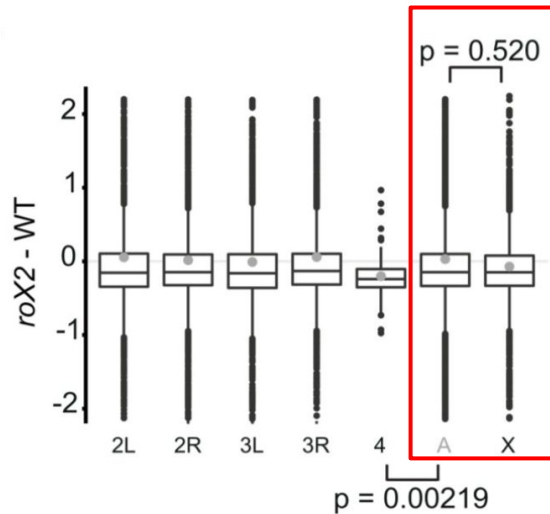
Chromosome-specific effects in roX mutants



➔ Average expression ratio for **X-chromosome genes slightly lower** than that of autosomal genes

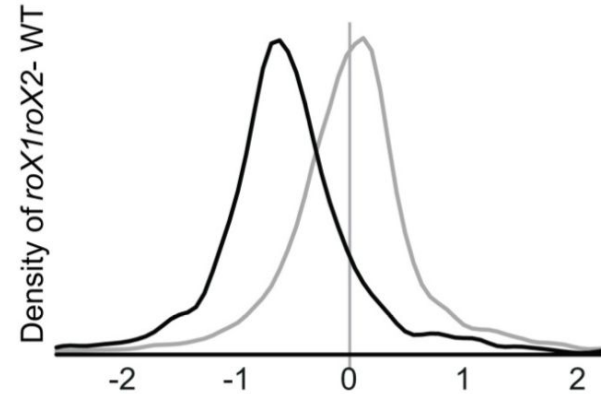
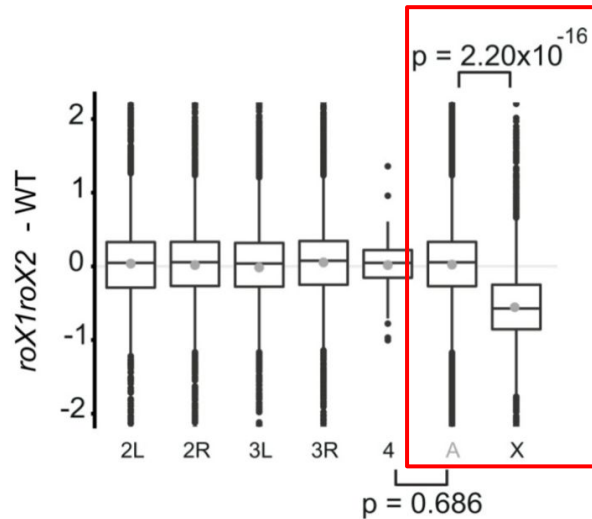
➔ Density distribution for X and autosomal expression ratios are **very similar**

Chromosome-specific effects in *roX* mutants



Global X-chromosome transcription is not significantly affected in the *roX2* mutant, and *roX2* mutant shows no lack of compensation

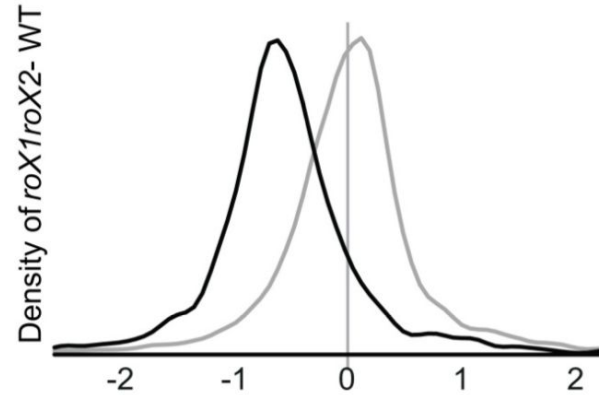
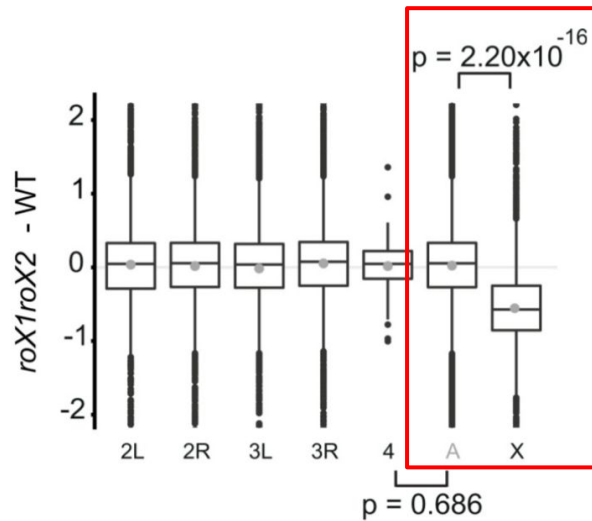
Chromosome-specific effects in roX mutants



➔ Average expression ratio for **X-chromosome genes really low** compared to autosomal genes

➔ Density distribution for X and autosomal expression ratios are **completely different**

Chromosome-specific effects in *roX* mutants

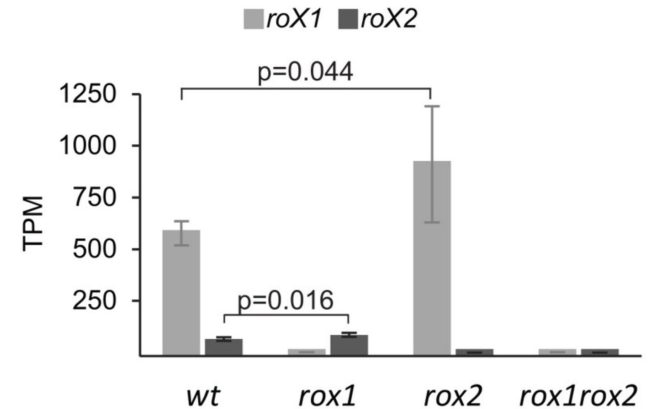


Global X-chromosome transcription is highly affected in the *roX1rox2* mutant

Chromosome-specific effects in *roX* mutants

Global X-chromosome transcription is :

- **slightly affected** in the *roX1* mutant
- **not significantly affected** in the *roX2* mutant
- **highly affected** in the *roX1roX2* mutant



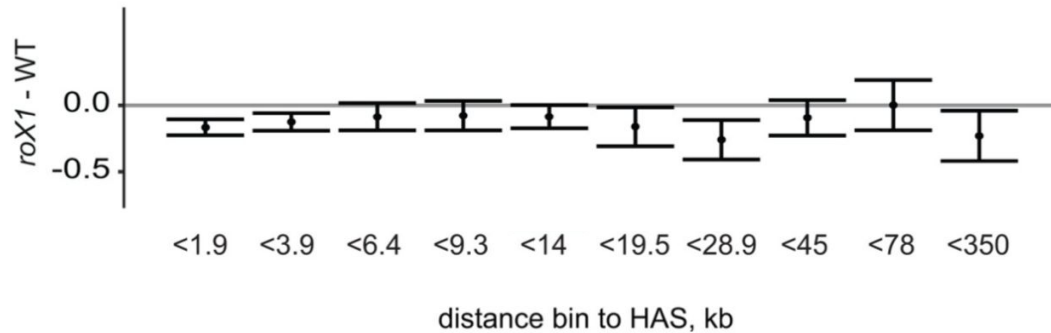
Dosage compensation not affected in *roX2* mutant compared to *roX1* and *roX1roX2* mutants



Does dosage compensation has a distinct spatial pattern along the X-chromosome related to High Affinity Sites (HAS)?

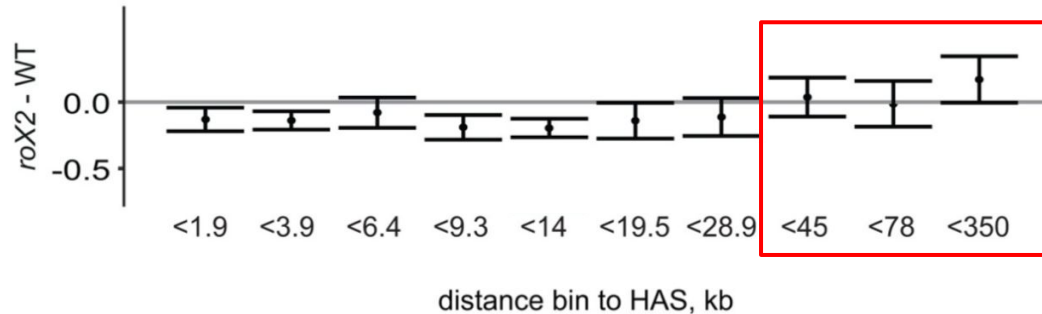


Dosage compensation of genes in roX mutants



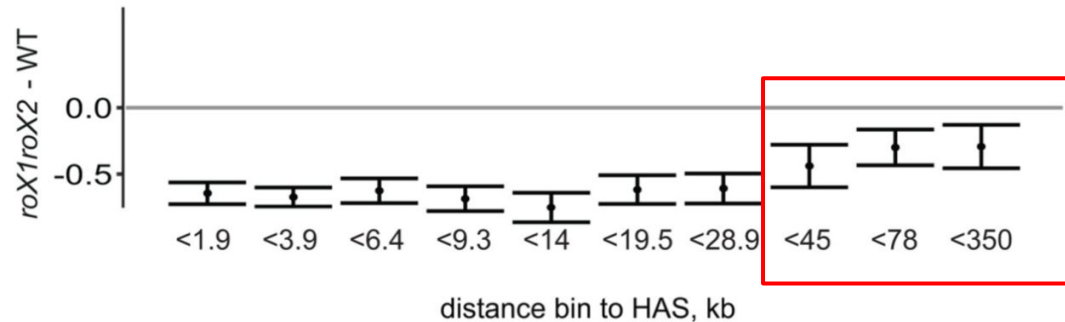
Average expression ratio **not significantly affected by the distance from HAS**

Dosage compensation of genes in roX mutants



Average expression ratio **not significantly affected by the distance from HAS within approximately 30kb**, then the ratio becomes higher than the **WT** for some remote genes (upregulation)

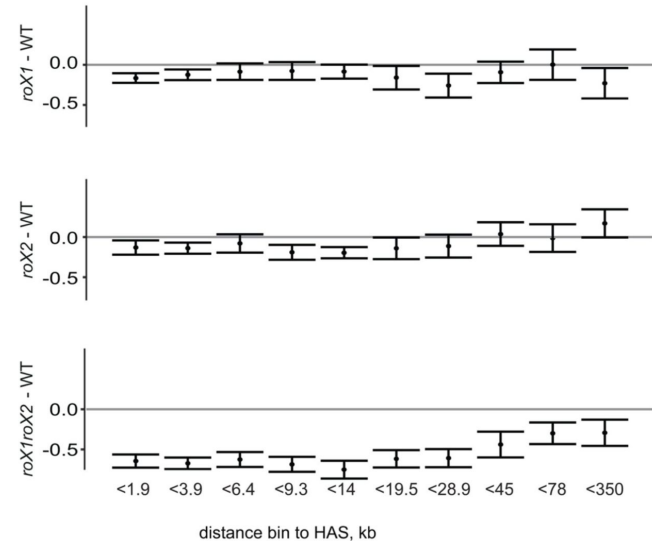
Dosage compensation of genes in roX mutants



Average expression ratio **not significantly affected by the distance from HAS within approximately 30kb**, then remote genes seems **less suppressed**

Dosage compensation of genes in *roX* mutants

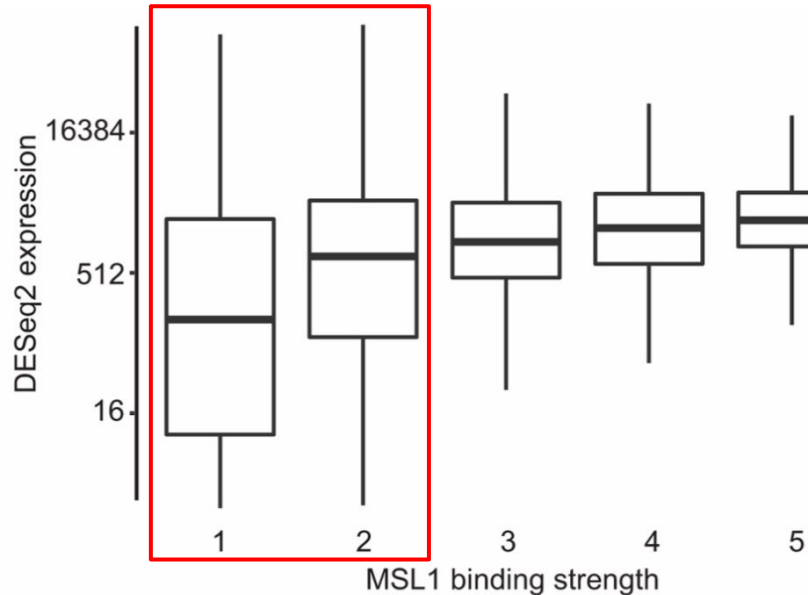
- Genes within approximately 30 kb from HAS are strongly and equally affected
- Genes more distant to HAS are less sensitive to the absence of *roX2* and *roX1roX2*



Distant genes may be compensated by an MSL-independent mechanism

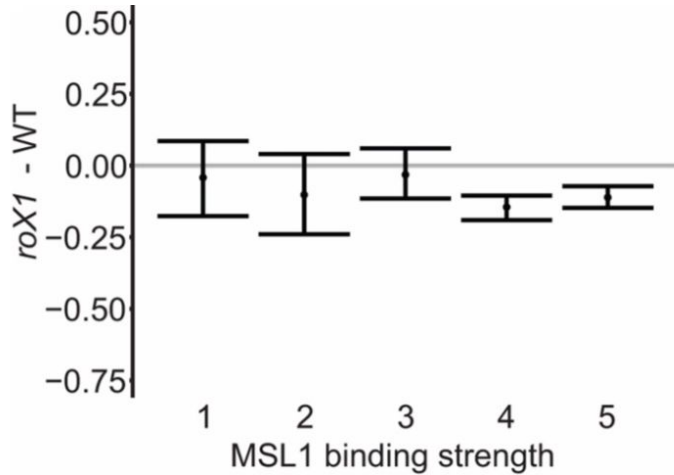
Does roX-dependent dosage compensation depends on the binding strength of the MSL complex ?

roX sensitivity of genes to the MSL complex binding strength



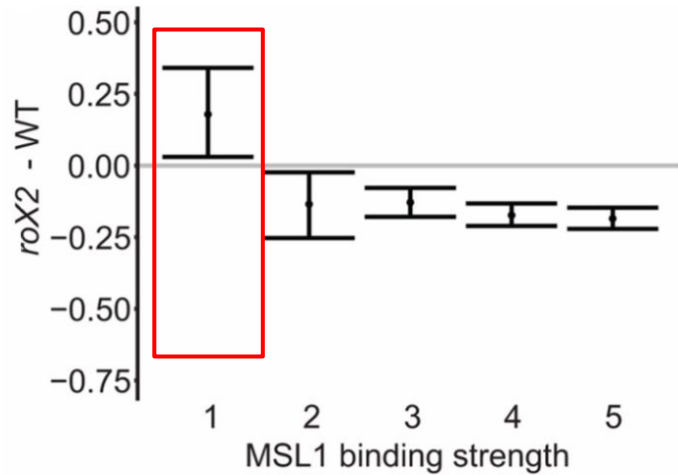
Bins 1 and 2 responded more variably to removal of either or both *roX* RNAs compared to the other bins, a pattern probably related to their low expression levels

roX sensitivity of genes to the MSL complex binding strength



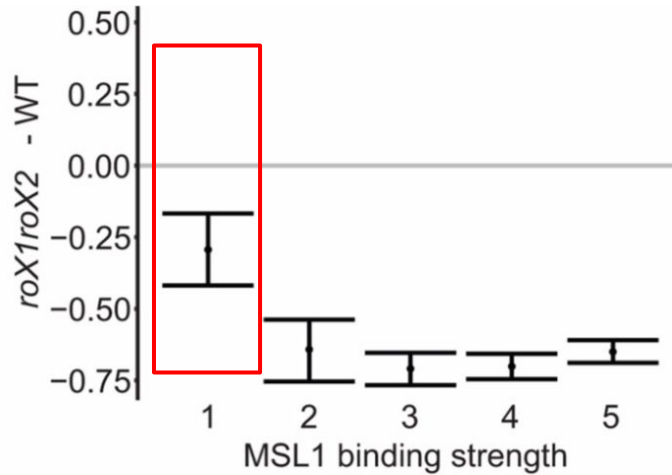
Expression ratios **not correlated**
with enrichment of MSL proteins,
no significant differences

roX sensitivity of genes to the MSL complex binding strength



- Expression ratios **not correlated with enrichment of MSL proteins**
- **Strong and significant upregulation of genes classified as non or weakly MSL complex-binding**

roX sensitivity of genes to the MSL complex binding strength



Weakly MSL complex-binding genes are suppressed, but much less than strongly binding genes

roX sensitivity of genes to the MSL complex binding strength

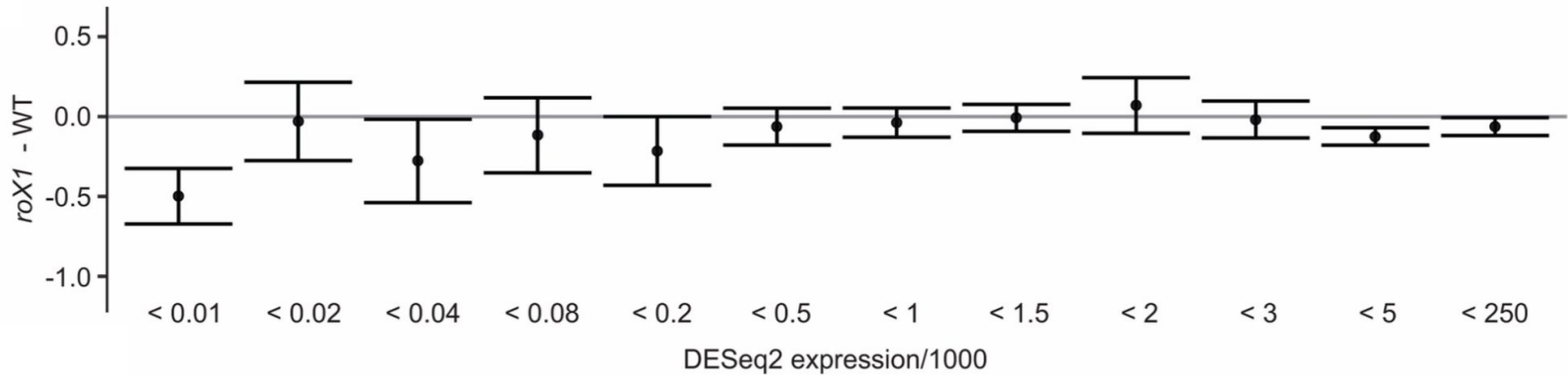
- **Single roX mutant** : Expression ratios **not correlated with enrichment of MSL proteins**
- ***rox2*** : **Strong and significant upregulation** of genes classified as **non or weakly MSL complex-binding**
- ***rox1rox2*** : **Weakly MSL complex-binding genes** are **suppressed**, but much **less than strongly binding genes**



As for distant HAS genes, weak MSL binding genes may be compensated by an MSL-independent mechanism

Does dosage compensation depends on genes' expression level in the absence of *roX* ?

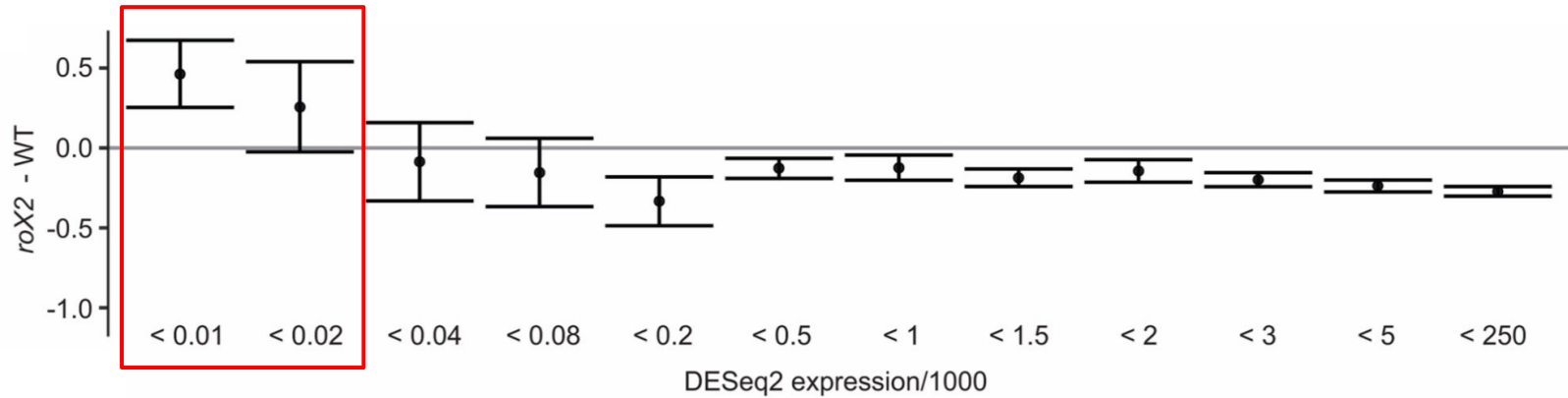
roX sensitivity of genes to the MSL complex binding strength



X chr : 12 equally sized bins
according to their expression levels

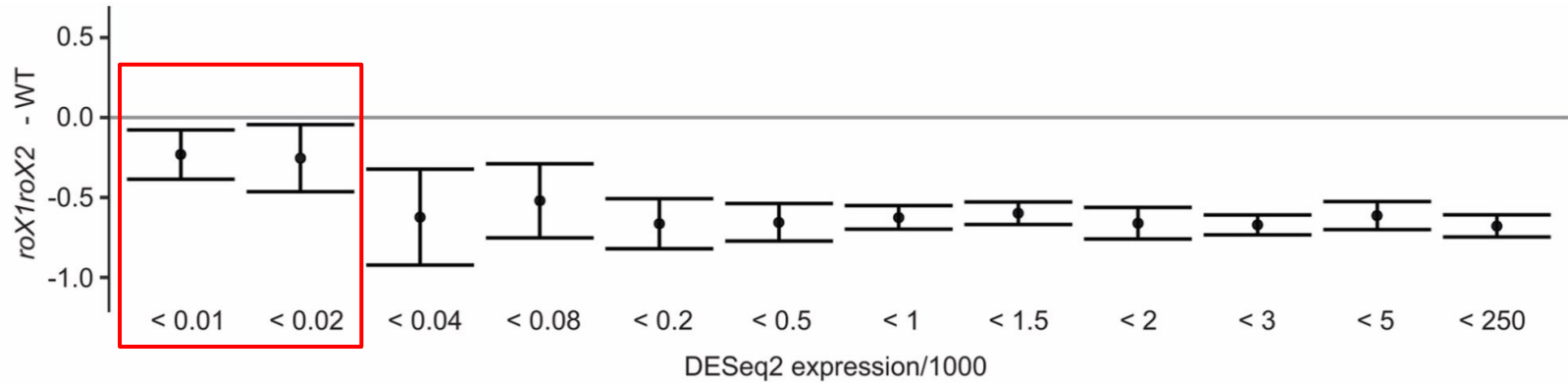
➔ **No significant gene expression changes between *rox1* mutant and the WT**

roX sensitivity of genes to the MSL complex binding strength



Upregulation of weakly expressed genes

roX sensitivity of genes to the MSL complex binding strength



Less pronounced reduction of weakly expressed genes

roX sensitivity of genes to the MSL complex binding strength

- ***rox1* mutant : No significant gene expression changes compared to the WT**
- ***rox2* mutant : Upregulation of weakly expressed genes**
- ***rox1rox2* mutant : Less pronounced reduction of weakly expressed genes**



Weakly expressed genes may be compensated by an MSL-independent mechanism

Weakly expressed genes



Greater distant to HAS



Less targeted by the MSL complex

All overexpressed in *roX2* mutant and less repressed in *roX1roX2* mutants



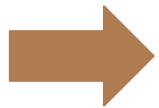
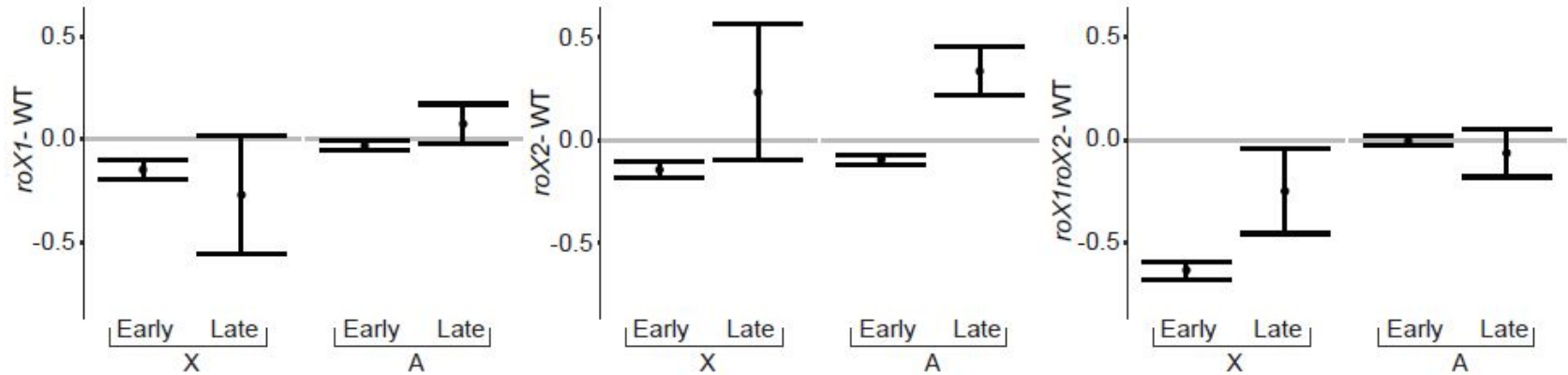
Those genes may be compensated by an MSL-independent mechanism that works with *roX2*

Does *roX* sensitivity correlate with replication timing?

roX sensitivity and replication timing

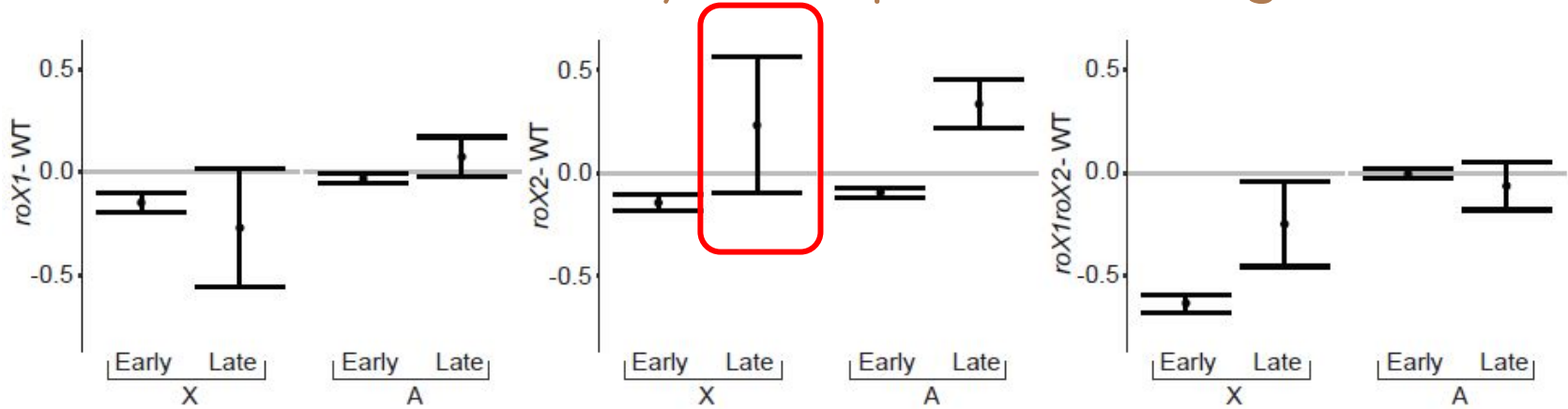
→ S2 and DmGB cells (male)

→ Kc167 cells (female)



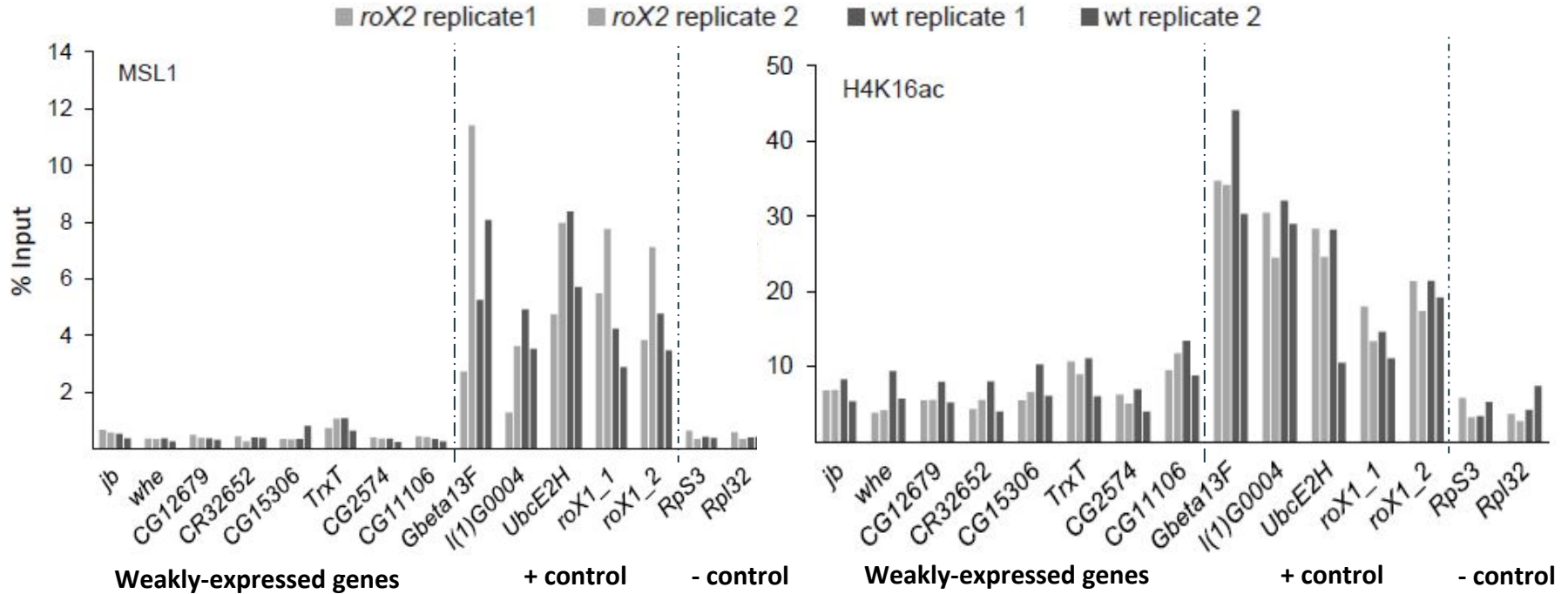
Early and late replication domains - bound and unbound genes by MSL complex - are affected in similar manners by *roX* mutations.

roX sensitivity and replication timing



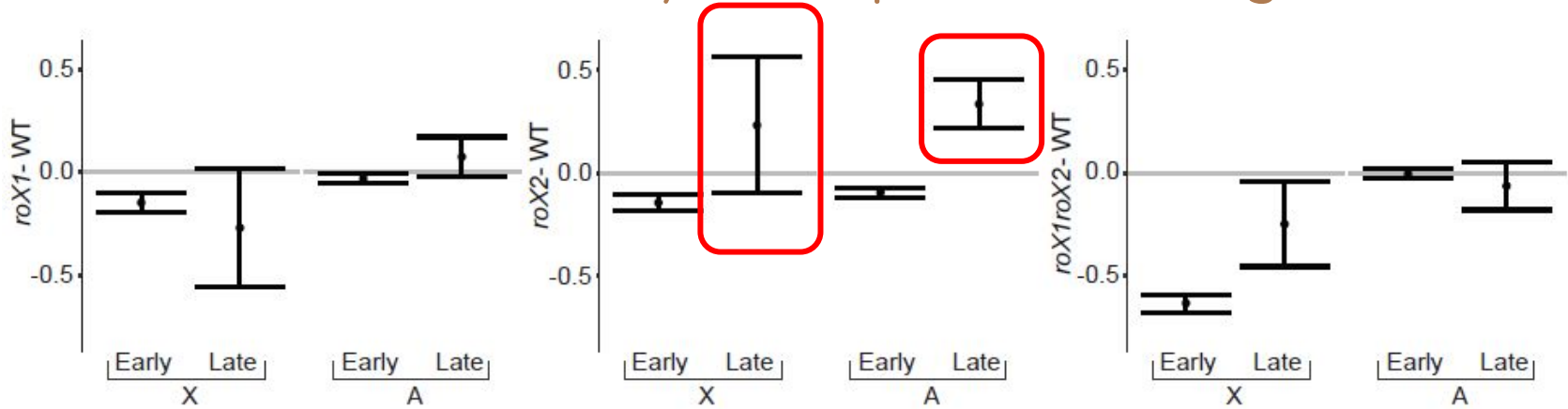
Is this upregulation caused by mis-targeting of MSL complexes associated with excess of *roX1* (and/or loss of *roX2*)?

Upregulation in *roX2* mutants



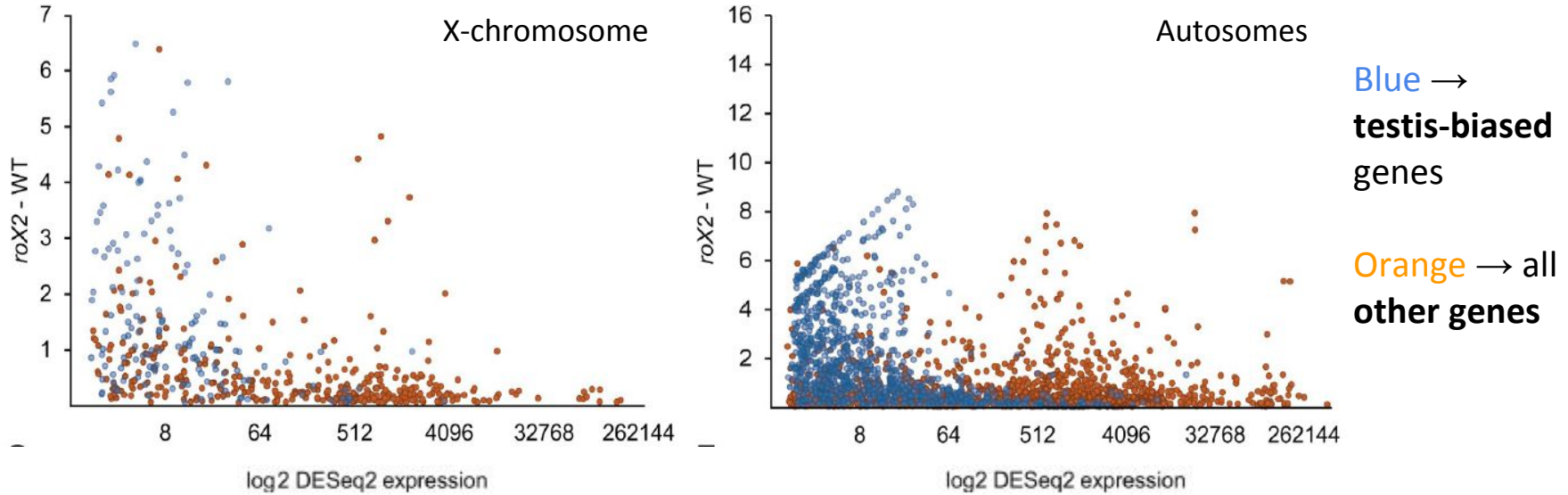
Stimulation of weakly expressed X-chr genes in *roX2* mutant is not mediated by MSL complex.

roX sensitivity and replication timing



Analysis of upregulated genes in *roX2* mutants showed that they included also some late-replicating autosomal genes.

Testis-biased genes are derepressed in *roX2* mutants



These upregulated genes in the *roX2* mutants include high proportions of genes (X-chromosomal and autosomal) with male-biased testis-specific transcription.

Conclusions

- ❑ ***roX1* and *roX2* fulfill separate functions in Dosage compensation in *D. Melanogaster*** (the two RNA species differ in both transcriptional level and cell-cycle regulation)
- ❑ **High tolerance for mis-expression of X-chromosome genes** has evolved, maybe in parallel with dosage compensation mechanisms; it may be a property of current and ancient sex-chromosomes.
- ❑ The function of MSL complex is compromised in *roX1roX2* mutants and the **dosage of distant genes is compensated by an alternative, unknown, mechanism.**
- ❑ Dosage compensation is a **stochastic process** that depends on **HAS distribution** and is correlated with **expression levels.**

THANKS TO JAN LARSSON FOR THE HELP !

Thanks for your mail. Please find my responses below and good luck with your presentation project.

Best wishes,

Jan

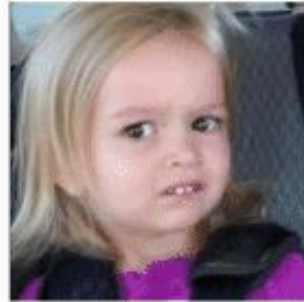
THANKS FOR YOUR ATTENTION !

After my awful presentation

Me



The class



My teacher



My friends

