Nucleolar Dominance of the Y Chromosome in *Drosophila melanogaster*

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ABSTRACT The rDNA genes are transcribed by RNA polymerase I to make structural RNAs for ribosomes. Hundreds of rDNA genes are typically arranged in an array that spans megabase pairs of DNA. These arrays are the major sites of transcription in growing cells, accounting for as much as 50% of RNA synthesis. The repetitive rDNA arrays are thought to use heterochromatic gene silencing as a mechanism for metabolic regulation, since repeated sequences nucleate heterochromatin formation in eukaryotes. *Drosophila melanogaster* carries an rDNA array on the X chromosome and on the Y chromosome, and genetic analysis has suggested that both are transcribed. However, using a chromatin-marking assay, we find that the entire X chromosome rDNA array is normally silenced in *D. melanogaster* males, while the Y chromosome rDNA array is dominant and expressed. This resembles “nucleolar dominance,” a phenomenon that occurs in interspecific hybrids where an rDNA array from one parental species is silenced, and that from the other parent is preferentially transcribed. Interspecies nucleolar dominance is thought to result from incompatibilities between species-specific transcription factors and the rDNA promoters in the hybrid, but our results show that nucleolar dominance is a normal feature of rDNA regulation. Nucleolar dominance within *D. melanogaster* is only partially dependent on known components of heterochromatic gene silencing, implying that a distinctive chromatin regulatory system may act at rDNA genes. Finally, we isolate variant Y chromosomes that allow X chromosome array expression and suggest that the large-scale organization of rDNA arrays contribute to nucleolar dominance. This is the first example of allelic inactivation in *D. melanogaster*.

A variety of mechanisms cause differential expression of alleles in diploid organisms (Sha 2008). Allelic differences in expression may be due to parental imprinting (where inheritance from one parent predisposes an allele to expression or silencing) or to random inactivation of one allele in the diploid. These effects are critical in certain examples of gene regulation, including dosage compensation in mammals and immunoglobulin diversity in the immune system. Indeed, more recent surveys have indicated that complete or partial differential allelic expression affects up to 20% of genes in mammals (Serre *et al.* 2008; Milani *et al.* 2009). Differential allelic expression also occurs in many diverse organisms, suggesting that it is a widespread feature of gene regulation.

Drosophila melanogaster has been used extensively to explore the mechanics of gene regulation. Experiments with deletions throughout the genome argued that *Drosophila*

lacks parentally imprinted loci (Lindsley and Grell 1969), and no inactivated alleles are known. However, some cases of imprinting affecting reporter genes on aberrant chromosomal rearrangements or in transposon insertions have been described (Golic *et al.* 1998; Lloyd 2000). Furthermore, a phenomenon akin to allelic exclusion occurs in interspecies hybrids of *D. melanogaster* and *Drosophila simulans*. In these hybrid animals, nucleolar dominance occurs where the rDNA genes from *D. melanogaster* are exclusively expressed while those inherited from *D. simulans* are silenced (Durica and Krider 1977). Nucleolar dominance has also been observed in interspecies hybrids of many sibling species of both animals and plants (McStay 2006). Although recent studies have implicated chromatin modifications, DNA methylation, and small RNAs in nucleolar dominance, the basis for preferring genes from one species for expression remains unknown.

The rDNA genes are usually arranged in multicopy arrays and transcribed by RNA polymerase I to produce a long primary transcript that is processed into ribosomal rRNAs. These RNAs are extremely conserved, but species-specific differences have been used to assess nucleolar dominance in

hybrids. Within a species, measuring expression from a specific rDNA array is more challenging. In *D. melanogaster* both the X and the Y chromosomes carry an rDNA array, and the transcripts generated from these loci are identical (Tautz *et al.* 1988). Genetic experiments have demonstrated that either of these loci is sufficient for full function (Hawley and Marcus 1989), but the lack of a transcribed polymorphism between the X and the Y rDNA genes has precluded the measurement of array-specific expression.

We report here a cytogenetic method to assess transcription of the rDNA genes in *D. melanogaster*. Using this method, we find that nucleolar dominance is a normal feature of the rDNA arrays in *D. melanogaster* males. Whereas in females the rDNA arrays of the two X chromosomes are both transcribed, in males only the Y chromosome array is active. Our work suggests that somatic pairing of the rDNA arrays of the sex chromosomes influences the silencing of the X chromosome array, resulting in nucleolar dominance.

Materials and Methods

Stocks and chromosomes

All crosses were performed at 25°. Mutations and chromosomal rearrangements not detailed here are described in Flybase (<http://www.flybase.org>). To eliminate X-linked variability in tests for Y chromosome nucleolar dominance, we used a single X chromosome derived from a *w¹¹¹⁸ Oregon-R* stock. To extract Y chromosomes from heterochromatin modifier stocks into an *Oregon-R* background, we crossed males to a *w¹¹¹⁸* second chromosome balancer stock, then crossed males to a *w¹¹¹⁸* third chromosome balancer stock, and finally crossed to *w¹¹¹⁸* and selected against all autosomal balancers. The Y chromosomes were designated as follows: *Y^{OR}* from the *w¹¹¹⁸ Oregon-R* stock; *Y^B* from *Bethulie*; *Y^C* from *Canton-S*; *Y^H* from *Harwich*; *Y^K* from *Kalahari*; *Y^S* from *Samarkand*; *Y^{sv254}* from *y w*; *Su(var)2-5⁰⁵/CyO*, *actGFP*; *Y^{sv391}* from *In(1)w^{m4}*; *Su(var)3-9¹/TM3*; and *Y^{sv392}* from *In(1)w^{m4}*; *Su(var)3-9²/TM3*. Tests of nucleolar dominance with *Y^B*, *Y^C*, *Y^H*, *Y^K*, and *Y^S* were performed by crossing males from these strains to *w¹¹¹⁸* females. Tests of the X chromosome from *Kalahari* and *Samarkand* strains (*X^K* and *X^S*, respectively) were performed by crossing females of these strains to *w¹¹¹⁸/Y^{OR}* males.

Immunocytology and FISH

To detect H3.3 incorporation at active rDNA arrays, we heat-shocked third instar larvae carrying an inducible H3.3-GFP construct on the X chromosome or on chromosome 2 (Schwartz and Ahmad 2005). Larvae were heat-shocked at 37° for 1 hr and then allowed to recover at 25° for 2 hr to produce a pulse of H3.3-GFP. Larval brains were then dissected and fixed as described (Pimpinelli *et al.* 2000), using the methanol:acetic acid:water (5: 2: 3) fixative. Immunodetection of GFP (Abcam) and the mitotic marker histone H3^{S10phospho} (Millipore) was performed as described (Schwartz and Ahmad 2005), except using 1% fish gelatin

(Sigma-Aldrich) in PBS as a blocking agent. Mitotic figures from at least three brains, where all chromosomes could be recognized, were selected by H3^{S10phospho} staining and scored for the presence of strong GFP foci on the X or Y chromosomes.

For combined *in situ* detection of proteins and DNA sequences, larval brains were dissected and processed as described (Lavrov *et al.* 2004) with the following adaptations: dissected brains were incubated in 0.5% sodium citrate for 10 min, fixed in 3.7% formaldehyde and 1% Triton-X in PBS for 1 min, and then fixed again in 3.7% formaldehyde and 50% acetic acid in water for 150 sec before squashing on slides. After immunostaining for the nucleolar component Fibrillarin (Abcam), slides were incubated in ethanol:chloroform:acetic acid (6:3:1) for 30 sec, and then processed for DNA hybridization.

A probe for the 18S rDNA was prepared by amplifying an 800-bp fragment by PCR from genomic DNA using the primers GCAGTTTGGGGGCATTAGTA and TTCACAATCCCAA GCATGAA. This product was labeled with Alexa Fluor 488-5-dUTP (Molecular Probes) by nick translation. A locked nucleic acid (LNA) oligonucleotide (TttTccAaaTttCggTcaT caAatAatCat, where capital letters are LNA bases; Integrated DNA Technologies) conjugated with rhodamine red was used as probe for the 359-bp satellite repeat. LNAs are analogs of ribonucleotides that contain a bridging linkage between the 2' and 4' positions of the ribose ring that improves oligonucleotide annealing in FISH experiments (Vester and Wengel 2004). No additional DNA was added to the hybridization mix.

R2 retrotransposon expression assays

RNA was isolated from 10–20 adult flies with Trizol (Invitrogen) and cDNA pools were generated by random priming of purified RNA with Superscript III reverse transcriptase (Invitrogen). The *w¹¹¹⁸* X chromosome carries a unique truncated R2 retrotransposon that is 167 bp long (referred to as *R2¹⁶⁷*; Eickbush and Eickbush 2003), and we used the primers AAAGCATTGTGATGGCCCTA and TGATCGCGGAGGTATG GAAA in qPCR reactions to measure abundance of transcripts from this insertion. Abundance of *Adh* transcripts was measured in the same cDNA pools using the primers TCTACCC CTATGATGTGACC and AGTGTAGTTGACGGCAATG. qPCR was performed using EvaGreen dye (Biotium) in an ABI 7500 thermocycler (Applied Biosystems). Reactions were performed in triplicate. Expression of *R2¹⁶⁷* was calculated as $-(Ct^{Adh} - Ct^{R2167})$ and normalized to the *w¹¹¹⁸ X/Y^{OR}* genotype in each experiment. Standard errors were calculated as the root mean square of the error for each primer pair. Unique PCR products for each primer pair were confirmed on agarose gels stained with ethidium bromide.

rDNA copy number determination

To measure the number of rDNA units on different Y chromosomes, we prepared DNA from adult flies carrying each Y in combination with the rDNA-deficient *C(1)DX* chromosome.

The number of rDNA units on the w^{1118} X chromosome was measured in females. The primers AATGGATGTGATGCCAAT GTA and TTCAGTGGATCGCAGTATGG (in the 3' end of the 28S rDNA) were used to measure the total number of rDNA units, and the primers TTCAAGTAAGCGCGGGTCAAC and ATTCCAAGCCCGTCCCTTG (spanning the insertion sites of the *R1* and *R2* retrotransposons) were used to measure the number of uninterrupted rDNA copies. qPCR was performed using EvaGreen dye (Biotium) in a SmartCycler (Cepheid). Equal amounts of DNA were loaded in each reaction, and reactions were performed in triplicate. Unique PCR products for each primer pair were confirmed on agarose gels stained with ethidium bromide. Estimates of the number of rDNA units were calculated from standard curves generated from qPCR with known amounts of PCR products.

Results

Nucleolar dominance in regular *D. melanogaster* males

Transcription within the eukaryotic nucleus causes nucleosome turnover and in most organisms drives the enrichment of the H3.3 histone variant in active chromatin (Workman 2006). Most new histones are deposited into chromatin during S phase of the cell cycle, but the H3.3 variant is deposited in active chromatin during gap phases as well. We have previously observed transcription-dependent nucleosome turnover by producing a pulse of epitope-tagged H3.3 in *Drosophila* cell lines and visualizing chromatin-bound H3.3 in nuclei and mitotic chromosome spreads (Ahmad and Henikoff 2002). Epitope-tagged H3.3 becomes widespread throughout the active gene-rich euchromatin, but the most intense site of H3.3 deposition is the rDNA arrays. This is consistent with the idea that transcription results in histone displacement and H3.3 replacement, as the rDNA genes are the most heavily transcribed sites in the genome. Similarly, epitope-tagged H3.3 produced in *Drosophila* larvae labels actively transcribed chromatin (Schwartz and Ahmad 2005). However, in these experiments, localization of H3.3 to rDNA genes was not examined. The rDNA genes can be visualized in mitotic chromosome spreads from larval neuroblasts. The rDNA genes are present on both the X and the Y chromosome (Figure 1A). To determine if the rDNA genes in these cells are also subject to nucleosome turnover, we produced a pulse of epitope-tagged H3.3 in larvae and then visualized the protein on mitotic chromosome spreads. In the limited time between the induction of the tagged histone and fixation of the samples, only H3.3 incorporated into chromatin during transcription appears on chromosome spreads. We first examined spreads from female larvae from an Oregon-R strain carrying the w^{1118} mutation. As observed in *Drosophila* cell culture, the major sites of H3.3 coincide with the rDNA genes on the X chromosome, with a much lower signal throughout the active euchromatin arms of chromosome spreads (Figure 1B). Both X chromosomes typically show a bright H3.3 spot at the rDNA genes, indicating that both arrays are active in these cells.

We then examined chromosome spreads from male larvae after an H3.3 pulse. Since males carry rDNA genes on both the X chromosome and on the Y chromosome, we expected that both chromosomes would show an intense H3.3 spot at their rDNA locus. However, we observed intense H3.3 signals only on the Y chromosome rDNA, and no signal on the X chromosome array (Figure 1C). As the X chromosome in males is identical to that in females, the rDNA of the X chromosome must be epigenetically silenced in males. We refer to this as nucleolar dominance of the Oregon-R Y chromosome (designated the Y^{OR} chromosome) in these males. Nucleolar dominance of the Y^{OR} rDNA array is nearly invariant in males (Table 1). In the rare cases where the X chromosome array is expressed, it is strongly labeled, suggesting that its expression is an all-or-none phenomenon. Expression of the silencing of rDNA by the Y chromosome is not a unique property of the X chromosome in the Oregon-R strain, because Y^{OR} is also dominant to X chromosomes from the Kalahari and Samarkand strains (Table 1). Furthermore, nucleolar dominance of the Y is independent of whether the chromosome is inherited paternally or maternally (Table 1). Thus, the rDNA in *Drosophila* males shows allelic differences in expression, but this does not appear to be due to gametic imprinting. In contrast, expression of both X chromosome rDNA arrays (nucleolar codominance) is typical of females from this strain (100% of spreads, $n = 84$).

While the main transcripts from the rDNA genes on the X and Y chromosomes cannot be distinguished, some genes are disrupted by insertions of the *R1* and *R2* non-LTR retrotransposons (Eickbush *et al.* 1997). These retrotransposons specifically insert at a ~30-bp target sequence near the end of the rDNA transcribed region, and both X and Y chromosome arrays carry many inserted elements. However, a fraction of these elements are deleted at their 5' ends, and a single 167-bp *R2* element ($R2^{167}$) is uniquely present on the X chromosome of the Oregon-R strain (Figure 2A; Eickbush and Eickbush 2003). This short insertion can be cotranscribed from the rDNA promoter, and we used it as a unique tag to measure expression from the X chromosome rDNA array by qPCR, using extension times and conditions that allow only extension of this short insertion (see *Materials and Methods*).

We first tested genomic DNA by PCR to determine that the $R2^{167}$ insertion is present on the X chromosome and absent from the Y chromosomes we use here. Thus, $R2^{167}$ -containing transcripts specifically measure expression of the X chromosome rDNA array. Previous work had shown that $R2^{167}$ is expressed at lower levels in Oregon-R males than in females (Eickbush and Eickbush 2003). We confirmed that females express approximately eight times more $R2^{167}$ -containing transcripts than males in our stock (Figure 2B, genotypes 1 and 2). This is consistent with our cytological assay of rDNA expression, where the X chromosome rDNA array is largely silenced in males (Figure 1C), although males do have some detectable amount of $R2^{167}$ expression relative

Figure 1 H3.3 marks the active rDNA genes on the sex chromosomes of *Drosophila*. Mitotic chromosome spreads from larval neuroblasts were fixed and stained with DAPI (red). Signals from hybridization probes to 18S rDNA (blue) and H3.3-GFP protein (green) are shown. (A) A spread from a male larva hybridized with an 18S rDNA probe shows the location of the rDNA arrays on the X and the Y chromosome. (B–F) Larvae carrying an H3.3-GFP construct were induced to produce a pulse of tagged histone, and the localization of the protein was assessed on mitotic spreads. The active rDNA genes are the major sites

of H3.3-GFP deposition after the pulse. (B) Spreads from a female larva show H3.3-GFP deposition on the rDNA arrays of both X chromosomes. The X chromosomes always show equivalent signals. (C) Spreads from a male sibling show H3.3-GFP only on the Y^{OR} chromosome rDNA array, indicative of Y nucleolar dominance in this strain. (D and E) Males carrying variant Y chromosomes show H3.3-GFP on both sex chromosomes, indicative of nucleolar codominance of the X and these Y chromosomes. (F) Males carrying the variant Y^{sv392} chromosome show H3.3-GFP only on the X chromosome. (G) Hybridization with an 18S rDNA probe (blue) to spreads from a male carrying the Y^{sv392} chromosome demonstrates that this variant Y is deleted for rDNA genes.

to animals that genetically lack the X chromosome array (Figure 2B, genotype 3).

Nucleolar dominance is a genetic property of the Y chromosome

To determine if Y chromosome nucleolar dominance was a peculiar feature of the Oregon-R strain, we used the $R2^{167}$ expression assay to survey a collection of Y chromosomes from diverse geographical locations. We generated males with the same Oregon-R X chromosome to compare only the effects of Y chromosomes from each strain. $R2^{167}$ expression in all these males was comparable to or less than that in regular Oregon-R males (Figure 2B, genotypes 4–8). Thus, we conclude that the X chromosome rDNA array is repressed in all these genotypes, and nucleolar dominance is a typical property of the Y chromosome in *D. melanogaster*.

The rDNA genes are embedded in repetitive regions of the X and Y chromosomes. These regions are enriched for heterochromatic proteins that repress transcription and compact chromatin (Hilliker *et al.* 1980). As the rDNA arrays are themselves repetitive, these genes have molecular features of heterochromatin (Blattes *et al.* 2006). Mutations in heterochromatin proteins relieve gene silencing of reporters in heterochromatic regions (Schotta *et al.* 2003), so we tested whether these mutations affected silencing of the X chromosome rDNA array in males. We first crossed w^{1118} females to males carrying mutations in the $Su(var)2-5$ (encoding HP1) or $Su(var)3-9$ (encoding the heterochromatic histone H3-K9 methyltransferase) genes. We then measured $R2^{167}$ expression in progeny males carrying our standard X chromosome and the $Su(var)$ mutations. These genotypes showed dramatic derepression of $R2^{167}$ (not

Table 1 H3.3 localization at X and Y chromosome rDNA arrays in males

Genotype ^b	Y parent stock	n ^c	Labeled mitotic spreads (%) ^a			Dominance
			$X^A Y^S$	$X^A Y^A$	$X^S Y^A$	
X / Y^{OR}	w^{1118}	49	0 (0)	4 (8)	45 (92)	Y dominant
$X^{paternal} / Y^{OR maternal}$	w^{1118}	42	0 (0)	0 (0)	42 (100)	Y dominant
X^K / Y^{OR}	w^{1118}	26	0 (0)	1 (4)	25 (96)	Y dominant
X^S / Y^{OR}	w^{1118}	30	0 (0)	0 (0)	30 (100)	Y dominant
$X / Y^{sv254} ; Su(var)2-5^4 / +$	$w^{m4} ; Su(var)2-5^4 / CyO$	42	1 (2)	21 (50)	20 (48)	Codominant
X / Y^{sv254}	$w^{m4} ; Su(var)2-5^4 / CyO$	33	0 (0)	16 (48)	17 (52)	Codominant
$X / Y^{sv391} ; Su(var)3-9^1 / +$	$w^{m4} ; Su(var)3-9^1 / TM3$	30	0 (0)	9 (30)	21 (70)	Codominant
X / Y^{sv391}	$w^{m4} ; Su(var)3-9^1 / TM3$	38	0 (0)	15 (39)	23 (61)	Codominant
$X / Y^{sv392} ; Su(var)3-9^2 / +$	$w^{m4} ; Su(var)3-9^2 / TM3$	20	20 (100)	0 (0)	0 (0)	Deleted Y
X / Y^{sv392}	$w^{m4} ; Su(var)3-9^2 / TM3$	56	54 (96)	2 (4)	0 (0)	Deleted Y

^a X^A is an X chromosome with an active rDNA locus labeled with H3.3-GFP, Y^A a Y chromosome with an active rDNA locus, and X^S and Y^S indicate chromosomes with silent rDNA loci with no H3.3-GFP labeling.

^b All males carry an inducible H3.3-GFP transgene.

^c Number of mitotic spreads scored from more than three larvae.

Figure 2 Transcription of an X-specific rDNA retrotransposon fragment is repressed by nucleolar dominance. (A) Schematic of two rDNA units showing the transcribed region (arrow) and the position of the 167-bp-long 5'-truncated R2 element (solid triangle, called $R2^{167}$). A single copy of $R2^{167}$ is present on the X chromosome used in this study (Eickbush and Eickbush 2003). The indicated primers (red) were used to measure abundance of the $R2^{167}$ transcript. (B) $R2^{167}$ transcript abundance measured by qPCR on cDNA pools generated from 10–20 adult animals. Ct values for the $R2^{167}$ transcript were normalized to Ct values for the *Adh* mRNA in the same samples and to the value for X/Y^{OR} males. The y-axis shows the fold change in expression. The standard error determined by RMS of each measurement is shown as black bars. The genotypes are (1) w^{1118} females, (2) w^{1118}/Y^{OR} males, (3) $C(1)DX/Y^{OR}$ females, (4) w^{1118}/Y^B males, (5) w^{1118}/Y^C males, (6) w^{1118}/Y^H males, (7) w^{1118}/Y^K males, (8) w^{1118}/Y^S males, (9) w^{1118}/Y^{sv254} males, (10) w^{1118}/Y^{sv391} males, and (11) w^{1118}/Y^{sv392} males. The parent stock for each Y chromosome is listed in the *Materials and Methods*. (C) $R2^{167}$ transcript abundance in males carrying *Su(var)* mutations. ΔCt is displayed as in B. The genotypes are (1) w^{1118} females, (2) w^{1118}/Y^{OR} males, (3) $w^{1118}/Y^{OR}; Su(var)2-5^{4/+}$ males, (4) $w^{1118}/Y^{OR}; Su(var)3-9^{1/+}$ males, (5) $w^{1118}/Y^{OR}; Su(var)3-9^{1}/Su(var)3-9^{2}$ males.

shown); however, sibling males without the *Su(var)* mutations also showed derepression (Figure 2B, genotypes 9–11). Thus, derepression of $R2^{167}$ could not be attributed to the *Su(var)* mutation. Some other factor in these *Su(var)* stocks must allow expression of the X chromosome rDNA in males.

Backcrosses revealed that the failure to silence the X chromosome rDNA in males mapped to the Y chromosomes of these stocks. We therefore designated the Y chromosomes in the *Su(var)2-5⁴*, *Su(var)3-9¹*, and *Su(var)3-9²* stocks as Y^{sv254} , Y^{sv391} , and Y^{sv392} , respectively. We conclude that these variant Y chromosomes are genetically distinct from the regular Y chromosome of wild-type strains.

To definitively test if heterochromatin mutations affect nucleolar dominance, we measured expression of $R2^{167}$ in males with the Y^{OR} chromosome and a *Su(var)* mutation (Figure 2C). We found that silencing of the X chromosome rDNA array was not dominantly affected by heterozygous *Su(var)2-5* or *Su(var)3-9* mutations. However, heterochromatic silencing does partially contribute to nucleolar dominance, because males completely deficient for the *Su(var)3-9* methyltransferase showed a moderate increase in $R2^{167}$ expression from the X chromosome (Figure 2C).

Finally, we assessed rDNA expression in *Su(var)* crosses using the epitope-tagged H3.3 cytological assay. In crosses with *Su(var)2-5⁴* and *Su(var)3-9¹* stocks we found that a significant number of cells showed labeling of both the X and the Y chromosome rDNA arrays, regardless of whether they carried the *Su(var)* mutation (Table 1 and Figure 1, D and E). Although many cells in these males continue to show Y nucleolar dominance, the substantial number of cells that show expression of both rDNA arrays indicates that the arrays can be codominant. In crosses with a *Su(var)3-9²* stock, only the X chromosome rDNA array was expressed,

with no H3.3 labeling on the Y chromosome (X nucleolar dominance). Again this was independent of whether these males carried the *Su(var)3-9²* mutation (Table 1 and Figure 1F). Therefore, derepression of the X chromosome rDNA in these genotypes is not due to the *Su(var)* mutations themselves, but is a genetic property of the Y chromosomes in these stocks.

Y chromosome dominance is not determined by rDNA copy number

What distinguishes the Y chromosomes of wild-type strains from the Y^{sv254} , Y^{sv391} , and Y^{sv392} chromosomes? Males carrying an X chromosome deficient for the rDNA array rely on the Y chromosome rDNA for rRNA synthesis and viability. However, we noted in crosses with the *Su(var)3-9²* stock that the Y^{sv392} chromosome appeared to lack rDNA genes, because it was inviable with an X chromosome rDNA deficiency. *In situ* detection of rDNA sequences of an outcrossed stock revealed that Y^{sv392} lacks any detectable rDNA (Figure 1G). Thus, the exclusive expression of the X chromosome rDNA in males carrying Y^{sv392} is simply due to the lack of any other rDNA genes in this genotype.

We therefore assessed if Y^{sv254} and Y^{sv391} also differ in rDNA copy number from the Oregon-R Y chromosome. We performed qPCR with primers to count both all rDNA copies and the number of functional rDNA copies (lacking *R1* or *R2* insertions). Our Oregon-R X chromosome carries a relatively small number of rDNA copies, and most of these are disrupted by retrotransposon insertions (Figure 3). In contrast, the Y chromosome rDNA arrays are much larger. Both the nucleolar dominant Y^{OR} and the codominant Y^{sv254} chromosomes have similar numbers of functional rDNA copies (approximately five times more than the X chromosome) and disrupted rDNA copies. Thus, there is no correspondence

Figure 3 rDNA copy number polymorphism does not account for nucleolar dominance. The copy number of intact rDNA units and those carrying *R1* or *R2* retrotransposon insertions were assessed by qPCR. Copy numbers for a single *w¹¹¹⁸* X chromosome were measured in females and that of the variant Y chromosomes in *C(1)DX/Y* females.

between silencing of the X chromosome and the total copy numbers of the Y chromosome array. In contrast, the rDNA array on *Y^{sv391}* is twice as large as the other Y chromosomes, and ~85% of its rDNA copies are disrupted by retrotransposon insertions (Figure 3). We suggest that in this case the high-insertion load in the rDNA array may abolish nucleolar dominance of the Y chromosome rDNA array.

Previous experiments have linked rDNA copy number on the Y chromosome to the severity of heterochromatic silencing (Paredes and Maggert 2009). This study showed that heterochromatin throughout the nucleus is affected by reductions in rDNA copy number. We therefore tested if the codominant Y chromosomes also altered silencing. We used two different chromosomal rearrangements that juxtapose a reporter gene to heterochromatin: a transposition of heterochromatin into the *bw⁺* gene on chromosome 2 (*bw^D*; Henikoff and Dreesen 1989) and an inversion on the X chromosome (*In(1)w^{m4}*; Muller 1930). One difference between these rearrangements is that the X chromosome inversion places the *white⁺* reporter gene close to its rDNA genes (Tartof *et al.* 1984). Strikingly, we found that the *Y^{sv254}* and *Y^{sv391}* chromosomes specifically derepressed silencing of the X chromosome rearrangement (Figure 4), while silencing on the chromosome 2 rearrangement was unaffected. Y chromosomes with extra heterochromatin are known to generally derepress heterochromatic gene silencing (Schultz 1936); this does not account for specific effects on *In(1)w^{m4}*. Additionally, the *Y^{OR}*, *Y^{sv254}*, and *Y^{sv391}* chromosomes have cytologically similar amounts of heterochromatin (Figure 1, C–E). As the codominant Y chromosomes allow expression of the X chromosome rDNA, and the *w⁺* reporter is adjacent to rDNA in *In(1)w^{m4}*, we suggest that an

Figure 4 Codominant Y chromosomes derepress heterochromatic silencing near the X chromosome rDNA array. Effects of three Y chromosomes on silencing with *In(1)w^{m4}* (left) and with *bw^{D/+}* (right). Silencing of *w^{m4}* chromosome is severe in males carrying the nucleolar dominant *Y^{OR}* chromosome, but is derepressed in males carrying either of the codominant *Y^{sv254}* or *Y^{sv391}* chromosomes. In contrast, silencing associated with *bw^D* is similar with all three Y chromosomes.

active rDNA array reduces heterochromatic silencing of neighboring euchromatic genes.

Codominant Y chromosomes are often paired with the X chromosome

In a number of mammalian systems, allelic exclusion is triggered by the physical association of two allelic loci (Anguera *et al.* 2006; Wutz and Gribnau 2007). We hypothesized that the rDNA arrays on the *Drosophila* X and Y chromosomes might interact, thereby directing silencing of the X chromosome rDNA array. We tested this hypothesis by measuring how often the rDNA arrays are somatically paired in interphase neuroblasts nuclei. We used a FISH probe to the 18S rDNA sequence to visualize the rDNA arrays and a second probe to the 359bp satellite repeat sequence that is unique to the X chromosome (Figure 5, A and B). Nuclei with one 18S rDNA signal were scored as paired rDNA arrays, whereas nuclei with two separated signals were scored as unpaired arrays (Figure 5, C and D).

The rDNA arrays were paired in 96% of nuclei from Oregon-R females (Figure 5E), as expected from the complete sequence homology between these chromosomes. This implies that the rDNA genes are somatically paired throughout the cell cycle in females. The X and the Y chromosomes have little

Figure 5 Codominant X and Y chromosomes are persistently paired. (A) Schematic of the X and Y chromosomes showing the rDNA arrays (blue) and the adjacent 359-bp satellite block (yellow) on the X. (B) Hybridization with probes to the rDNA (blue) and to the 359-bp satellite (yellow) of a mitotic chromosome spread from a male larva shows the position of these sequences on the X and Y chromosomes. DAPI is in red. (C and D) Examples of interphase nuclei from larval brains hybridized with rDNA (blue) and 359-bp satellite (yellow) probes, combined with antibody detection of Fibrillarin (red, a component of the nucleolus). In C there is only one block of rDNA signal, indicating that the two rDNA arrays are paired. In D two blocks of rDNA signal are apparent, and the Y chromosome array coincides with the nucleolus. (E) Quantitation of rDNA pairing in females and males with different Y chromosomes. At least 500 nuclei were scored in each genotype. * indicates comparisons that are statistically significant (<0.0001 , χ^2 test).

DNA sequence homology except for the rDNA genes, but in spite of this in Oregon-R males the rDNA arrays are paired in 61% of nuclei (Figure 5E). While this may indicate that the two arrays pair only in some cells, we favor the interpretation that pairing between the X and the Y chromosomes is disrupted during mitosis in all cells and then gradually reestablished as the cell cycle progresses. We thereby infer that the rDNA arrays are paired for only half of interphase in somatic cells.

We also examined where rDNA arrays were relative to the nucleolus, where active rDNA transcription occurs. We

found that when rDNA arrays in males are unpaired, only the Y chromosome array was associated with the nucleolus. This is consistent with the Y array being the only active one in these cells. However, we noted that paired arrays also associate with the nucleolus (Figure 5C). These must be pairing interactions between active Y chromosome arrays and silent X chromosome arrays (92% of the X chromosome arrays are silent in this strain).

We then examined rDNA pairing with the codominant Y^{sv254} and Y^{sv391} chromosomes. Surprisingly, we observed a high frequency of pairing in males carrying these chromosomes, with ~88% of nuclei showing association between rDNA arrays. This suggests that high frequencies of pairing between the X and Y chromosome rDNA arrays may promote activation of the X chromosome genes. As our FISH assay provides a snapshot across all stages of the cell cycle, we infer that the arrays are paired through most of the cell cycle. In contrast, the dominant Y array must be paired only with the X array for about half of interphase. This suggests that a longer period of pairing is associated with a higher degree of codominance.

Discussion

A chromatin-marking system detects nucleolar dominance

D. melanogaster males have two very similar rDNA arrays, one each on the X and on the Y chromosome. Deletions of either array are recessive mutations, indicating that only one is needed for viability. Here, we show that in regular *Drosophila* males only the Y chromosome array is active, while the X array is silenced. We find that silencing of the X array requires the presence of a wild-type Y chromosome rDNA array. We refer to this phenomenon as “Y nucleolar dominance” because of its similarity to the nucleolar dominance observed between rDNA arrays from different species in hybrid animals. Furthermore, we identify two variant Y chromosomes that carry functional rDNA arrays but are unable to induce silencing of the X chromosome rDNA. Previous reports of the copy number of rDNA genes in polytenized cells in *Drosophila* showed that the Y rDNA array is preferentially overreplicated, implying that there is some dominance relationship between the X and Y rDNA arrays (Endow and Glover 1979). Our observation that dominance between rDNA arrays is a normal feature in diploid cells within the *D. melanogaster* species provokes a reconsideration of replicative dominance and interspecies nucleolar dominance.

Interspecies nucleolar dominance refers to the observation that in a hybrid organism, the rDNA genes from one species are typically expressed, while the rDNA genes from the other parent are not (Reeder 1985). This phenomenon occurs in diverse species pairs, including plants and animals. Nucleolar dominance is thought to result from competition between the heterologous rDNA genes for transcription factors available in the hybrid. However, more recent work has

indicated that an entire rDNA array from one species is repressed by chromatin-mediated gene silencing. Silencing is triggered by small RNAs that originate in the rRNA promoter that then direct gene-silencing chromatin modifications (Lawrence and Pikaard 2004). Gene silencing of arrays is thought to be part of the normal downregulation of rRNA production according to the metabolic activity of the cell (Preuss and Pikaard 2007). Our observation of *Y* nucleolar dominance can be understood as a preference to silence the *X* chromosome array when the demand for rRNA synthesis is low. Similarly, interspecies nucleolar dominance may result from normal growth-regulated processes that occur within each species.

What distinguishes dominant *Y* chromosome arrays from codominant ones? Studies of nucleolar dominance in *D. melanogaster*/*D. simulans* hybrids have implicated a heterochromatic region adjacent to the *D. melanogaster* *Y* rDNA array as required for dominance in interspecies hybrids (Durica and Krider 1978). Potentially the *Y^{sv254}* and *Y^{sv391}* chromosomes we isolated might show codominance because they are deleted for the adjacent critical region. However, this neighboring element was not molecularly characterized, and the rDNA arrays of these deleted chromosomes might also differ from wild-type *Y* chromosomes.

An alternative possibility is that rDNA arrays themselves differ in their ability to induce silencing of another rDNA array. Certainly the *X* chromosome array can be expressed when the *Y* chromosome rDNA is deleted. If some *Y* chromosome arrays are slow to induce, the *X* chromosome array may also be induced. In this scenario, we imagine that there are rDNA arrays that may appear functional when no other rDNA array is present, but are less responsive to metabolic signals stimulating rRNA synthesis.

While the rDNA transcripts produced from the *X* and the *Y* chromosome arrays are identical, there are differences in the structure of the arrays. The promoter of each rDNA gene lies in a nontranscribed repetitive spacer. Spacers with varying numbers of repeats have been identified, and long variants are enriched in the *Y* chromosome rDNA array (Hawley and Marcus 1989). Additionally, *X* chromosome arrays are known to contain a higher proportion of genes disrupted by the *R1* and *R2* retrotransposons than *Y* chromosome arrays (Hawley and Marcus 1989).

Could these differences affect nucleolar dominance? If one rDNA array is more easily activated as growth conditions change, production of ribosomal RNAs may attenuate activating signals before a second, more resistant array is induced. The rDNA spacer repeats are known to stimulate transcription (Grimaldi *et al.* 1990), and perhaps longer spacers result in efficient activation of some rDNA genes in the *Y* chromosome array. Alternatively, previous work in *Drosophila* has shown that retrotransposons within the rDNA arrays may affect rDNA transcription (Eickbush and Eickbush 2003). The *R1* and *R2* elements insert within the transcribed portion of the rDNA repeat unit and strongly reduce transcription of the unit by stimulating early tran-

scriptional termination (Ye and Eickbush 2006). The wild-type *X* chromosome array has 80% of its ~100 rDNA units disrupted by retrotransposons, while the dominant wild-type *Y* chromosome array has 60% of its ~300 units disrupted. Perhaps there is a critical density of retrotransposons in an rDNA array that determine whether it is dominant or not. This hypothesis can explain why the *Y^{sv391}* chromosome shows codominance, as 85% of its ~650 units are disrupted. However, we found that a *Y* chromosome rDNA array that appears similar in size and insertion load to the wild-type *Y^{OR}* chromosome (*Y^{sv254}*) is also codominant. Thus, other factors in addition to total insertion load must influence nucleolar dominance. For example, perhaps the organization of inserted and uninserted units within an array may determine the overall responsiveness of the array, if interactions between neighboring rDNA units affect activation. A similar effect of large-scale array organization has been proposed to account for differences in interspecies nucleolar dominance (Eickbush *et al.* 2008).

Notably, the three variant *Y* chromosomes we found that fail to silence the *X* chromosome rDNA array were all isolated from stocks with mutations in heterochromatin proteins, and such mutations are known to stimulate recombination within rDNA arrays (Peng and Karpen 2007). Potentially recombination within these *Y* chromosome rDNA arrays has changed their structures. Indeed, one of these chromosomes now carries a deletion of the rDNA. We emphasize that this possibility for rearrangement within arrays makes it likely that nucleolar dominance relationships between arrays are more complicated than we have detailed here. *X* chromosomes are known to vary greatly in array size, retrotransposon load, and large-scale organization (Lyckegaard and Clark 1991). These features may allow them to outcompete *Y* arrays in males or generate nucleolar dominance relationships between *X* chromosomes in females.

While our work establishes that nucleolar dominance is a normal feature of rDNA regulation within *D. melanogaster*, the molecular mechanism remains largely unknown. Specialized chromatin modification complexes that target rDNA genes have been described in budding yeast (Straight *et al.* 1999), *Arabidopsis* (Lawrence *et al.* 2004; Earley *et al.* 2006; Preuss *et al.* 2008), and in mammals (Murayama *et al.* 2008). In *Arabidopsis* and mammals, these complexes reduce overall histone acetylation and direct histone methylation at lysine-9 of the histone H3 (H3K9me), leading to silencing of rDNA genes. These histone modifications are features of heterochromatin. *Drosophila* rDNA genes also show moderate levels of H3K9me (Blattes *et al.* 2006; Peng and Karpen 2007), arguing that heterochromatin-mediated repression is a general feature of rDNA regulation. While the Su(var)3-9 enzyme is one of the major H3K9 methyltransferases for *Drosophila* heterochromatin (Brower-Toland *et al.* 2009), it is unknown how much histone methylation at the rDNA genes is due to the Su(var)3-9 enzyme. In any case, our genetic analysis shows that this enzyme only

partially contributes to silencing of the *X* chromosome rDNA array, suggesting that rDNA silencing may be molecularly distinct from other forms of heterochromatic gene silencing.

Regardless of how individual rDNA genes are silenced, our work implies that cells can direct large-scale silencing of entire rDNA arrays. Similar large-scale silencing of rDNA occurs in mammalian cells (Schlesinger *et al.* 2009). Our results suggest that persistent somatic pairing between *X* and *Y* chromosome rDNA arrays may promote activation of both arrays. In this way, rDNA codominance may be a form of transvection, where pairing between alleles enhances transcriptional activity (Wu and Morris 1999). However, pairing cannot be an all-or-none parameter of rDNA dominance, because dominant *Y* chromosome arrays are paired with the silenced *X* chromosome array ~60% of the time. Somatic pairing is disrupted during mitosis and must be reestablished again in each daughter cell (Fung *et al.* 1998). Perhaps there is a critical time shortly after mitosis where paired rDNA arrays are activated together, while unpaired arrays allow one to be activated and the other silenced.

If repetitive features predispose genes to allelic inactivation, other repeated loci in the *Drosophila* genome may also be subject to allelic inactivation. For example, the histone genes are contained in a ~100 copy array that is embedded in heterochromatin. Perhaps a competition between induction and heterochromatic gene silencing at these arrays regulates the number of genes that are activated. This competition may be a general feature of allelic inactivation systems, as repeats tend to be enriched around loci that are subject to differential allelic expression (Walter *et al.* 2006).

Acknowledgments

We thank Welcome Bender and Keith Maggert for useful suggestions and comments. This work was supported by National Institutes of Health grant R01GM068696 to K.A. and a Netherlands Organisation for Scientific Research Rubicon fellowship to F.G. F.G. and K.A. conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the article.

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Communicating editor: P. K. Geyer