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Andrea Rachel Marcadis April 19, 2011

The Role of Topoisomerases in Dosage Compensation of the Male X Chromosome of *Drosophila melanogaster*

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An abstract of
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Abstract

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In fruit flies (*Drosophila melanogaster*), X chromosome dosage compensation is achieved by twofold upregulation of transcription of the male X chromosome. This increased level of transcription is the responsibility of the Male Specific Lethal (MSL) complex, which functions by modifying the chromatin of dosage compensated genes, enhancing the elongation step of transcription. Topoisomerases are enzymes that resolve the torsional tension and topological hindrance that occur when DNA is being actively transcribed. Because of their function in the elongation step of transcription, as well as experimental evidence revealing topoisomerase interaction with the MSL complex, a role for topoisomerases in the dosage compensation process was suspected. Using RNA interference to knock down topoisomerases and a plasmid model system to reproduce dosage compensation, this study reveals a role for topoisomerase II in *Drosophila* X chromosome dosage compensation. This observation helps to further elucidate the mechanism of action of the MSL complex.

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Background

XY Sex Determination

The chromosomes in the nucleus of every cell of an organism contain all of the information that makes that organism what it is; what proteins it makes, how it functions, its physical appearance, and its sex. Sex determination is almost always genetic, however the chromosomal differences that lead to sexual differentiation between males and females vary widely between species. In some organisms, there is no structural difference between the chromosomes containing the sex-determining alleles, while in others the sex-determining chromosomes are structurally and genetically different [1]. One of the most common sex determination systems is the XY system, which is seen in many species including all mammals, some insects such as the fruit fly (*Drosophila melanogaster*), as well as many plants [1,2]. In species with an XY sex determination system, female organisms inherit two X chromosomes, one from the male parent and one from the female parent, while male organisms inherit an X chromosome from the female parent and a morphologically and genetically different Y chromosome from the male parent [2].

While in mammals and other organisms it is the Sex-determining Region Y (*SRY*) on the male Y chromosome that causes development of the male sex organs and leads to sexual differentiation between males and females, *Drosophila* has a very different mechanism. In *Drosophila*, it is the ratio of X chromosomes to sets of autosomes (X:A ratio) in each cell of the organism that determines its sex. In normal cells, there are always two sets of autosomes, one set being all of the chromosomes inherited from the father, and the

other set all of those inherited from the mother. If the ratio of the number of X chromosomes to the sets of autosomes in a cell is 0.5 (1 X chromosome : 2 sets of autosomes), it will become a male. If that ratio is 1 (2 X chromosomes : 2 sets of autosomes), it will be female [3]. The difference in the X:A ratio leads to sex differences in *Drosophila* because an X:A ratio of 0.5 (male) causes the inactivation of the Sex-lethal gene (*Sxl*) while an X:A ratio of 1 (female) causes activation of *Sxl*. The presence of the SXL protein activates a pathway that leads to female specific gene expression and female characteristics [4].

While the Y chromosome in both mammals and *Drosophila* contains very few genes, all of which are male-specific, the X chromosome encodes for several non-sex-specific genes. These non-sex-specific genes are equally important for the development and maintenance of male and female organisms. In order for normal organismal function, these non-sex-specific gene products need to be present in equal quantities in both males and females. Because female organisms have two X chromosomes in each cell, and males only have one, there needs to be a mechanism to account for this genetic difference [5].

Dosage Compensation in *Drosophila*

Dosage compensation is a regulatory mechanism that serves to equalize the amount of X chromosome-linked gene products in males and females. Different species have different mechanisms for achieving dosage compensation, however they all function by remodeling chromatin through covalent and/or ATP-dependent mechanisms [6]. In mammals, one of the X chromosomes in every cell of a female organism condenses to form an inactive Barr body that does not get transcribed or translated into protein. Therefore,

both male and female organisms have one transcribed X chromosome per cell, and both male and female organisms have the same amount of X chromosome-linked gene products. In *C. elegans*, each of the hermaphrodite X chromosomes are hypotranscribed by one-half, leading to X chromosome gene products in the female equal to those of the one normally transcribed X chromosome in males. In *Drosophila*, dosage compensation is achieved by twofold upregulation of the male X chromosome, transcribing the male X chromosome at twice the rate of each of the two female X chromosomes, and bringing up the levels of X chromosome-linked gene products in males to those of the females [2].

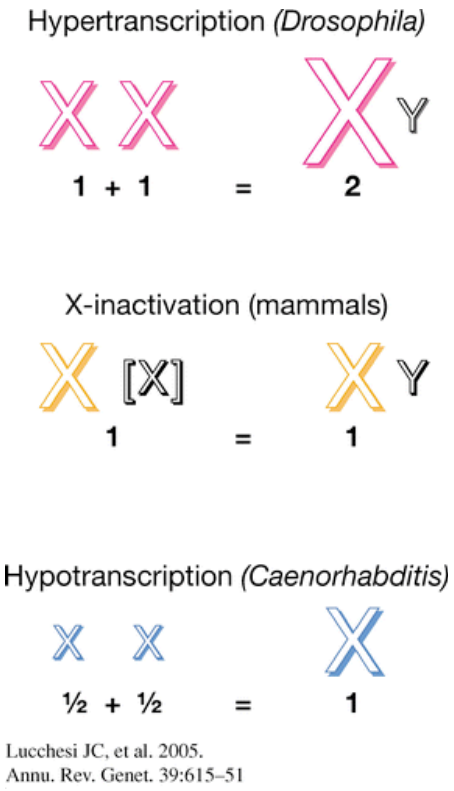
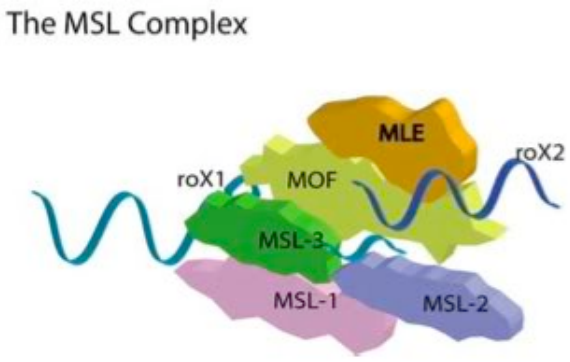


Figure 1: X chromosome dosage compensation mechanisms of *D. melanogaster*, mammals, and *C. elegans*.

The Male Specific Lethal (MSL) complex is responsible for this twofold upregulation of the genes on the male X chromosome in *Drosophila*. The MSL complex localizes at the 3' ends of the genes on the male X chromosome, and is made up of at least five proteins (MSL-1, MSL-2, MSL-3, MOF, and MLE) as well as one of two long non-coding RNAs (roX1 and roX2) [2]. Each of the subunits of the MSL complex plays an important role in its function. MSL-1 has been shown to act as an assembly platform for the complex, physically interacting with each of the MSL complex proteins (except for MLE) and allowing them to bind together [7]. MSL-2 is a critical component of the complex, because without it, MSL-1 is degraded, and the complex cannot assemble. The reason that

dosage compensation does not occur in female fruit flies is due to an absence of MSL-2 protein and thus an absence of the MSL complex in their cells. This absence of MSL-2 protein in females is the result of a genetic pathway activated by *Sxl*, which inhibits translation of the MSL-2 protein [7]. In males, *Sxl* pre-mRNA is spliced in a way that a premature stop codon is retained and the translated product is a non-functional short peptide. In the absence of the SXL protein, the MSL complex assembles and dosage compensation occurs. While not as much is known about MSL-3 as the other proteins of the MSL complex, evidence suggests that MSL-3 is involved in the spreading of the MSL complex along the X chromosome, rather than the initial attachment at the high affinity sites [8].



Mendjan S & Akhtar A. 2007. *Chromosoma* 116 (2): 95-106.

Figure 2: The components of the Male Specific Lethal (MSL) complex: MSL-1, MSL-2, MSL-3, MOF, MLE, roX1, and roX2.

The two long noncoding RNAs that associate with the MSL complex, roX1 and roX2, are essential in assuring that the MSL complex acts on the proper genes on the X chromosome. Because the roX RNAs are unstable unless they associate with some of the protein subunits of the MSL complex, the MSL complex only forms at loci where there are sequences coding for roX1 or roX2. From these loci, the MSL complex associates with the DNA at hundreds of sequences on the *Drosophila* male X chromosome, where dosage compensation occurs [2, 10].

Maleless (MLE) and Males absent on the first (MOF) are the two subunits of the MSL complex that act enzymatically to perform the functions that allow for dosage

compensation. MLE is thought to act as an ATP dependent RNA/DNA helicase. It is related to the ATPases present in complexes that remodel chromatin by altering the positioning between nucleosomes and chromatin, freeing the DNA for higher levels of transcription. The ATPase function of MLE is needed for MLE's role in transcription enhancement, while its helicase function is essential for the MSL complex to spread to its hundreds of attachment sites on the male X chromosome of *Drosophila* [9].

MOF is a histone acetyltransferase (HAT) protein that acetylates histone H4 at lysine 16 (H4K16ac), and is responsible for enrichment of this modification at the 3' ends of the compensated genes. While MOF (in the context of other complexes) also facilitates 5' enrichment of H4K16 acetylation throughout the genome of both males and females, this 3' enrichment is only found on the male X chromosome and is MSL dependent. A current hypothesis is that the MSL complex binds MOF already present on the promoters of genes, and skews its location towards the ends of genes for specific acetylation and upregulation of genes on the male X chromosome [7].

Acetylation of histones on the promoter regions of genes has long been associated with relaxed chromatin and increased levels of transcription [7]. The 3' histone acetylations facilitated by MOF and the MSL complex appear to function in the same way; to increase transcription levels on dosage compensated genes. Instead of enhancing transcription by promoting initiation, as the 5' histone acetylations are thought to do, the 3' histone acetylations increase transcription by enhancing the elongation step, decreasing the time necessary to complete a gene transcript [10]. By enhancing elongation, re-initiation (assembly of a new pre-initiation complex after the previous one leaves the promoter) can also be enhanced [11].

Topoisomerases and Dosage Compensation

In order for DNA to be transcribed, both during normal transcription as well as during dosage compensation, it needs to be locally unwound. DNA helicases, such as MLE, are enzymes that unwind the DNA for both transcription and replication. This local unwinding causes torsional tension and topological hindrance in the DNA, and DNA topoisomerases are enzymes that resolve those issues [12]. There are two classes of DNA topoisomerases, type I and type II, which differ in their structure and mechanism of action. Type I DNA topoisomerases (topo I) are monomeric and create transient single-strand breaks in the DNA [13]. Type II DNA topoisomerases (topo II) are dimeric and generate double-strand breaks in the DNA, using energy derived from ATP. It has been shown that topo I is primarily involved in releasing the torsional stress caused by RNA polymerase during transcription, while topo II is mainly involved in resolving topological problems that arise during DNA replication, allowing sister chromatids to separate from each other. While the main role of topo II is in replication, it has also been shown to play a role in transcription [12].

Because topoisomerases play such an essential role in the elongation step of DNA transcription [12], the same step that is enhanced in dosage compensated genes, it can be inferred that they play a role in *Drosophila* dosage compensation. Experimental evidence shows that topoisomerase II interacts with components of the *Drosophila* MSL complex *in vitro* [14]. It has also been shown to be part of a complex with DNA Supercoiling Factor (SCF), which is involved in dosage compensation and colocalizes with the MSL complex [15]. This evidence points to a possible role for topoisomerases in *Drosophila* dosage compensation.

Experimental Approach

In order to test for a possible role of topoisomerases in *Drosophila* dosage compensation, RNA interference (RNAi) was used to knock down the function of endogenous topoisomerases in *Drosophila* Schneider line 2 (S2) cells, and the effect of this absence of topoisomerases on dosage compensation was evaluated using a plasmid system containing a firefly luciferase reporter gene.

Drosophila S2 cells were chosen for this experiment because they are "male" cells in that they do not express Sex-lethal (*Sxl*), the regulatory gene for female sex determination, and contain a fully functional MSL complex [16]. It was therefore possible to utilize their endogenous MSL complex to test for dosage compensation on the plasmid system. In addition, they are easy to grow and maintain in the lab, are highly susceptible to RNAi gene inhibition, and are easily visualized using high-resolution light microscopy [17].

The plasmid system utilized in the experiment was developed in 2007 [16] in order to facilitate the study of the mechanism underlying dosage compensation. One plasmid (roX2 plasmid) reproduces dosage compensation by containing a fragment of the *roX2* gene immediately downstream of the reporter gene firefly luciferase. The presence of this sequence allows assembly of the endogenous MSL complex on the plasmid and twofold upregulation of the firefly luciferase gene. The plasmid used as a control (Nesprin plasmid) contains the firefly luciferase reporter gene, but the *roX2* sequence is replaced by a DNA fragment from an intron of the human Nesprin gene. Because it is missing the *roX2* sequence, which is critical for assembly of the MSL complex, the Nesprin plasmid cannot be dosage compensated.

By measuring the relative luciferase activity in topo I and/or topo II knockdowns versus control S2 cells, this study aims to discover differences in dosage compensation, thus establishing a role for topoisomerases in the dosage compensation mechanism of *Drosophila melanogaster*.

Materials and Methods

S2 Cells

The S2 cells used for this experiment were grown in HyQ SFX-insect medium (HyClone) with penicillin-streptomycin antibiotic at 25^{o}C without CO_2. On the first day of the experiment, 800,000 S2 cells were transferred to a six well culture dish and grown in 2 mL of medium.

RNAi Knockdown of Topoisomerases

1-2 hours after transferring the cells to the culture dish, they were treated with 10 μg/mL of double stranded RNA (dsRNA). Cells were treated with either topo I dsRNA (dstopoI) topo II dsRNA (dstopoII), or GFP dsRNA (dsGFP, control).

The primers used to make the double strand RNA are: GFP forward: 5' ACGTAAACGGCCACAAGTTC 3' reverse: 5' TGCTCAGGTAGTGGTTGTCG 3', topo Ia forward: 5' GCCCTTTACTTCATCGACAA 3' reverse: 5' GCCCTTTACTTCATCGACAA 3', topo Ib forward 5’ CGCAATGTACGGTTCTACTACG 3’ reverse 5’ TTATCGATTACCTTGTCCAGGC 3’, topo II forward: 5' TAGTGGCTCGATCTTTTGGC 3' reverse: 5' TTGCCAGAGCGATATCTCTACA 3'. The kit used to synthesize the dsRNA is MEGAscript T7 by Ambion.

Plasmids

Plasmids used were: ptTA, copia-*Renilla* luciferase (R), pBluescript (pBS) by Stratagene, and a plasmid containing a firefly luciferase gene; either roX2-FF (roX2) or Nesprin-FF (Nesprin).

Both of the firefly luciferase plasmids (FF) contain the tetracycline resistance operator (*tetO*) inserted upstream of the firefly luciferase gene of the pGL3-Basic plasmid (Promega). The roX2 plasmid contains a 1,087-base pair fragment of the *Drosophila roX2* gene (nucleotides 158-1244 of GenBank sequence U85981) inserted downstream of the firefly luciferase gene, in the BamHI unique pGL3-Basic site. The Nesprin plasmid is the FF plasmid with 1,140 base pairs from the Nesprin human intron (nucleotides 99300 to 100440 of the emb AL359235 sequence) inserted in the BamHI unique pGL3-Basic site to replace the *roX2* gene sequence.

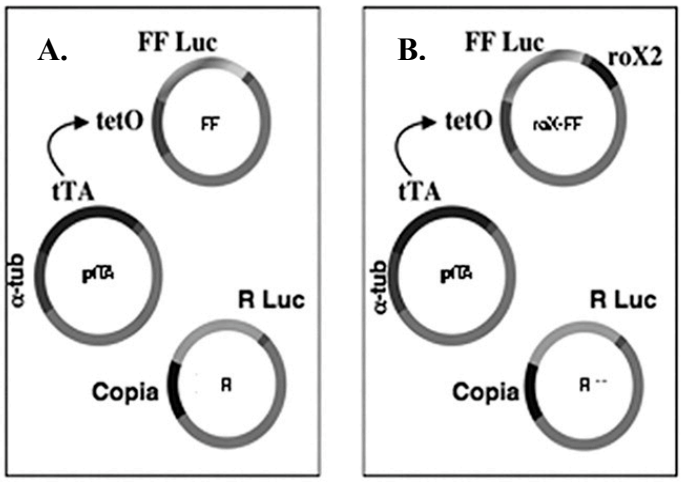


Figure 3 [16]: Plasmid model of dosage compensation. (A) Plasmids transfected in Nesprin (control) cells: FF (Nesprin-FF), ptTA, copia-*Renilla* luciferase. (B) Plasmids transfected in roX2 model of dosage compensation: roX2-FF, ptTA, copia-*Renilla* luciferase.

The ptTA plasmid has the transcriptional activator tTA encoding the tetracycline repressor protein (TetR in a Tet-Off system), which induces transcription of the firefly luciferase genes to very high levels. Transcription of the *tTA* genes is driven by the *D. melanogaster* constitutive alpha-tubulin 1 promoter (cloned as an XhoI-EcoRI fragment) replacing the cytomegalovirus promoter in the plasmid pUHD15.1

The R plasmid contains the *Renilla* luciferase gene under the control of the *Drosophila* copia promoter inserted into pRL-null plasmid (Promega). It was utilized as an internal control for the levels of transfected roX2 vs. Nesprin plasmid.

The pBS plasmid was used as a DNA carrier for the transfection.

Plasmid Transfection

One, three, or four days after treatment with dsRNA, the cells were transferred to 5 mL flasks and transfected. Transfection was carried out following the QIAGEN Effectine protocol with 1.0 ng ptTA, 5.4 ng R plasmid, 0.6 μg pBS, and 30 ng roX2 or Nesprin plasmid. The next day the cells were diluted to a final concentration of 0.6 X 10^6 cells/mL.

Luciferase Assay

Four days after transfection, the cells were collected for the luciferase assay and protein isolation. Luciferase activity was determined by using the dual luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

Western Blot Analysis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using 7.5% Tris-HCl precast gel (BIO-RAD), and the samples were run in 1X Tris-Glycine SDS (TGS). The proteins were transferred to a PVDF membrane (BIO-RAD) using a semi-dry method in the following buffer: 10% methanol-Tris-glycine transfer buffer following Bio-Rad's Criterion protocol. The membrane was blocked for 1 hour in 5% milk in PBS-Tween. The

primary antibodies used were topo I (1/2000, provided by Hsieh TS), topo II (1/200, TopoII (T22C5) by Santa Cruz Biotech), and Lamin*Dm*0 (1/750, ADL195-c by DSHB (Developmental Studies Hybridoma Bank)) as a loading control. The secondary antibodies used were anti-mouse labeled with horseradish peroxidase (HRP) (1/10,000, Pierce) and anti-rabbit HRP (1/10,000, Pierce). All of the antibodies were diluted in PBS-Tween. Western blots were developed by using enhanced chemiluminescence (ECL-Plus; GE Healthcare).

Results

Calculation of Relative Dosage Compensation

In each experiment that was performed, dosage compensation was measured by determining the ratio of firefly luciferase/*Renilla* luciferase in cells transfected with the roX2 plasmid and in cells transfected with the Nesprin plasmid. Although full dosage compensation should yield a ratio of exactly 2, previous experience in our laboratory using this plasmid model suggested that values in the range of 2.0 ± 0.3 should be considered as evidence of dosage compensation. Therefore, in the experiments described below, values within the range of 1.7-2.3 were considered dosage compensated, and values lower than 1.7 were considered to show a significant reduction in dosage compensation. The effect of topoisomerases on dosage compensation was determined by comparing the level of dosage compensation in cells treated with topoisomerase dsRNA to that of cells treated with GFP dsRNA (control). Wild type, untreated controls were included in several experiments.

In the plasmid model system used, dosage compensation of the roX2 plasmid is not observed until the fourth day after transfection (see for example [16]). Therefore, cells were always incubated with the plasmids for four full days; with the luciferase assay and western blot analysis taking place on the fourth day post-transfection. To determine the optimal time for dsRNA treatment, exposure to the dsRNA was varied in different experiments (Table 1).

Experiment	dsRNA treatment prior to plasmid transfection	# of days cells were incubated with plasmids	Luciferase assay/Cells collected for western blot
1 + 4	1 day	4 days	5 days after dsRNA addition
3 + 4	3 days	4 days	7 days after dsRNA addition
4 + 4	4 days	4 days	8 days after dsRNA addition

Table 1: Summary of experimental set up for all experiments performed. Experiments are listed according to their duration, from shortest to longest, and not according to the order in which they were performed.

1 + 4 Experiment

In the first experiment that was performed, cells were treated with double stranded topo II siRNA (dstopoII), double stranded Green Fluorescent Protein siRNA (dsGFP, control) or left untreated, (wild type, control) for one day prior to plasmid transfection. Four days post-transfection, the luciferase assay was performed and cells were collected for western blot analysis.

In this experiment, the wild type and dsGFP-treated controls were both dosage compensated as expected, while in the cells treated with dstopoII, there was a reduction in dosage compensation (roX2/Nesprin=1.48) (Table 2).

Sample	Firefly	Renilla	Firefly/Renilla	Average FF/R	X/N
X WT	2,667,131	470,072	5.67		
X WT	4,480,802	692,440	6.47	6.07	
N WT	1,148,468	410,037	2.80		2.15
N WT	1,290,035	454,404	2.84	2.82	
X GFP	437,688	81,477	5.37		
X GFP	412,868	77,633	5.32	5.35	
N GFP	211,102	71,309	2.96		1.95
N GFP	187,253	74,643	2.51	2.74	
X TOPOII	855,497	109,823	7.79		
X TOPOII	910,339	125,050	7.28	7.54	
N TOPOII	402,185	85,627	4.70		1.48
N TOPOII	432,637	78,868	5.49	5.10	

Table 2: Luciferase assay results, 1+4 experiment. Firefly (FF)=Firefly luciferase activity. Renilla (R)=*Renilla* luciferase activity. X=roX2 plasmid. N=Nesprin plasmid. WT=Wild type cells (control, not treated with dsRNA). GFP=cells treated with dsGFP (control). Topo II=Cells treated with dstopoII.

Western blot analysis was performed in order to determine the amount of topo II present in the cells on the day of the luciferase assay (5 days after treatment with dsRNA), and revealed almost undetectable levels of the protein (Figure 4).

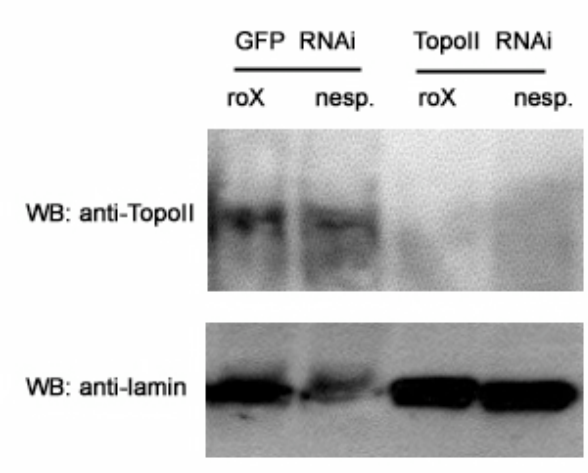


Figure 4: Western blot showing knockdown of topoisomerase II 5 days after treatment with dsRNA. dsGFP is the experimental control and lamin is the loading control.

Even though a good knockdown of topo II was evident five days after treatment with dstopoII (Figure 4), this may not have been the case when the plasmids were first transfected, one day after dsRNA treatment. A time course experiment performed in our laboratory had shown that maximal reduction of topo II levels occurred three days after dstopoII treatment. Therefore, in order to achieve a greater decrease in dosage compensation, I decided to incubate the cells with the dsRNA for a longer period of time before the plasmid transfection.

4 + 4 Experiment

In the 4+4 experiment, cells were treated with dstopoII or dsGFP (control) four days before plasmid transfection. The luciferase assay was still performed four days after the plasmid transfection. Two independently dstopoII-treated groups of cells were compared to the same set of dsGFP-treated cells as a control.

Sample	Firefly	Renilla	Firefly/Renilla	Average FF/R	X/N
X GFP	796,612	17,046	46.73		
X GFP	1,542,562	33,142	46.54	46.64	
N GFP	439,409	17,513	25.09		1.90
N GFP	738,672	30,899	23.91	24.50	
X TOPOII (1)	85,588	2,236	38.28		
X TOPOII (1)	264,591	6,174	42.86	40.57	
N TOPOII (1)	232,407	9,397	24.73		1.70
N TOPOII (1)	856,963	37,400	22.91	23.82	
X TOPOII (2)	660,265	18,244	36.19		
X TOPOII (2)	1,321,194	32,054	41.22	39.19	
X TOPOII (2)	1,309,019	32,600	40.15		1.62
N TOPOII (2)	154,815	7,291	21.23		
N TOPOII (2)	317,965	13,090	24.29	24.19	
N TOPOII (2)	575,835	21,285	27.05		

Table 3: Luciferase assay results, 4+4 experiment. 2 separate experiments were performed, each using dsGFP as a control. (1) and (2) labeled samples are independent experiments.

Again, the control cells were dosage compensated, as expected (Table 3). The first set of experimental cells treated with dstopoII (labeled (1) in Table 3) were on the lower limit of what is considered dosage compensated (roX2/Nesprin=1.70). The second set of experimental cells treated with dstopoII (labeled (2) in Table 3) had a slight reduction in dosage compensation (roX2/Nesprin=1.62), however this reduction was not as significant as the reduction seen in the 1+4 experiment (Table 2). The average reduction in dosage compensation for the two 4+4 experiments was 1.90 to 1.66 ± 0.06 (Figure 5).

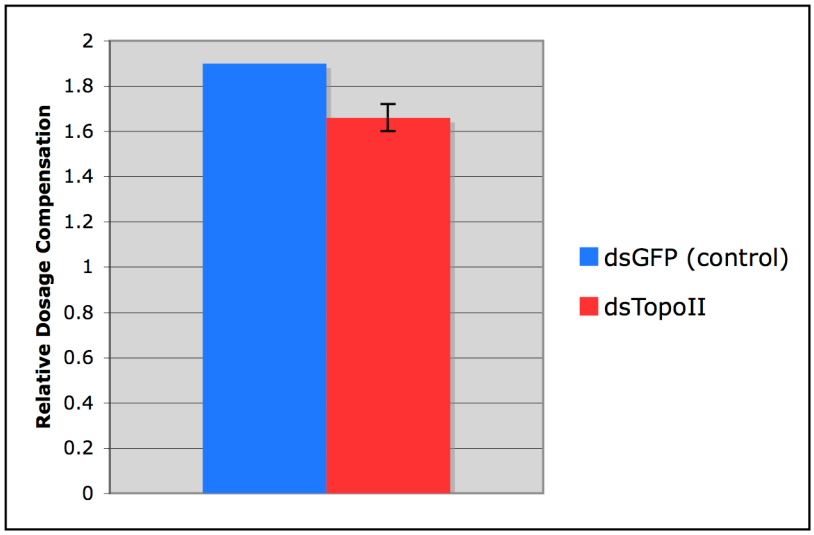


Figure 5: Average of relative dosage compensation for both 4+4 experiments. Error bar represents standard deviation of the mean. roX2/Nesprin values decreased from 1.90 (control) to 1.66±0.06 (experimental).

Because in the 4+4 experiments the cells were treated with RNAi eight days prior to the collection of cells for the luciferase assay and for western blot analysis, I suspected that this increase in the level of dosage compensation compared to the 1+4 experiment was due to a depletion in the concentration of RNAi and an increase in the levels of topoisomerase II present in the cells. In order to prevent the levels of topo II from rising back up during the last few days of the experiment, but still ensure that it is completely knocked down before

the plasmid transfection, I decided to decrease the length of time of dsRNA incubation before plasmid transfection, and thus the length of the experiment, by one day.

3 + 4 Experiment

In the 3+4 experiment, cells were treated with dstopoII or dsGFP (control) three days before plasmid transfection. As in the previous experiments, the luciferase assay and collection of cells for western blot analysis were performed four days after the plasmid transfection.

Sample	Firefly	Renilla	Firefly/Renilla	Average FF/R	X/N
X GFP	4495591	309716	14.51		
X GFP	4508261	319013	14.32	14.32	
N GFP	1892337	317760	5.96		2.34
N GFP	1935222	309260	6.26	6.11	
X TOPOII	1265751	150189	8.43		
X TOPOII	1263993	148185	8.53	8.49	
N TOPOII	544979	86196	6.32		1.38
N TOPOII	999915	167482	5.97	6.14	

Table 4: Luciferase assay results, 3+4 experiment. dsGFP served as the control.

This method resulted in the best decrease in dosage compensation with topo II knockdowns attained yet (roX2/Nesprin=1.38) (Table 4). The experiment was repeated three times, however the second (roX2/Nesprin = 1.56) and third (roX2/Nesprin=1.51) trials resulted in a lesser decrease in dosage compensation. Nevertheless, the average reduction in dosage compensation for the three 3+4 experiments was 2.11 ± 0.2 to 1.48 ± 0.09 and appears to be significant (Figure 6). This average roX2/Nesprin value was similar to the roX2/Nesprin value attained in the 1+4 experiment (Table 2). Western blot analysis performed seven days after dsRNA treatment revealed a good knockdown of topo II (Figure 7).

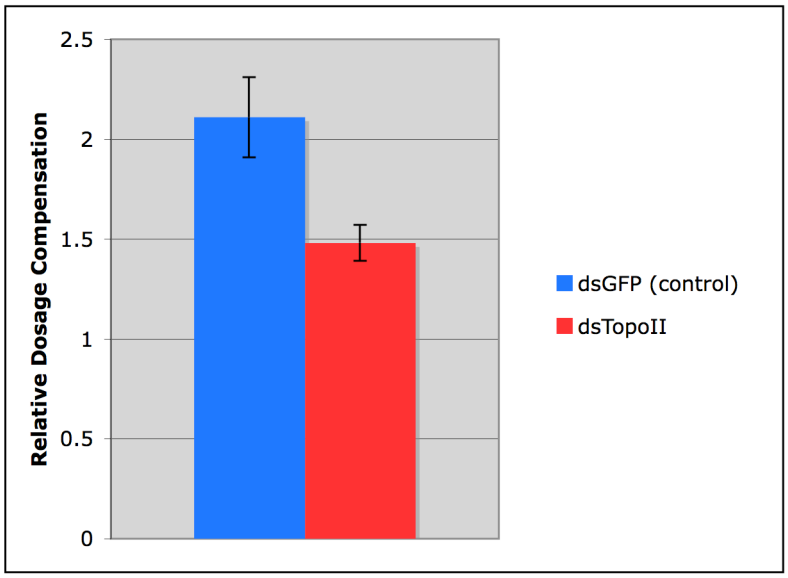


Figure 6: Average of relative dosage compensation for both 3+4 experiments. Error bars represent standard deviation of the means. roX2/Nesprin values decreased from 2.11±0.20 (control) to 1.48±0.09 (experimental).

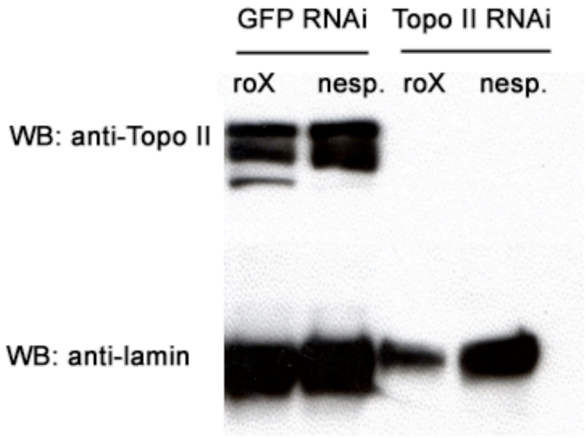


Figure 7: Western blot showing knockdown of topoisomerase II 7 days after treatment with dsRNA. dsGFP is the experimental control and lamin is the loading control.

Topoisomerase I

Because topoisomerase I plays an integral role in gene transcription, I hoped to perform the same experiments with topo I that I did with topo II, in order to check for its involvement in *Drosophila* dosage compensation. I treated the cells with two different double strand RNAs for topo I: dstopoIa and dstopoIb. Using dstopoIa I saw some reduction of the protein, while dstopoIb was much less effective. To attempt a better knockdown, I treated the cells with both dsRNAs at the same time (Figure 8). Even using both dsRNAs I could not attain a significant knockdown of the enzyme, therefore it was not possible to investigate if topo I is involved in dosage compensation.

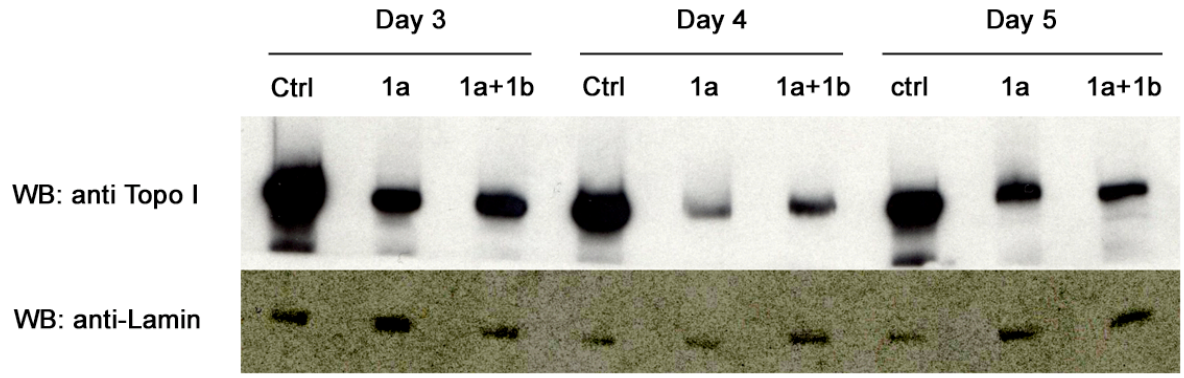


Figure 8: Western blot showing time course for cells treated with dstopoI. Cells were collected 2 (Day3), 3 (Day4) and 4 (Day5) days after dsRNA treatment. Control=dsGFP-treated cells. 1a=cells treated just with dstopoIa. 1a+1b=cells treated with both dstopoIa and dstopoIb.

Discussion

The decreases in dosage compensation levels observed in the topoisomerase II knockdown cells suggest that this enzyme does play a role in the X chromosome dosage compensation mechanism of *Drosophila melanogaster*. This result agrees with what we

already know about topoisomerases; that they are involved in the elongation step of gene transcription, the same step that is enhanced in *Drosophila* dosage compensation.

Topo II's role in dosage compensation agrees with preliminary results from a recent experiment performed in the Lucchesi lab, which suggests that in dosage compensated roX2 plasmids, the DNA is more relaxed and less negatively supercoiled. This result was surprising because negative supercoiled DNA is found in the promoter regions of genes, and is related to increased levels of transcription initiation [18].

It is interesting that throughout the course of my experiments with topo II knockdown cells, I could never attain a roX2/Nesprin value of less than 1.38, with the average for the cells incubated with dsRNA for one day, as well as those incubated with dsRNA for three days prior to plasmid transfection being 1.48. In addition to treating the cells with dsRNA for various lengths of time prior to plasmid transfection, I tried several 3+4 experiments where a constant concentration of dsRNA was maintained throughout the eight-day experiment. This was accomplished by adding dsRNA to the medium every time the cells were transferred to a larger container (immediately before and one day after plasmid transfection). In almost all of these experiments, the cells did not replicate at the rate that they normally do, and by the end of the eight days there were not enough cells to perform the luciferase assay. In addition, in the experiments where I was able to perform the luciferase assay, the decrease in dosage compensation was of a magnitude similar to the 3+4 experiments in which the dsRNA concentration was not kept constant.

It is important to note that a knockdown of MOF (one of the catalytic subunits of the dosage compensation complex) resulted in decreasing dosage compensation to a level similar to the one that I was able to achieve. Because MOF's histone acetyltransferase

activity is critical for *Drosophila* dosage compensation, MOF knockdown cells should ideally have a roX2/Nesprin value of 1. In the experiments performed with the plasmid model system, however, dosage compensation in MOF dsRNA treated cells was only reduced from 2.0 ± 0.07 to 1.44 ± 0.15 [16], similar to what was seen with the topo II knockdowns. These results bolster the confidence that the decreases in roX2/Nesprin values seen in topo II knockdowns were the result of true differences in dosage compensation.

A possible explanation for the failure to attain a complete lack of dosage compensation with the topo II knockdowns is that the transcriptional function of topo I "overlaps" with that of topo II: though they work through different mechanisms, they are both involved in reducing supercoils and other torsional stress in the DNA.

Another possibility is that the function of topo II may not be crucial for dosage compensation of the genes directly next to a roX sequence (the entry site for the MSL complex), even if it is required for dosage compensation of X chromosome genes further away from the roX sequence. If this were the case, the topo II knockdowns may not result in a significant decrease in dosage compensation because the firefly luciferase gene in this plasmid system is directly next to the roX2 sequence.

Future Work

One of the first things that I would like to check is the effect of topoisomerase I on dosage compensation. In order to do that, however, I would need to attain a successful knockdown of topo I. I would like to try using a different sequence of the topo I gene in the

dsRNA, and maybe that sequence would result in a better knockdown of the enzyme. In addition, because the functions of topoisomerase I and topoisomerase II have been shown to somewhat overlap, I would like to test dosage compensation in cells that are knocked down for both topo I and for topo II.

One of the major limiting factors in this experiment is the long amount of time that the cells need to be kept alive after knocking down topoisomerases, which are essential to the functioning of the cells. Topo II's involvement in cell replication causes additional problems, because once it is knocked down, I have found that the cells divide at a much lower rate than normal S2 cells. Because of this limitation, I would like to try to carry out real-time reverse-transcription polymerase chain reaction (qRT-PCR) on the endogenous X chromosome genes in topo I and/or topo II knockdown S2 cells. This way, I wouldn't have to keep the cells alive for more than 3-4 days after treating them with dsRNA, and could attain a more definite answer on whether or not their genes, not the plasmid model, are dosage compensated.

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