Restricting Dosage Compensation Complex Binding to the X Chromosomes by H2A.Z/HTZ-1

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Abstract

Dosage compensation ensures similar levels of X-linked gene products in males (XY or XO) and females (XX), despite their different numbers of X chromosomes. In mammals, flies, and worms, dosage compensation is mediated by a specialized machinery that localizes to one or both of the X chromosomes in one sex resulting in a change in gene expression from the affected X chromosome(s). In mammals and flies, dosage compensation is associated with specific histone posttranslational modifications and replacement with variant histones. Until now, no specific histone modifications or histone variants have been implicated in Caenorhabditis elegans dosage compensation. Taking a candidate approach, we have looked at specific histone modifications and variants on the C. elegans dosage compensated X chromosomes. Using RNAi-based assays, we show that reducing levels of the histone H2A variant, H2A.Z (HTZ-1 in C. elegans), leads to partial disruption of dosage compensation. By immunofluorescence, we have observed that HTZ-1 is under-represented on the dosage compensated X chromosomes, but not on the non-dosage compensated male X chromosome. We find that reduction of HTZ-1 levels by RNA interference (RNAi) and mutation results in only a very modest change in dosage compensation complex protein levels. However, in these animals, the X chromosome–specific localization of the complex is partially disrupted, with some nuclei displaying DCC localization beyond the X chromosome territory. We propose a model in which HTZ-1, directly or indirectly, serves to restrict the dosage compensation complex to the X chromosome by acting as or regulating the activity of an autosomal repellent.

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Introduction

Many species—such as humans, mice, flies, and worms—utilize a chromosome-based mechanism to establish sex. This results in a difference in sex chromosome number between the sexes that, left uncorrected, puts one sex at a great selective disadvantage. In order to combat this, these organisms employ a second mechanism to ensure that the same amount of sex chromosome-linked gene expression occurs in both sexes. This mechanism is called dosage compensation [1–4].

In flies and mammals, specific posttranslational histone modifications and/or replacement of core histones with variants are key features of the dosage compensated X chromosomes [5]. Dosage compensation in flies is brought about by the MSL (male-specific-lethal) complex that localizes to the single X chromosome in males resulting in a two-fold increase in gene expression [6,7]. The MSL complex is made up of at least five proteins (MSL1 [8], MSL2 [9], MSL3 [10], MLE [11], and MOF [12]), and one of two non-coding RNAs (roX 1 and roX2, RNA on the X [13,14]). The hypertranscribed male X is enriched for histone H4 lysine 16 acetylation (H4K16ac) [15]. MOF (male-absent on the first) places the H4K16ac mark on the male X and this function is essential for dosage compensation [16–18]. In mammals, one of the two female X chromosomes is transcriptionally inactivated [3]. The inactive X is targeted for silencing by the non-coding RNA, Xist in mice [19,20], XIST in humans [21], that coats the inactive X chromosome [22]. This is followed by chromosome-wide histone H3 lysine 27 tri-methylation (H3K27me3) by Polycomb repressor complex 2 (PRC2) [23], and histone H2A and H2A.Z mono-ubiquitylation by Polycomb repressor complex 1 (PRC1) [24,25]. On the inactive X chromosome, there is also an enrichment of histone H3 lysine 9 dimethylation (H3K9me2) [26] and an enrichment of the histone variant macroH2A2 [27]. However, other modifications and variants are specifically under-represented on the inactive X: de- and trimethylation of histone H3 lysine 4 (H3K4me) [26,28], dimethylation of histone H3 arginine 17 (H3R17me2) [29], acetylation of the N-terminal tails of histones H2A, H3 and H4 [30–32], and the phosphorylated form of macroH2A1 [33]. These and other modifications are thought to be vital for the resulting essential change in X-linked gene expression in male flies and female mammals.

In C. elegans, dosage compensation is achieved by the dosage compensation complex (DCC), which binds both X chromosomes in hermaphrodites to downregulate gene expression two-fold [2]. DPY-27 [34], MIX-1 [35], DPY-26 [36], DPY-28 [37], and CAPG-1 [38] form a condensin-like complex, condensin IβC. SDC-2 [39], SDC-3 [40], and DPY-30 [41] are thought to be
Author Summary

In organisms where females have two X chromosomes and males only have one, a mechanism called dosage compensation ensures that both sexes receive the same amount of information from their X chromosomes. Disruption of dosage compensation leads to lethality in the affected sex. While the precise mechanisms of dosage compensation differ between organisms, changes to the structure of the X chromosomes are involved in each case. The DNA of all chromosomes is packaged into a complex protein–DNA structure called chromatin. The most basic level of packaging involves wrapping DNA around a group of small proteins called histones. In both mammals and flies, dosage compensation is associated with specific changes to the histones on the dosage compensated X chromosome. Until now, no such change has been associated with dosage compensation in worms. Here we present evidence that the histone variant HTZ-1/H2A.Z plays a role in dosage compensation in the worm. Specifically, we suggest that HTZ-1 functions to ensure that only the X chromosomes, and not the other chromosomes, are subjected to dosage compensation. This suggests that, despite different mechanisms, one common theme of dosage compensation is a change at the level of the histones associated with the chromosomal DNA.

Responsible for recruitment of the condensin-like complex, as well as DPY-21 [39] and SDC-1 [42,43], to the X chromosomes in hermaphrodites. All DCC proteins, except for SDC-2, are supplied maternally to the oocyte, and are initially present in both male and hermaphrodite embryos [2]. SDC-2 is not contributed maternally, expressed only in hermaphrodites and is thought to confer both sex-specificity and X-chromosome specificity to dosage compensation [39]. The DCC initially binds to sex [recruitment (S) elements on the X], which represent sites of DCC enrichment on the X chromosome, and spreads in cis along the lengths of both X chromosomes in the hermaphroditic [44–47]. As a result, gene expression from the two hermaphroditic X chromosomes is down-regulated by half, thus limiting X-linked gene products to levels produced in XO males [48]. Condensin complexes are well known for their roles in affecting chromosome architecture during mitosis and meiosis [49], so it is believed that the DCC may be altering the overall organization of the X chromosomes to dampen gene expression during interphase. A chromosome-wide architectural change by the DCC condensin may require or lead to specific modifications to the basic organizational unit of chromatin, the nucleosome. However, no nucleosomal changes, such as postranslational modification of histones or histone variants, have been previously implicated to play a role in C. elegans dosage compensation.

While in somatic cells of hermaphrodites the X chromosome is subject to dosage compensation, in the postembryonic germ line of both sexes the X is subject to a distinct form of chromosome-wide regulatory process: global repression throughout meiosis in males and during early meiosis in hermaphrodites [50]. Maternal effect sterility (MES) proteins mediate silencing of the germ line X chromosome and their function is required for germ line viability [50–52]. Three of the mes genes (mes-2, mes-3, and mes-6) encode proteins that function together in a PRC2-like complex, which localizes to the germ line X-chromosome(s) and leads to enrichment of H3 lysine 27 trimethylation on the X [53–56]. By contrast, an additional MES protein, MES-4, localizes only to the autosomes and not X, and its function is necessary for germ line X silencing [51,57]. Additionally, the silenced germ line X chromosomes show a significant depletion of activating marks such as acetylation of the N terminal tail of histone H4 and methylation of lysine 4 on H3 [50,58].

From studies of dosage compensation in other organisms and of germ line X chromosome silencing in C. elegans, there are many well-documented links between different forms of chromosome-wide gene regulation and specific nucleosome characteristics. This led us to explore whether we might find a similar link between C. elegans dosage compensation and nucleosome composition. We were interested to see if any histone modifications or histone variants play a functional role in dosage compensation in worms. In this paper we report on the role of the C. elegans histone H2A.Z variant (HTZ-1).

The histone variant H2A.Z is conserved from yeast to humans and has been implicated in diverse biological processes. Interestingly, depending on its histone partner in the nucleosome core particle, H2A.Z can either stabilize or destabilize the nucleosome [59]. When partnered with histone H3, the H2A.Z-containing nucleosome becomes more stable, but when partnered with the histone variant H3.3, the nucleosome becomes destabilized. Unstable H2A.Z/H3.3 nucleosomes may function to poise genes for activation. Consistently, studies in several organisms implicate H2A.Z in various aspects of transcription activation. In Tetrahymana, hvl1/H2A.Z associates with the transcriptionally active macronucleus [60–62]. Genome-wide localization studies in yeast [63–66], worms [67], flies [68], plants [69], and humans [70], revealed that H2A.Z preferentially localizes to 5’ ends of genes, consistent with a role in transcription activation. Loss of Htz1 has been shown to diminish RNA Pol II binding to promoters, slow the activation of regulated genes, or prevent rapid reactivation of recently repressed genes [66,71,72]. A role of HTZ-1 to poise genes for rapid activation has also been observed in a study of the C. elegans H2A.Z homolog, HTZ-1 [73].

However, H2A.Z also localizes to regulatory regions not corresponding to promoters to exert other functions. In budding yeast, Htz1 also associates at boundary elements to protect genes from heterochromatinization by antagonizing the spread of silencing complexes [74]. This antisilencing functions at the global level, not just locally [75]. Consistent with an antisilencing role, in plants, H2A.Z antagonizes DNA methylation [69]. H2A.Z also localizes to insulator elements in chicken [76], and to functional regulatory elements in human cells [70]. It has been proposed that in this context, the presence of an H2A.Z/H3.3 labile nucleosome prevents the spreading of heterochromatic marks [59].

On the other hand, H2A.Z also plays a role in heterochromatin formation. In this context, H2A.Z most likely partners with H3 to form stable nucleosomes [59]. In mammals and in flies, H2A.Z associates with pericentric heterochromatin and interacts with heterochromatin protein HP1 [77–80]. Mammalian H2A.Z also becomes incorporated into the inactive XY body following meiosis [81]. However, H2A.Z is significantly underrepresented and differentially modified on the mammalian inactive X chromosome in somatic cells, indicating that H2A.Z enrichment is not a general feature of all heterochromatin [25,79,82]. Consistent with this, H2A.Z is not enriched at heterochromatic chromocenters in plants [69,83].

Here we show that in C. elegans the histone variant H2A.Z/HTZ-1 functions in dosage compensation. Consistent with previous reports [67], we find that HTZ-1 is under-represented on the dosage compensated X chromosomes in somatic nuclei of hermaphrodites. However, we do not observe HTZ-1 depletion on the non-dosage compensated X chromosome in male somatic nuclei. We also see an underrepresentation of HTZ-1 on the silent DNA.
X chromosomes of both male and hermaphrodite germ nuclei. Partial depletion of HTZ-1 does not lead to an overall decrease in DCC protein levels. Instead we see mislocalization of the DCC away from the X chromosomes and onto autosomes. These results reveal an HTZ-1 dependent activity that serves to repel the DCC away from autosomes. We propose that HTZ-1 plays a role in dosage compensation by directly or indirectly restricting binding of the DCC to the X chromosomes.

**Results**

HTZ-1 function promotes dosage compensation

To search for chromatin modifiers involved in worm dosage compensation, we utilized two RNAi-based assays in a genetic background sensitized for detecting disturbances in dosage compensation. We tested genes encoding *C. elegans* homologs of histone variants, genes implicated in modifying chromatin via posttranslational histone modifications (such as acetylation or methylation) or chromatin remodeling [84], as well as genes annotated to contain chromo-, bromo- or SET domains (Wormbase [http://www.wormbase.org], release WS201).

The first assay was completed in the *sex-1(y263)* mutant background. *sex-1* functions genetically as an X signal element by repressing *xol-1*, the master switch regulating both sex-determination and dosage compensation [85,86]. In addition, *sex-1* plays a role downstream of *xol-1*, promoting dosage compensation in hermaphrodites [87]. In *sex-1(y263)* mutant hermaphrodites, dosage compensation is partially impaired, resulting in 15–30% embryonic lethality. In these worms, partial loss-of-function due to feeding RNAi of a gene important for dosage compensation leads to increased lethality [87]. A second genetic assay was based on the rescue of males that inappropriately turn on dosage compensation due to a *xol-1(y9)* mutation. Expression of *sex-1* in males is essential to prevent dosage compensation of the single X chromosome [88]. Mutations in *xol-1* are male lethal due to ectopic dosage compensation, leading to abnormally low levels of X-linked gene expression. The *sex-1(y263)* mutation partially weakens dosage compensation, as described above. *xol-1(y9) sex-1(y263)* males die, but they can be rescued by feeding RNAi of dosage compensation genes [38,87]. To ensure a consistent proportion of males in our test strain, we perform these assays in a strain that also carries the *him-8(e1489)* allele. Mutations in *him-8* cause X chromosome nondisjunction in meiosis and results in a predictable 38% of XO progeny each generation [89].

RNAi of DCC components show near complete *sex-1* lethality and results in 33–60% rescue of *him-8(e1489); sex-1(y9) sex-1(y263)* males in these two assays [38]. One candidate, the histone variant *htz-1* (*C. elegans* H2A.Z homolog) showed a similar genetic interaction. RNAi in the wild type background leads to little to no lethality, while *htz-1* RNAi in the *sex-1(y263)* background leads to near complete embryonic lethality (Figure 1A). In the *him-8(e1489); sex-1(y9) sex-1(y263)* background, RNAi of the histone variant *htz-1* resulted in over 15% rescue (Figure 1B). To ensure that these phenotypes are not caused by general disruption to chromatin, we also tested two genes encoding H3 histone variants (*his-71* and *his-72*) [90], and genes encoding linker histones (*his-24* [H1.1], *hil-3* [H1.3], *hil-4* [H1.4], *hil-5* [H1.3], *hil-6* [H1.6], and *hil-7* [H1.Q]) [91]. RNAi of these genes did not show similar genetic interactions. RNAi of many other chromatin factors also failed to result in significant male rescue (for a complete list, see Table S1). The chromatin remodeling enzyme *isw-1*, and the histone deacetylase *let-418* are shown as examples (Figure 1B). We conclude that depletion of HTZ-1 leads to disruption of dosage compensation.

**HTZ-1 is underrepresented on hermaphrodite X chromosomes but not the male X chromosome**

A previous study found that in worms HTZ-1 preferentially localizes to promoters, as in other organisms [67]. Furthermore, fewer peaks of HTZ-1 incorporation were found on the X chromosome, rather than a direct role in dosage compensation [67]. Our RNAi data above indicates that HTZ-1 function is needed for wild type levels of dosage compensation, but does not address whether this role is direct or indirect. That is, *htz-1*
may directly regulate some aspect of DCC function, or \( hzt-1 \) may indirectly affect dosage compensation by regulating expression of known or unknown dosage compensation genes. To begin to distinguish between these possibilities, we analyzed the distribution of HTZ-1 in male and hermaphrodite nuclei. We reasoned that if HTZ-1 functions in dosage compensation, its distribution in the nucleus may be different in males (dosage compensation inactive) and hermaphrodites (dosage compensation active).

To analyze HTZ-1 distribution, we took advantage of a strain expressing a YFP-HTZ-1 fusion protein, or used an HTZ-1 specific antibody. The specificity of our HTZ-1 antibody is demonstrated by recognition of a protein of the predicted size on western blots and reduction of signal after HTZ-1 depletion on both western blots and by immunofluorescence (IF) (Figure S1). We marked the X-chromosome territory with an antibody specific to DPY-27 (marks the X chromosomes in hermaphrodites only) or X-paint fluorescent in situ hybridization (FISH) (to mark the X chromosomes in both sexes). Consistent with a previous report [67], we observed reduced HTZ-1 staining on the dosage compensated X chromosomes in mid-late stage hermaphrodite embryos after the onset of dosage compensation by DPY-27. HTZ-1 IF (Figure 2A and 2B), and combined X-Paint FISH/HTZ-1 IF (Figure 2C). We also observed reduced levels of YFP-HTZ-1 in the territory of the X-chromosomes in transgenic hermaphrodite embryos (Figure S2). However, in males we did not observe a decrease in HTZ-1 staining intensity in the X chromosome territory of somatic nuclei (Figure 2C). These results indicate that reduced HTZ-1 levels are specific to dosage compensated X chromosomes and not a general feature of X chromosomes in both sexes in adult animals.

**HTZ-1 depletion does not lead to a decrease in DCC protein levels**

The results of the genetic assays and localization assays appeared contradictory: reduced \( hzt-1 \) expression disrupts dosage compensation, yet the protein itself is depleted on the dosage compensated X chromosomes. Therefore, we wanted to explore how dosage compensation is affected in \( hzt-1 \) depleted animals. If HTZ-1 functions in dosage compensation indirectly (by regulating expression of dosage compensation genes) we would predict to see a decrease in DCC protein levels upon HTZ-1 depletion. We analyzed worms carrying the \( hzt-1 \) deletion allele \( tm2469 \) that removes 345 of 885 base pairs from \( hzt-1 \) and likely represents a null allele. \( hzt-1(tm2469) \) homozygous progeny of heterozygous mothers (\( m^{hzt-1}\)) develop into healthy adults but are sterile, as reported [67]. However, the \( tm2469 \) deletion appears to affect expression of not just \( hzt-1 \), but the neighboring gene as well (Figure S3). It was therefore important to obtain \( hzt-1 \)-depleted worms using an alternate method and to confirm that phenotypes are due to HTZ-1 depletion, and not depletion of the neighboring gene product. As an alternative method, we depleted HTZ-1 levels by feeding worms bacteria expressing double stranded RNA corresponding to \( hzt-1 \). As a control, worms were fed bacteria carrying an empty vector. Feeding RNAi in wild type animals greatly reduced HTZ-1 as detected both by immunofluorescence and quantitative Western blot analyses (89% reduction) (Figure 3B and Figure S1).

To investigate the possibility that HTZ-1 depletion leads to a decrease in DCC protein levels, we quantified protein levels by western blotting of HTZ-1 depleted and control animals. Although HTZ-1 levels were clearly reduced after \( hzt-1 \) RNAi, we did not observe a dramatic change in DCC protein levels (Figure 3A and 3C). Levels of DPY-27 and CAPG-1 show a very slight decrease while MIX-1, DPY-26, and DPY-28 show very slight increases after \( hzt-1 \) RNAi. Our results suggest that, HTZ-1 reduction does not lead to a significant defect in overall DCC protein levels. However, we cannot exclude the possibility that the timing of DCC gene expression is changed (delayed) in HTZ-1 depleted cells, as was observed for genes involved in foregut development [73]. It is also possible that a small amount HTZ-1 that remains after feeding RNAi is sufficient for DCC gene expression, but more complete HTZ-1 depletion would result in a significant decrease in DCC protein levels.

SDC-2, the primary determinant of hermaphrodite fate, is the only DCC protein whose expression in the zygote is essential [39]. The remaining DCC proteins are maternally loaded into the oocyte and this maternal load is sufficient to carry out dosage compensation in the developing embryo. Therefore, it was important to determine whether \( sdc-2 \) transcript levels are affected after HTZ-1 depletion. We analyzed \( sdc-2 \) mRNA levels in HTZ-1 depleted and control animals by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) and observed no significant change in \( sdc-2 \) expression (Figure 3D). We conclude that the changes observed in DCC protein and RNA levels are not likely to be sufficient to explain the observed requirement for HTZ-1 to maintain wild type levels of dosage compensation.

**DCC localization is disrupted in HTZ-1-depleted animals**

An alternative possibility is that HTZ-1 has a more direct role in dosage compensation by affecting DCC localization or function. To explore this possibility, we used immunofluorescence to observe DCC localization in HTZ-1-depleted worms. The DCC was clearly present in nuclei of \( hzt-1(RNAi) \) animals, again suggesting that HTZ-1 depletion does not lead to a significant reduction in DCC protein levels. However, the territory occupied...
by the DCC in these nuclei was significantly more diffuse in appearance than in wild type nuclei (Figure 4).

We hypothesized that the diffuse appearance of the DCC reflected mislocalization of the DCC away from the X chromosome. To observe DCC localization relative to X chromosomes, we combined DCC immunofluorescence with X-paint FISH in \textit{htz-1} (RNAi) (Figure 4) and mutant (Figure 5) animals. We used intestinal nuclei because they are 32-ploid, allowing for easier visualization of sub-nuclear regions by FISH [92]. To determine the degree of colocalization between X-Paint and DPY-27 signals we determined Pearson's correlation coefficient ($R_r$) values (see Materials and Methods). An $R_r$ value of +1 indicates a complete and positive correlation between two signals within a region of interest while a value of 0 indicates no linear relationship between the two signals.

In vector control RNAi animals we observed that the DCC was highly restricted to the X chromosomes and the mean $R_r$ was 0.65 ± 0.14. $R_r$ was greater than 0.5 in the vast majority of nuclei observed (58%), and only a minority of nuclei had $R_r$ values between 0.5 and 0.2 (~12%). No correlation values of less than 0.2 were observed in these animals. Representative nuclei and corresponding $R_r$ values are shown in Figure 4A. By contrast, after \textit{htz-1} RNAi, the mean $R_r$ for \textit{htz-1} RNAi nuclei was 0.44 ± 0.20, significantly lower than control ($p = 5.79E-8$). The majority of nuclei (58%) had DPY-27/X-paint correlation values below 0.5, and 27% of nuclei had values below the lowest value observed in the control. Representative \textit{htz-1} (RNAi) nuclei and corresponding $R_r$ values are shown in Figure 4B. A summary of DPY-27/X-Paint colocalization quantification after vector and \textit{htz-1} RNAi treatment is shown in Figure 4C.

DCC mislocalization was also observed in intestinal nuclei of homozygous \textit{htz-1} (tm2469) hermaphrodite progeny of heterozygous mothers (\textit{m+\textit{z}2}) (Figure 5) and in HTZ-1-depleted embryos (Figure S4). The mislocalization phenotype observed in \textit{htz-1} (tm2469) (Figure 5) mutant animals was very similar to the observations made after \textit{htz-1} RNAi. In wild-type hermaphrodites only 5% of nuclei observed had $R_r$ values below 0.5, but a majority of \textit{htz-1} (tm2469) nuclei (59%) had values below 0.5. Additionally, 45% of nuclei
HTZ-1 Restricts DCC to X

The portion of DCC which is not associated with the X chromosome appears nonetheless bound to chromatin. When we combined X-Paint FISH/DPY-27 IF with a protocol previously shown to extract nucleoplasmic proteins [97], we were unable to remove the non-X associated DCC within intestinal nuclei of htz-1 RNAi animals (Figure 6A). This suggests that the non-X associated DCC is associated with autosomal chromatin.

To confirm DCC association with autosomes, we analyzed prophase chromosomes in both vector control and htz-1 depleted embryos. We reasoned that individualized mitotic chromosomes would allow for more conspicuous visualization of DCC localization. To mark mitotic nuclei, embryos were co-stained with α-Phospho-Histone H3 Serine 10. In control embryos, DCC localization was largely restricted to two chromosomes in prophase nuclei. After htz-1 RNAi, however, 32% of prophase nuclei had low-level DCC staining on more than two chromosomes (Figure 6B). These data indicate that the DCC associates not only with the X chromosome, but also with autosomes in HTZ-1-depleted animals. Taken together, these results suggest the existence of an HTZ-1 dependent autosomal repellent activity. In wild type animals, this activity restricts localization of the DCC to the X chromosome. Loss of htz-1 reduces the efficiency of this repellant, allowing the DCC to bind other chromosomes.

HTZ-1 levels are also reduced on the X chromosomes in the male and hermaphrodite germ lines

In different organisms, H2A.Z has been observed to be either enriched in silent chromatin, (such as mammalian and Drosophila centromeres [77–80], or the XY sex body in the mammalian germ line [81]) or depleted in silent chromatin (such as heterochromatic chromocenters in plants [69,83], or the transcriptionally inactive micronucleus in Tetrahymena [60–62]). Dosage compensation in worms is thought to involve two-fold downregulation of gene expression, but not complete silencing [2]. To explore whether HTZ-1 localizes to silent chromatin in worms, we examined its distribution in the germ line, where the X chromosomes are subject to chromosome-wide silencing by a mechanism unrelated to dosage compensation [50]. In the male germ line, the single X chromosome is subject to meiotic silencing of unpaired chromatin and is silent throughout meiosis. In the hermaphrodite germ line, the paired X chromosomes are silent during early meiosis, but become transcriptionally active in later stages [50]. To test HTZ-1 levels on the silent X chromosome in the germ line, we performed immunofluorescence experiments on dissected male and hermaphrodite gonads. To distinguish the X from autosomes we used antibodies specific to MES-4, H3K27me3, or H4K16ac, all of which have been used in previous studies to distinguish the X from autosomes in the germ line. MES-4, a SET-Domain protein, is enriched on autosomes and markedly depleted from the X chromosome in the germ line [51,57]. Conversely, H3K27me3 is enriched on the silent X chromosomes in the germ line [53]. In the male germ line, H4K16ac is present on autosomes but absent from the unpaired X chromosome [50]. We found that HTZ-1 levels observed had values below the lowest value observed in wild-type nuclei (Figure 5B and 5C). We also analyzed HTZ-1-depleted embryos after the 50-cell stage in development (after the onset of dosage compensation). We observed 16% of htz-1-depleted embryos with a diffuse nuclear DCC localization pattern (as opposed to 2% of vector RNAi control embryos), confirming that DCC mislocalization is not tissue specific (Figure S4).

Finally, we analyzed DCC distribution in nuclei of ssl-1 (n4077) mutant animals. ssl-1 encodes a homolog of Swr1, the catalytic subunit of Swr1-com, the complex responsible for exchanging H2A for H2A.Z [93–96]. Consistent with this function, ssl-1 (n4077) m/z– homozygous animals have reduced HTZ-1 staining (Figure 5A). In ssl-1 (n4077) hermaphrodites, 43% of nuclei observed had $R_r$ values below 0.3, similar to what we observe after htz-1 RNAi and in htz-1 (tm2469) animals. Also, 33% of ssl-1 nuclei had $R_r$ values below the lowest value observed in wild type nuclei, confirming that reduced HTZ-1 disrupts the localization of DCC to the X chromosomes (Figure 5B and 5C). Together, these results strongly suggest that HTZ-1, a protein more abundant on autosomes, is important for restricting localization of the DCC to the X chromosomes.

DCC localization to autosomes upon HTZ-1 depletion

Together these results strongly suggest that HTZ-1, a protein more abundant on autosomes, is important for restricting localization of the DCC to the X chromosomes.
are much lower on the X chromosomes than on autosomes in both male and hermaphrodite germ lines (Figure 7). Thus, underrepresentation of HTZ-1 appears to be a general feature of both types of chromosome-wide repression in the worm: two-fold downregulation by dosage compensation and complete meiotic silencing. The possible involvement of HTZ-1 in germ line X chromosome silencing will be explored elsewhere.

Discussion

Dosage compensation in C. elegans is accomplished by the DCC, a complex of proteins that binds the two X chromosomes in hermaphrodites to down-regulate expression of genes two-fold. In this study we report on the role of the histone variant H2A.Z/HTZ-1 in this process. HTZ-1 is less abundant on the dosage compensated X chromosomes in hermaphrodites but is found at higher levels on autosomes and X chromosomes in male somatic cells. When htz-1 expression is reduced, levels of DCC proteins do not change. However, binding of the DCC is no longer restricted to the X chromosomes and dosage compensation is impaired.

Models for HTZ-1 function in dosage compensation

One of the intriguing challenges in the study of dosage compensation is to understand how the DCC machinery is able to specifically target the X chromosomes for regulation. Our studies indicate that when HTZ-1 is depleted, the DCC appears to be no longer targeted correctly to the X chromosome. Rather than binding solely to the X chromosomes, the complex now binds to autosomes as well. These results reveal that the normal function of HTZ-1 (or an HTZ-1 regulated factor) includes keeping the DCC away from autosomes. Previous studies indicated that specific DCC binding sites on the X chromosome, so-called rex sites (recruiting element on X), are important for attracting the DCC to the X chromosome ([44–47]). Taken together, these data suggest that positive forces (X-specific recruitment elements that attract the DCC) and negative forces (autosomal chromatin that repels the DCC) cooperate to discriminate the X from autosomes (Figure 8).

The mechanism of how HTZ-1 restricts DCC localization is unclear. We will consider three possible models. First, HTZ-1 may serve as a direct regulator of DCC binding. Targeting of the DCC to the X chromosome is believed to be a two-step process. The complex initially binds to an estimated 200 rex sites, followed by dispersal to numerous so-called dos sites (dependent on X) or “way

Figure 6. DCC associates with autosomes in HTZ-1-depleted cells. (A) DPY-27 (green) and X-chromosome (red) localization in vector and htz-1 RNAi treated hermaphrodite adult intestinal nuclei after detergent extraction of nucleoplasmic proteins. DCC staining not in association with the X chromosomes remains after detergent extraction in htz-1 RNAi animals. (B) DPY-27 (green) localization to prophase chromosomes (DAPI, blue) in vector and htz-1 RNAi treated embryonic nuclei (>50 cell stage). DPY-27 staining to more than two chromosomes is observed in 32% of htz-1(RNAi) prophase nuclei (n = 63) but only observed in 11% of vector prophase nuclei (n = 57). Mitotic nuclei were identified using an antibody that recognizes H3S10Ph (red). doi:10.1371/journal.pgen.1000699.g006

Figure 7. HTZ-1 is underrepresented on the silent X chromosomes of male and hermaphrodite germ lines. (A) Hermaphrodite germ nucleus in pachytene of prophase I of meiosis stained with antibodies specific to H3K27me3 (a mark enriched on the X, green), HTZ-1 (red), and DAPI (grayscale). (B) Hermaphrodite germ nucleus in diplotene of prophase I co-stained for MES-4 (enriched on autosomes, green), HTZ-1 (red), and DAPI (grayscale). (C) Male germ nucleus in pachytene of prophase I co-stained for H4K16ac (enriched on autosomes, green), HTZ-1 (red) and DAPI (grayscale). In all cases, HTZ-1 levels are lower on the X chromosome (arrows) than on autosomes. doi:10.1371/journal.pgen.1000699.g007

Figure 9. HTZ-1 Restricts DCC to X
stations" [44,46,47,98]. *Rex* sites coincide with the highest peaks of DCC binding and are characterized by the presence and clustering of short sequence motifs called MEX motifs [46,47,99]. MEX motifs are slightly enriched on the X chromosome, but are also present on autosomes [46]. In principle, HTZ-1 can affect either DCC targeting to *rex* sites, or dispersal to *dox* sites, or both.

It should be pointed out that this model is different from the interpretation of the HTZ-1 localization data presented in [67]. Using high-resolution analysis of HTZ-1 binding, the authors showed that a subset of DCC peaks on the X chromosome coincide with HTZ-1 peaks. One way to reconcile their data and ours is to point out that the DCC-HTZ-1 overlap tends to be at promoters (DCC *dox* sites), and so at the highest peaks of DCC binding (DCC *foci* or *rex* sites) [67]. Therefore, if HTZ-1 is a negative regulator of DCC binding, it is more likely that HTZ-1 affects the targeting step to *rex* sites, but not the dispersal step to *dox* sites. Another way to reconcile the data in the two studies is to suggest that HTZ-1 at *dox* sites is modified posttranslationally (see below) in such a way that permits DCC binding. According to this model, MEX motifs attract DCC to the X chromosome, whereas HTZ-1 negatively regulates DCC recruitment to *rex* sites. If a MEX motif-containing sequence is not bound by HTZ-1 the DCC will be recruited. However, if a MEX motif-containing sequence is bound by HTZ-1, the DCC will be prevented from binding. From sites of entry, the DCC then may be dispersed to *dox* sites in a sequence and HTZ-1 independent manner. When HTZ-1 levels are reduced by RNAi or mutation, the DCC will be able to bind all MEX motif containing sites, whether they are on the autosomes or on the X chromosome. Ectopic DCC binding to autosomes will reduce the amount of DCC binding to the X chromosomes, and dosage compensation will be impaired as a result. To test this model, it will be important to observe DCC binding patterns genome-wide at high resolution upon *htz-1* depletion and to determine whether ectopic DCC binding sites contain a DNA sequence motif similar to MEX motifs.

An alternative possibility is that changes in the higher order chromatin organization imposed by HTZ-1 determine whether the DCC is able to bind the chromosome. H2AZ has been reported to alter the nucleosome surface, affect recruitment of other chromatin components, and thereby modulate higher order features of the chromatin fiber [77]. High levels of HTZ-1 on the autosomes may result in alterations in the overall structure of the chromatin fiber, which preclude DCC binding. Low levels of HTZ-1 on the X chromosome would allow DCC binding. Upon reduction of HTZ-1 levels, general disruption of higher order chromatin folding would allow the DCC to bind both the X and the autosomes. According to this model, the changes in chromatin fiber folding are a direct consequence of HTZ-1 levels on the chromosome. However, the model does not require complete mutually exclusive binding of the DCC and HTZ-1 at high resolution, and therefore does not conflict with the data in [67].

Finally, HTZ-1 may regulate expression of a DCC component, or another gene needed for proper DCC localization. While HTZ-1 is an obvious candidate for the DCC-repelling activity, it should be noted that in principle another HTZ-1-regulated protein could also perform this function. Our evidence, as yet, does not support this model, as we do not observe a change in DCC protein levels or *sdc-2* RNA levels when *htz-1* expression is reduced. In addition, most DCC proteins are loaded into oocytes, and this maternal load of DCC proteins is sufficient for healthy development. Therefore, it is unlikely that the observed dosage compensation defects in m+z− *htz-1* mutant animals are due to defects in transcription of DCC genes. However, it remains possible that HTZ-1 plays more subtle roles in regulating the exact levels and timing of expression of dosage compensation genes. Nonetheless, the difference in HTZ-1 levels in male and hermaphrodite X chromosomes (Figure 2C) argue for a more direct role for HTZ-1 in the hermaphrodite specific-process of dosage compensation. High-resolution analysis of HTZ-1 binding to the male X chromosome may help distinguish between the models presented above.

HTZ-1 depletion on the X chromosome

A question that remains unanswered is how HTZ-1 is specifically targeted to autosomes, or conversely, how the X chromosomes become depleted of HTZ-1. The small number of developmentally important genes on the X chromosome relative to autosomes can certainly contribute to this difference [67]. However, this model does not explain why the male X in adult animals does not appear to be depleted of HTZ-1 at the chromosomal level (Figure 2C). The X chromosome in both the male and hermaphrodite germ lines is subject to silencing [50–52], and it is possible that the chromosome maintains some memory of this silencing after fertilization. Such effects have been seen on the sperm-derived X chromosome [100]. The differences between the sperm-derived X (which only contains the embryo's RNAi) and the oocyte-derived X (which both males and hermaphrodites receive) may contribute to the sex-specific differences in observed X-linked HTZ-1 levels. Comparison of HTZ-1 dynamics in male and hermaphrodite embryos in early development will be an important future area of investigation.

Different HTZ-1 pools for different functions

It is important to keep in mind that dosage compensation is a chromosome-wide gene regulation mechanism that is superimposed on the unique transcriptional programs of individual X-linked genes. While HTZ-1 levels on the dosage compensated X chromosomes are reduced overall, the protein is not completely absent. Indeed, HTZ-1 binds to the promoter of an X-linked gene...
dosage compensated gene, myo-2, and is needed for its proper temporal activation [73]. It is possible that a pool of HTZ-1 functions at promoters, including promoters on the X chromosome, to promote timely regulation of gene expression. Superimposed on that is the global repression of the X chromosome by the DCC. Thus, HTZ-1 may perform a double role: it regulates genes both individually (by binding to promoters) and chromosome-wide (by regulating DCC binding).

Different pools of HTZ-1 may differ in their histone partners and/or the level of posttranslational modifications. Unlike yeast (where the only histone H3 is most similar to H3.3), worms possess both H3 and H3.3 [90]. Therefore, in principle, one population of HTZ-1 in worms is able to form labile nucleosomes, while another population can form stable nucleosomes. Furthermore, both populations can be modified by various posttranslational modifications, increasing the number of potentially different ways in which HTZ-1 can affect genome activity. Consistent with this idea, acetylation of the N-terminal tail of Htz1 is necessary for the anti-silencing property of Htz1 in S. cerevisiae, as unacetylatable Htz1 shows no change in localization to anti-silenced genes, but Sir complex spreading and decreased expression of anti-silenced genes is observed [101]. Htz1 is also subject to C-terminal SUMOylation. SUMO-Htz1 is implicated in directing chromosomes with persistent double-strand breaks to re-localize to the nuclear periphery in budding yeast [102]. Posttranslational modification of H2A.Z has also been observed in mammalian dosage compensation [25]. H2AZ is under-represented on the inactive X in female mouse nuclei, but the small population of remaining H2A.Z is specifically mono-ubiquitylated by the Ring1B E3 ligase as part of the Polycomb repressor complex 1 (PRC1). Ring1B is also responsible for mono-ubiquitylation of histone H2A in X inactivation [24]. Although it is not currently understood how mono-ubiquitylation of H2A and H2A.Z function in X inactivation, the fact that this modification is largely specific to the inactive X suggests an important role. It is highly likely that C. elegans HTZ-1 is subject to posttranslational modification and it will be important to address how these modifications affect its role both in dosage compensation and in other processes.

Barriers to repressor complex binding

The proposed role of HTZ-1 in dosage compensation is similar to that of two proteins shown to function in germline X-chromosome silencing in C. elegans. The gene encoding MES-4 was originally identified in a forward genetic screen with several other genes whose mutations led to the same mes phenotype (maternal effect sterility) [51]. MES-2, MES-3 and MES-6, are the protein products of the other genes identified, and these form a Polycrom repressor-like complex that is responsible for enriching the X chromosomes with the silencing H3K27me3 mark [53]. Surprisingly, MES-4, a histone H3 lysine 36 methyltransferase (HMT), localizes to autosomes, not the X, and yet it has been shown to be important for germ line X-chromosome silencing [51,57]. MRG-1, an ortholog of the mammalian mortality factor related protein MRG15, is the second autosome-enriched protein that has been shown to play a role in germ line X chromosome silencing [103]. In both mes-4 and mg-7 mutants, de-silencing of X-linked genes is observed. It has been proposed that the activities of MES-4 and MRG-1 on autosomes prevent the binding of a repressor protein or complex and help limit repressor binding to the X chromosomes. The proposed mode of action of MRG-1 and MES-4 in germ line X chromosome silencing is similar to the model of HTZ-1 function in dosage compensation we have proposed.

Our model describing HTZ-1 as an autosomal DCC barrier is also similar to the role of Htz1 in yeast in blocking the spread of silencing complexes into euchromatic regions adjacent to telomeres [74]. In htz-1/4 cells, the Sir proteins spread into these regions, leading to silencing of genes. Recent evidence has shown that loss of Htz1 leads to ectopic Sir complex localization that is not limited to immediate anti-silenced regions, but, rather, is found throughout the genome [75]. Thus, Htz1p in yeast may serve a global, not just a local, anti-silencing function, similar to our proposed model of HTZ-1 action in worms. Furthermore, in Arabidopsis, H2AZ also plays a global anti-silencing role by protecting DNA from methylation [69]. When H2AZ incorporation is compromised, DNA methylation expands into regions once protected by H2A.Z-containing nucleosomes. Therefore, a function for H2A.Z in the protection against transcriptional repression may be a widely conserved role for this histone variant.

Htz1 functions in parallel with other nucleosomal elements to prevent heterochromatin spreading. The Set1 complex is responsible for histone H3 lysine 4 methylation (H3K4me) and also has an anti-silencing function [104]. A recent study found that Set1 and Htz1 cooperate to mediate global antisilencing in yeast [75]. This raises the possibility that there are other nucleosomal modifications or elements that might function in X-chromosome DCC restriction in parallel with HTZ-1 in C. elegans.

Materials and Methods

Strains and alleles

All strains used were maintained as described [105]. Strains include: N2 Bristol strain (wild type), TY2384 sex-1(y263) X; TY4403 him-8(e1489) IV; xol-1(y9) sex-1(y263) X; EKM11 htz-1 (tm2469) IV/x(tl)(gK51) IV; V; MT12963 sld-1(w4077) III/et1 (III;V); SM1353 cha-1(p1182) IV; exE214[HTZ-1promoter::TFP::HTZ-1 promoter::CFP::LacI + pRF4] [73].

RNA interference

E. coli HT115 bacteria expressing double stranded RNA for htz-1, dpy-27, capg-1, his-71 (coding region), his-24, hil-3, hil-4, hil-5, hil-6, hil-7, isw-1 or vector (polylinker), were used for feeding RNAi using the Ahringer feeding RNAi clones [106]. To generate RNAi vectors for let-418 and the 3’ UTR of his-71 and his-72, the regions were PCR amplified, digested with Bam HI and Bgl II (let-418) or Bgl II and Not I (his-71 and his-72), and cloned into the DT7 vector as described [106]. The following primers were used for amplification:

- his-71 3’UTR: cgaagatcttgtgatatagctagctgct and gagggagcctgacgctgtgctgctgaanac
- his-72 3’UTR: cgaagatctgtgatatagctagctgct and gagggagcctgacgctgtgctgctgaanac
- let-418: catgaggattcggcctccctcaattac and gctagactgtagctgacgtagacgagaac

RNAi in N2 was initiated at the L1–L2 stage. Adults were then transferred to new RNAi plates to produce progeny for 24 hours. For IF/ISH, western, and RT-PCR analysis, RNAi progeny were processed 24 hours post-L4. To score embryonic lethality in the sex-1 strain, adult animals were allowed to lay eggs for 24 hours and the number of embryos laid was counted. The next day the number of dead embryos and larvae were counted and the percentage of embryonic lethality was calculated by dividing number of dead embryos by the total number of embryos laid. To score male rescue in him-8(e1489) IV; xol-1(y9) sex-1(y263) X, adult animals were allowed to lay eggs for 24 hours. When adult animals
were removed from the RNAi plates, the number of embryos laid was counted. Three days later the number of male progeny on each plate was counted. Male viability was calculated by dividing the number of male progeny observed by the expected number of males. The\( \text{hs}-\text{trang}^{1489} \) mutation reproducibly results in 38% male self-progeny [99], so the expected number of males was determined to be 38% of total embryos laid. Male rescue = Number of males/total embryos ×0.30. All RNAi was conducted at 20°C.

### Immunostaining

Rabbit and rat \( \alpha \)-HTZ-1 antibodies were raised against the C-terminal 19 amino acids (NKKGAPVPGKPGAPGGQP) and affinity purified. Polyclonal rat \( \alpha \)-HTZ-1 was used at a dilution of 1:500, polyclonal rabbit \( \alpha \)-HTZ-1 at a dilution of 1:100 for immunofluorescence. Other primary antibodies used are: polyclonal rabbit \( \alpha \)-DPY-27 at a dilution of 1:100 [38], polyclonal rabbit \( \alpha \)-MES-4 [Susan Strome (UC Santa Cruz), [57] at 1:100, rabbit antisemur \( \alpha \)-acetyl-histone H4 (Lys16) (Upstate) at 1:100, rabbit polyclonal \( \alpha \)-trimethyl-histone H3 (Lys27) (Upstate) at 1:500, and mouse monoclonal \( \alpha \)-phospho-histone H3S10 (6G3) (Cell Signaling Technology) at 1:500. Secondary antibodies used are: Fluorescein (FITC) conjugated donkey \( \alpha \)-rabbit (Jackson ImmunoResearch) and Cy3 conjugated donkey \( \alpha \)-rabbit IgG (Jackson ImmunoResearch) both at a dilution of 1:100. Embryos were stained as described [34]. Adult animals were dissected and stained as described [44]. In adults, somatic non-intestinal nuclei near the cut site (vulval area) were observed. Images were captured with a Hamamatsu ORCA-ERGA CCD camera mounted on an Olympus BX61 motorized X-drive microscope using a 60× oil immersion objective. Captured images were deconvolved with 3i Slidebook imaging software. Projected images were taken at 0.2 μm intervals through samples. Adobe Photoshop was used for assembling images.

**Fluorescent in situ hybridization**

FISH probe templates were generated by degenerate oligonucleotide primed PCR to amplify purified yeast artificial chromosome DNA. The labeled X-paint probe was prepared and used as described [44]. Hybridization was performed on adult animals (24 hours post-L4) with or without previous RNAi treatment. For X-paint hybridization followed by DPY-27 immunostaining, sample and probe were denatured at 95°C for 3 minutes. For X-paint hybridization followed by HTZ-1 immunostaining, sample and probe were denatured at 78–80°C for 8–10 minutes in a Hybaid OmniSlide in situ Thermal Cycler System (Thermo Scientific). Imaging was conducted as described above.

**Quantification of colocalization**

3i Slidebook imaging software was used to measure colocalization of DPY-27 (FITC) and X-Paint (Cy3) signals on images obtained as described above. A FITC mask was set for each nucleus \( z \)-stack and the correlation between signals was calculated within this mask by the software. The FITC:CY3 correlation coefficient was recorded and used as an indication of colocalization between DPY-27 and X-Paint.

**Detergent extraction**

Detergent extraction of nucleoplasmic protein from dissected nuclei was performed by dissecting animals in 1× sperm salts plus 1% Triton detergent [97]. Dissected animals were then processed for either Fluorescent in situ hybridization or immunofluorescence.

### Western blot analysis

For each treatment described, 100 animals (all 24 hours post-L4) were picked into 1×M9, washed, and incubated for ten minutes at 95°C in 19 μl SDS-PAGE loading dye (0.1 M Tris-HCl pH 6.8, 75 M Urea, 2% SDS, Bromophenol Blue for color) plus 1 μl β-mercaptoethanol. The treated samples were then loaded into either 6% acrylamide (for detection of DPY-27, MIX-1, DPY-26, DPY-28, and CAPG-1) or 15% acrylamide gels (for detection of HTZ-1). SDS-PAGE was performed and protein was transferred onto nitrocellulose. The following antibodies and dilutions were used: rabbit \( \alpha \)-HTZ-1 at 1:500, rabbit \( \alpha \)-DPY-27 at 1:500, rabbit \( \alpha \)-CAPG-1 at 1:500 [38], rabbit \( \alpha \)-DPY-28 (gift of K. Hagstrom) at 1:5000, rabbit \( \alpha \)-X-Paint (gift of K. Hagstrom) at 1:5000, mouse \( \alpha \)-Tubulin (Sigma) at 1:1000, LI-COR IRDye 800CW Conjugated Goat (polyclonal) \( \alpha \)-Mouse IgG at 1:10000, LI-COR IRDye 800CW Conjugated Goat (polyclonal) \( \alpha \)-Rabbit IgG at 1:10000. Blots were scanned and band intensities were quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences). Protein levels for DCC proteins and HTZ-1 were normalized to \( \alpha \)-tubulin. Relative protein levels after \( \text{htz-}1 \) RNAi were calculated by dividing the normalized \( \text{htz-}1 \ \)RNAi level by normalized vector RNAi level.

**RT–PCR**

Trizol (Invitrogen) was used to extract RNA from all samples. Worms were washed from RNAi plates or normal OP50 plates 24 hours post-L4, washed with M9 and stored at −80°C until extraction. For RNA extraction, samples were thawed on ice and tissue was homogenized by grinding using a microcentrifuge tube pestle. Tissue was ground in three 60-second intervals and re-frozen in liquid nitrogen between each interval. During the final 60-second interval, 250 μl of Trizol was added to the tube, and when completed, another 250 μl was added for a total volume of 500 μl Trizol and the standard protocol was used to extract RNA from the homogenized samples (Invitrogen). DNA-Free kit (Applied Biosystems) was used to digest remaining DNA contamination.

Reverse transcription (RT) reactions were performed utilizing the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). 1 μl of DNase-treated RNA was used in each RT reaction.

PCR was used to observe relative levels of \( \text{htz-}1 \), R08C7.10, and \( \text{act-}1 \) (actin) expression levels. The following primers were used with an annealing temperature of 60°C normalizing vector RNAi level.

| Primer | Sequence | Description |
|--------|----------|-------------|
| \( \text{htz-}1 \) | gccagcagcctctctgtctgctg | \( \text{htz-}1 \) expression |
| \( \text{act-}1 \) | gccagcagcctctctgtctgctg | \( \text{act-}1 \) expression |

**Real-time PCR analysis**

Real-time PCR analysis was conducted as described [98]. The following primers were used with an annealing temperature of 59°C:

| Primer | Sequence | Description |
|--------|----------|-------------|
| \( \text{htz-}1 \) | gccagcagcctctctgtctgctg | \( \text{htz-}1 \) expression |
| \( \text{act-}1 \) | gccagcagcctctctgtctgctg | \( \text{act-}1 \) expression |

Relative \( \text{htz-}1 \) and \( \text{act-}1 \) expression levels were calculated using the Pfaffl method [107] incorporating the PCR efficiency for each primer set as determined by a 10-fold dilution series for each primer set in each reaction. Reactions were conducted in triplicate per experiment. Data shown are resulting averages from three experiments.
Supporting Information

**Figure S1** α-HTZ-1 antibody is specific. (A) HTZ-1 (red), DPY-27 (green), and DAPI (grayscale) staining of adult intestinal nuclei in vector and α-HTZ-1 RNAi treated hermaphrodites. The HTZ-1 signal is greatly reduced after α-HTZ-1 RNAi. (B) α-HTZ-1 Western blot. α-HTZ-1 recognizes a band of the expected size (~15 kDa) in both embryonic extract and adult protein samples. The α-HTZ-1 signal is reduced in α-HTZ-1 RNAi treated animals as compared to vector treated animals.

Found at: doi:10.1371/journal.pgen.1000699.s001 (7.19 MB TIF)

**Figure S2** Transgenic YFP-HTZ-1 is also under-represented on the dosage compensated X chromosomes. Embryos after the onset of dosage compensation were stained with α-GFP antibodies to observe YFP-HTZ-1 localization (red), α-DPY-27 to mark the X chromosome (green) and DAPI (grayscale). Arrows in the top panels indicate enlarged nucleus shown below. YFP-HTZ-1 levels are reduced in the region of the dosage compensated X chromosomes.

Found at: doi:10.1371/journal.pgen.1000699.s002 (7.78 MB TIF)

**Figure S3** tm2469 deletion affects htz-1 and R08C7.10 expression. (A) Reverse-transcription polymerase chain reaction (RT-PCR) analysis of expression of htz-1, R08C7.10 and actin (control in wild type (+/+), heterozygous (htz-1(tm2469)/+), or homozygous (htz-1(tm2469)) animals. Expression of both htz-1 and R08C7.10 is affected in homozygous animals. Contaminating amplification product from residual DNA in the RNA sample is indicated by a star. (B) Schematic showing relative positions of htz-1 and R08C7.10 on chromosome IV (not to scale). The tm2469 deletion removes most of the coding region of htz-1. In addition, it likely affects the promoter or other cis control elements of the R08C7.10, a gene located only 522 base pairs away from the deletion.

Found at: doi:10.1371/journal.pgen.1000699.s003 (5.72 MB TIF)

**Figure S4** htz-1-depleted embryos also show compromised DCC localization. Vector and α-HTZ-1 RNAi embryos were stained with α-Phospho-H3 Ser10 (red) to mark mitotic nuclei and α-DPY-27 (green). After htz-1 RNAi, 16% of embryos with Phospho-H3 Ser10 staining (>50 cell stage) had diffuse nuclear DPY-27 localization (n = 372), as opposed to 2% in vector embryos (n = 314).

Found at: doi:10.1371/journal.pgen.1000699.s004 (2.89 MB TIF)

**Table S1** RNAs of the following genes did not result in significant (>10%) male rescue.

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Author Contributions

Conceived and designed the experiments: ELP GC. Performed the experiments: ELP KSC AJC MJS. Analyzed the data: ELP GC. Wrote the paper: ELP GC.

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