Dosage Compensation in *Drosophila*
Dosage Compensation was discovered in Drosophila

- X chromosome carries many genes involved in housekeeping functions/developmental pathways.
- Loss-of-function mutations of \textit{msl1}, \textit{msl2}, \textit{msl3} and \textit{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.
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.
MLE shows RNA/DNA helicase, ATPase and single-stranded RNA/DNA binding activities.

MLE performs its function by interacting with the roX RNAs.
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.

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.
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 \textit{cis}
- full set of targets along the chromosome

\textit{roX} ncRNAs mostly act in \textit{cis}, and X-chromosome has additional targeting signals beyond the two \textit{roX}
Targeting signals of the MSL complex

Complete complex $\rightarrow$ numerous sites

Mutant or incomplete complex $\rightarrow$ fewer sites: CESs and HASs

MRE mutations abolish MSL recruitment $\rightarrow$ 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 $\rightarrow$ 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

MSL binding $\rightarrow$ H4K16ac $\rightarrow$ 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

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 transcripted 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 biological 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.
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 have 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

HAS = High Affinity Sites
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).
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 \textbf{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 depend 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?
Early and late replication domains - bound and unbound genes by MSL complex - are affected in similar manners by roX mutations.
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.
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 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