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A sex chromosome piRNA promotes robust dosage compensation and sex determination in *C. elegans*

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SUMMARY

In metazoans, Piwi-related Argonaute proteins engage piRNAs (Piwi-interacting small RNAs) to defend the genome against invasive nucleic acids, such as transposable elements. Yet many organisms—including worms and humans—express thousands of piRNA that do not target transposons, suggesting that piRNA function extends beyond genome defense. Here we show that the X chromosome-derived piRNA *2lux-1* down-regulates XOL-1 (XO Lethal), a master regulator of X-chromosome dosage compensation and sex determination in *C. elegans*. Mutations in *2lux-1* and several Piwi-pathway components sensitize hermaphrodites to dosage compensation and sex-determination defects. We show that the piRNA pathway also targets *xol-1* in *C. briggsae*, a nematode species related to *C. elegans*. Our findings reveal physiologically important piRNA–mRNA interactions, raising the possibility that piRNAs function broadly to ensure robust gene expression and germline development.

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Although piRNAs are known to defend the genome against transposons, it remains unclear whether they have additional functions beyond genome defense. Tang et al. show that an X chromosome-derived piRNA down-regulates XOL-1 expression to promote robust regulation of dosage compensation and sex determination in *C. elegans*.

INTRODUCTION

Piwi proteins and their piRNA co-factors silence transposons and promote fertility in diverse animals (Juliano et al., 2011; Siomi et al., 2011; Weick and Miska, 2014). The importance of transposon silencing for genome stability, and therefore fertility, seems obvious, but whether the role of piRNAs in fertility depends on transposon silencing alone remains a matter of debate. In worms, Piwi mutants show markedly reduced fertility that increases in severity over multiple generations—i.e., the mortal germline phenotype (Simon et al., 2014), but most piRNAs do not target transposons (Batista et al., 2008; Das et al., 2008; Ruby et al., 2006). Moreover, piRNAs can target with imperfect base-pairing (Bagijn et al., 2012; Goh et al., 2015; Gou et al., 2014; Lee et al., 2012), which dramatically increases their targeting potential. It seems reasonable therefore that transposon silencing is not the only way that piRNAs help ensure a fit germline and appropriate developmental outcomes.

Indeed, piRNA pathways have been linked to epigenetic programming, genome rearrangement, and developmental regulation in diverse organisms (Ross et al., 2014). For example, a Piwi-related Argonaute is required in the totipotent neoblast cells of planarians for the regeneration of body parts (Reddien et al., 2005). In ciliates, Piwi orthologs and parental piRNAs regulate DNA elimination during development of the new somatic macronucleus (Fang et al., 2012). The *C. elegans* Piwi protein, PRG-1, initiates heritable silencing of foreign genes, such as single-copy transgenes containing *gfp* sequences. Remarkably, PRG-1 is responsible for generating secondary small RNAs at a number of endogenous mRNAs (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012). The physiological relevance—if any—of this regulation has not been explored.

Here we show that a piRNA gene located on the X-chromosome, *21ux-1* (21U-RNA on the X chromosome), is complementary to sequences within the open-reading frame of the *xol-1* mRNA and functions to repress *xol-1* expression in the hermaphrodite germline. *xol-1* encodes a GHMP (Galacto, Homoserine, Mevalonate and Phosphomevalonate) kinase homolog that specifies male(XO) development when active and hermaphrodite(XX)

development when repressed (Luz et al., 2003; Miller et al., 1988; Rhind et al., 1995). Consistent with the idea that *2lux-1* promotes robust regulation of *xol-1*, we show that *2lux-1* mutations dramatically enhance the dosage compensation and sexual transformation phenotypes of animals with mutations in *sex-1* or *sdsc-2*. Moreover, these synergistic phenotypes are strongly suppressed upon loss of *xol-1*. We show *2lux-1* acts maternally to ensure the robust sensitivity of zygotes to the dose of X-signal elements. Furthermore, we show that the piRNA pathway regulates the expression of the *xol-1* ortholog in *C. briggsae*, a related nematode that separated from *C. elegans* 50–100 million years ago. Together, our findings provide evidence that piRNA-mediated mRNA surveillance has physiological consequences for the proper regulation of embryonic development across *Caenorhabditis* species.

RESULTS

***2lux-1* targets *xol-1* and represses its expression**

The X chromosome-derived piRNA locus *2lux-1* produces the single most abundant piRNA, yet its function is unknown (Gu et al., 2012). piRNA targeting recruits RdRP (RNA-dependent RNA polymerase) to generate secondary small RNAs termed 22G-RNAs that guide silencing by members of an expanded group of WAGOs (Worm Argonautes) (Bagijn et al., 2012; Lee et al., 2012). A parallel study (Seth et al., co-submitted), reprogrammed the *2lux-1* locus to target *gfp*, i.e., *2lux-1(anti-gfp)* using CRISPR/CAS9 genome editing. To identify candidate targets for *2lux-1* piRNA, we examined this *2lux-1(anti-gfp)* strain for endogenous genes whose levels of PRG-1-dependent 22G-RNAs were reduced. This analysis revealed sixteen genes, including the well-studied gene *xol-1* (Figures 1A and S1A). These findings were confirmed in our analysis of a second *2lux-1* allele that completely deletes the *2lux-1* piRNA, *2lux-1(deletion)* (Figure 1A). A search of the *xol-1* mRNA revealed a putative *2lux-1* target site that could base-pair perfectly with positions 2 to 15 of the piRNA (Figure 1A).

Based on published deep-sequencing data we deduced that the *xol-1*-targeted 22G-RNAs are dependent on both the PRG-1 and WAGO pathways. For example, mutations that disrupt these pathways including *prg-1* (Piwi), *rde-3* (putative polynucleotide polymerase required for 22G-RNA accumulation) (Chen et al., 2005), and the 12 *wago* genes (*mag-12*) (Gu et al., 2009; Yigit et al., 2006), led to significant reduction of *xol-1* 22G-RNAs. Moreover, these 22G-RNAs were enriched in the HRDE-1/WAGO-9 immunoprecipitation (Figure 1A; Buckley et al., 2012; Shirayama et al., 2012).

Because the PRG-1 and WAGO pathways typically silence their targets (Ashe et al., 2012; Bagijn et al., 2012; Gu et al., 2009; Lee et al., 2012; Shirayama et al., 2012), we measured *xol-1* mRNA levels in mutants that disrupt these pathways. In *2lux-1*, *prg-1*, and *rde-3* mutants, *xol-1* mRNA levels were increased by 2- to 3-fold (Figure 1B). Developmental analysis of these mutants revealed that *xol-1* mRNA levels were elevated by the L4 larval stage (before embryos are formed) and persisted throughout adulthood in hermaphrodites (Figure 1B). Thus, *2lux-1* along with piRNA pathway components appears to repress *xol-1* mRNA expression in the L4 and adult germline.

To examine XOL-1 protein expression, we engineered the *xol-1* locus to express a GFP::FLAG::XOL-1 fusion protein. In an otherwise wild-type background, *gfp::flag::xol-1* hermaphrodites rarely produce male offspring, and we did not observe GFP fluorescence in embryos (Figure 1C; Data not shown). In a *him-8* (high incidence of males) background, *gfp::flag::xol-1* hermaphrodites produced male progeny at the expected frequency (~40%; Figure S1B) (Phillips et al., 2005), indicating that the GFP::FLAG::XOL-1 fusion protein is functional. Moreover, GFP fluorescence was readily detected in some embryos (Figure S1C), as expected for XO-specific expression of XOL-1 (Rhind et al., 1995).

In *21ux-1(deletion)* hermaphrodites, GFP fluorescence was detected in the oocytes and in early embryo (Figure 1C), and *gfp::flag::xol-1* mRNA was derepressed (Figure 1B). Furthermore, GFP::FLAG::XOL-1 fusion protein levels as measured by western blotting were significantly increased at both the L4 and adult stages (Figure 1D). The re-specified *21ux-1(anti-gfp)*, which can silence a GFP transgene (Seth et al., co-submitted), was sufficient to silence *gfp::flag::xol-1* expression (Figures 1B–D). Together, these data indicate that a single piRNA, *21ux-1*, directly regulates *xol-1* mRNA and protein expression in the hermaphrodite germline.

***21ux-1* promotes dosage compensation and sex-determination**

xol-1 negatively regulates the activity of the hermaphrodite-specific *sdc* (sex determination and dosage compensation) genes (Dawes et al., 1999; Strome et al., 2014). Activation of *xol-1* is thus essential for male development, but dispensable for hermaphrodite development (Miller et al., 1988; Rhind et al., 1995). Hermaphrodites with abnormally elevated *xol-1* expression exhibit a range of phenotypes that include embryonic and larval death, partial sexual transformation (psuedomale formation), abnormal shortened body morphology (dumpy), and vulval and egg-laying defects (Carmi et al., 1998; Miller et al., 1988; Rhind et al., 1995). Although XOL-1 expression was increased in *21ux-1* and piRNA pathway mutants, these single mutants showed at most a mild reduction in hermaphrodite viability (Figure 2A and Table 1).

X-signal elements (XSEs) that count the X-chromosome-to-autosome ratio and repress *xol-1* are partially redundant, and mutations in multiple XSEs are required to fully activate *xol-1* (Carmi et al., 1998; Farboud et al., 2013; Gladden and Meyer, 2007; Hodgkin et al., 1994; Nicoll et al., 1997; Skipper et al., 1999). We therefore tested whether the piRNA pathway acts redundantly with XSEs to repress *xol-1* expression. The nuclear hormone receptor SEX-1, for example, directly binds to the *xol-1* promoter and represses *xol-1* transcription (Carmi et al., 1998; Farboud et al., 2013). As previously reported (Carmi et al., 1998), depleting *sex-1* via RNA interference (RNAi) or loss-of-function mutations reduced hermaphrodite viability by 50% to 60% (Figure 2A and Table 1), with survivors showing dumpy, egg-laying defective, and masculinized phenotypes (Table 1). Strikingly, simultaneous depletion of *sex-1* in *prg-1*, *rde-3*, or *21ux-1* mutants led to nearly 100% lethality in hermaphrodites (Figure 2A and Table 1). Moreover, the XX survivors showed strong sexual transformations, including male-like tail morphogenesis, and became very dumpy (Figures 2B and 2C). The *21ux-1(deletion)* and *21ux-1(anti-gfp)* alleles exhibited

identical synthetic phenotypes when combined with *sex-1*, in the context of a wild-type *xol-1* locus (Table 1).

Since *21ux-1* targets mRNAs other than *xol-1*, the synthetic phenotypes we observed could result from misregulation of one or more of these other targets. We showed above that the re-specified *21ux-1(anti-gfp)* piRNA can repress the *gfp::flag::xol-1* allele, but it should not repress other *21ux-1* targets. In the presence of endogenous *xol-1*, *sex-1(RNAi)* is synthetic lethal with both *21ux-1(deletion)* and *21ux-1(anti-gfp)* alleles. In the *gfp::flag::xol-1* background, however, *sex-1(RNAi)* was still strongly synthetic lethal with the *21ux-1(deletion)* but was no longer synthetic lethal with *21ux-1(anti-gfp)* (Table 1). Thus, *21ux-1(anti-gfp)* mediates functional repression of *gfp::flag::xol-1* by targeting the *gfp* sequences of the fusion gene. These findings confirm that piRNA mediated regulation of *xol-1* function is important for robust dosage compensation and sex determination.

The above findings suggest that *21ux-1* and *sex-1* repress *xol-1* expression independently, or in parallel. Consistent with this idea, we found that *xol-1* loss-of-function mutations partially rescued the viability of *21ux-1 sex-1* double mutant animals (Table 2). The degree of rescue was comparable to the levels observed in previously described *xol-1 sex-1* double mutant strains (Gladden et al., 2007).

To further explore the relationship between *21ux-1* and *xol-1* expression, we examined XX lethality in combination with *sdc-2* depletion. XOL-1 down-regulates dosage compensation genes, such as *sdc-2* (Dawes et al., 1999). Hermaphrodites with a complete loss of *sdc-2* function are inviable (Dawes et al., 1999; Gladden et al., 2007). However, RNAi-mediated depletion of *sdc-2* caused an intermediate phenotype, reducing XX viability to ~78% (Table 1), which allowed us to explore potential synthetic interactions between *sdc-2(RNAi)* and *21ux-1*. As previously shown (Gladden et al., 2007), we found that *sdc-2(RNAi)* in a *sex-1* null mutant results in a near complete loss of hermaphrodite viability (to ~1%, Table 1). Similarly, *sdc-2(RNAi)* in *21ux-1* mutants caused a marked loss of viability (to ~18%, Table 1). For both double mutants, the survivors showed a high incidence of sexual-transformation and body morphological defects (Table 1). If *21ux-1* functions through *xol-1*, then mutations in *xol-1* should suppress the synergistic lethality caused by the combination of *21ux-1* and *sdc-2 (RNAi)*. Indeed, loss of *xol-1* activity strongly suppressed the synthetic lethality caused by loss of both *21ux-1* and *sdc-2(RNAi)* (Table 2). Together these findings suggest *21ux-1* acts directly on *xol-1* to regulate dosage compensation and sex determination.

21ux-1 has a maternal effect

PRG-1 and piRNAs are expressed in the maternal and paternal germlines (Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008), while *xol-1* levels are thought to be regulated in response to X-signal elements in the early embryo. We therefore explored the parental and zygotic roles of *21ux-1* expression in dosage compensation and sex determination. Mating *sex-1* single-mutant males to homozygous *21ux-1 sex-1* double-mutant hermaphrodites failed to rescue the viability of the resulting *21ux-1/+ sex-1* hermaphrodite progeny, indicating that paternal and zygotic expression of *21ux-1* are insufficient to rescue viability (Figures 3A and S2). Conversely, hermaphrodites homozygous for *sex-1* and heterozygous for *21ux-1* (*21ux-1/+ sex-1/-*) produced viable

2lux-1^{-/-} self-progeny at the same frequency as *2lux-1*^{+/+} self-progeny (Figures 3B and S2). Thus, maternal expression of *2lux-1* is necessary and sufficient for the viability of homozygous *2lux-1 sex-1* progeny.

Finally, we mated wild-type males to homozygous *2lux-1 sex-1* hermaphrodites to generate hemizygous *2lux-1 sex-1* male and heterozygous *2lux-1/+ sex-1/+* hermaphrodite progeny. As expected, we found that *sex-1* and *2lux-1* activities were not essential for male viability or development (Figure S3A). By contrast, we observed a partial rescue among the XX progeny from this cross presumably due to zygotic expression of *sex-1*, but we observed significantly fewer viable (*2lux-1/+ sex-1/+*) XX progeny relative to XO progeny (Figure S3B). This latter result suggests that both *2lux-1* and *sex-1* may contribute maternally to the viability of hermaphrodite progeny, or that the reduction in maternal *2lux-1* uncovers a zygotic haploinsufficiency for *sex-1*. Consistent with the former possibility, a previous report has shown that SEX-1 activity is contributed in part maternally (Carmi et al., 1998; Gladden et al., 2007). Taken together, these findings suggest that *2lux-1* is required maternally for robust *xol-1* regulation and for the proper development of hermaphrodite progeny.

piRNA regulation of *xol-1* is conserved in the related nematode *C. briggsae*

We wished to study if piRNAs have conserved functions in regulating dosage compensation and sex determination in other *Caenorhabditis* species. *C. briggsae* is an ideal model for this comparative study as its whole genome is sequenced and well annotated (Stein et al., 2003). *C. briggsae* and *C. elegans* are thought to have separated from a common ancestor 50~100 million years ago (Coghlan and Wolfe, 2002). Both species have the same chromosome numbers, similar genome sizes, a large number of orthologous protein coding genes (Stein et al., 2003). Yet sex-related genes and genetic pathways have rapidly evolved across *Caenorhabditis* species (Civetta and Singh, 1998; Haag et al., 2002).

The *C. elegans xol-1* ortholog has been identified in *C. briggsae*. Structural and genetic analysis has suggested that it could function as a regulator of sexual development and dosage compensation in *C. briggsae* (Luz et al., 2003). To the best of our knowledge, factors acting upstream of *xol-1* are completely unknown in *C. briggsae*. Although we could not identify a homolog of *2lux-1*, *C. briggsae* is known to express a prg-1/Piwi homolog, RdRP and an array of WAGOs (Shi et al., 2013). To ask if *C. briggsae xol-1* is regulated by piRNAs, we mutated the *C. briggsae Piwi* gene and monitored 22G-RNAs genome-wide. We found that 22G-RNAs targeting *xol-1* mRNA were greatly diminished in the Piwi mutants (Figure 3C). Furthermore, we found that the *xol-1* transcript level was increased by 3- to 4- fold in the mutant animals at the stage of L4 larvae and adult. Thus in both *C. elegans* and *C. briggsae* the piRNA pathway functions to repress *xol-1* expression in the germline (Figure 3D).

DISCUSSION

The piRNA pathway is often discussed in the context of transposon and transgene suppression. Our findings identify a physiological role for a piRNA in regulating an endogenous gene in nematode. Specifically, we have shown that an X-chromosome piRNA regulates the maternal level of *xol-1* mRNA and protein. *xol-1* encodes a master regulator of

dosage compensation and sex determination (Miller et al., 1988; Rhind et al., 1995). The *2lux-1* piRNA acts along with other maternally contributed factors, including SEX-1 mRNA or protein, to establish a delicate balance of XOL-1 and its regulators, such that X-signal elements can robustly specify sexual fates in response to the karyotype of XX and XO progeny (Figure 3E). This remarkably subtle, but very pathway-specific function of *2lux-1*, demonstrates that a piRNA can confer physiologically important regulation on a germline mRNA.

piRNAs in gene regulation

The piRNA pathway is thought to initiate silencing by recruiting RdRP which templates 22G-RNA synthesis directly from the target mRNA. These 22G-RNAs are loaded onto WAGOs and deposited into mature gametes. In concert with chromatin modifiers, WAGOs/22G-RNA complexes can maintain transgenerational gene silencing independently of the Piwi pathway (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012). Nevertheless, Seth et al. (co-submitted) have shown that some transgenes require both the WAGO and Piwi pathways to maintain silencing, and that full repression of transgenes can be piRNA-dependent. The *xol-1* locus provides an example of an endogenous gene that depends on both WAGO and Piwi pathways for its regulation.

Because of the extreme diversity of piRNA sequences and their ability to recognize targets with partial complementarity (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012), piRNAs should, in principal, target all germline transcripts. This notion has led to the proposal that endogenous mRNAs are protected (at least partially) by other mechanisms (See Discussion Below, Shirayama et al., 2012; Wedeles et al. 2013; Seth et al., 2013). Conceivably, over evolutionary time, germline mRNAs sample piRNA targeting through random nucleotide changes that strengthen or weaken base-pairing. Some piRNA-target interactions might lead to stable silencing (as seen for most transposons and some protein coding genes), while other interactions—e.g., between the *2lux-1* piRNA and the *xol-1* mRNA—may lead to partial down-regulation. The degree of targeting and silencing at each transcript could in turn provide a source of heritable variation upon which natural selection can act.

It is interesting that *xol-1* is not completely or permanently silenced upon piRNA targeting. Although the details are not understood, it is clear that mechanisms exist that oppose piRNA-induced silencing. At the DNA level, A/T rich clusters have been shown to counteract silencing and to promote *C. elegans* germline gene expression (Frokjaer-Jensen et al., 2016). At the RNA level, the CSR-1 Argonaute has been shown to promote the transitive activation of a silent transgene, and has been proposed to protect mRNAs from piRNA-mediated silencing (Seth et al., 2013; Wedeles et al., 2013). The *csr-1* gene is essential for fertility and viability, and *csr-1* mutants exhibit changes in the levels of many germline mRNAs (Claycomb et al., 2009; Gerson-Gurwitz et al., 2016; Yigit et al., 2006). Indeed, a recent study proposed that CSR-1 tunes germline gene expression broadly (Gerson-Gurwitz et al., 2016). The emerging picture from our studies and others is that there are many influences on germline gene expression and that these are likely to include, as yet, entirely undiscovered pathways. The present findings and those of Seth et al., (co-submitted) suggest

that variations in the level of piRNA-target complementarity—within the context of other mechanisms that govern sensitivity/resistance to piRNA silencing—provides a rich regulatory milieu within which Argonautes have the potential to broadly shape (or tune) the profile of germline gene expression.

Non-coding RNAs in dosage compensation and sex determination

A role for *2lux-1* in dosage compensation and sex determination provides yet another example of an independently evolved non-coding RNA that functions to equalize sex-chromosome gene expression (Amrein and Axel, 1997; Brockdorff et al., 1992; Brown et al., 1992; Meller et al., 1997). McJunkin and Ambros (2017) recently showed that the *mir-35* microRNA family exerts maternal control of sex determination in the early *C. elegans* embryo (McJunkin and Ambros, 2017). Thus, multiple small RNA pathways contribute to sex determination in *C. elegans*.

The regulation of *xol-1* by *2lux-1* exhibits striking parallels to the likely independently evolved relationship between the *Masculinizer* (*Masc*) gene and the feminizing (*Fem*) piRNA in the Lepidopteran *Bombyx mori* (Kiuchi et al., 2014). *Fem* originates from the W chromosome and specifies female (ZW) development by silencing the Z-derived *Masculinizer* (*Masc*) mRNA. *Masc* encodes a CCCH-type zinc finger protein that controls dosage compensation and sex determination in *Bombyx mori* males (ZZ) (Kiuchi et al., 2014). Both the nematode and insect *B. mori*—separated by at least 700-million years of evolution—use piRNAs to regulate this dose-sensitive developmental mechanism, providing further support for the idea that piRNAs play important physiological roles in gene regulation in animals.

STAR★METHODS

• CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Craig C. Mello (Craig.Melloumassmed.edu)

• EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans—The Bristol strain N2 was designated as the wild-type *C. elegans* strain. Other strains used in this study are listed in Table S1 and KEY RESOURCES TABLE. The *C. elegans* and *C. briggsae* strains were maintained with E. coli OP50 as diet on Nematode Growth Media (NGM) (Brenner, 1974). All animals were maintained at 20 °C unless otherwise indicated.

METHOD DETAILS

Generation of transgenic strains by CRISPR/CAS9—*2lux-1* (*anti-gfp*) and *C. briggsae* piwi mutants were generated by a co-CRISPR strategy and *unc-22* sgRNA served as a co-injection marker to enrich CRISPR/CAS9-mediated genome editing events (Kim et al., 2014). *2lux-1* deletion, *xol-1* with *N terminal gfp::2XFlag::auxin-based degron sequences*, *2lux-1 sex-1* double, and *2lux-1 xol-1 sex-1* triple mutant strains were generated using CRISPR/CAS9 ribonucleoprotein complexes (Paix et al., 2015). The transgenic strains

made by CRISPR/CAS9 are listed in Table S1. The vector pRF4 expressing *rol-6* (*su1006*), a dominant allele conferring a roller phenotype, served as a co-injection marker.

Scoring viability and masculinized animals—Five gravid hermaphrodites (wild-type and mutants) were placed on individual plate and allowed to lay embryos for six to eight hours. The total number of eggs was scored. Surviving animals were counted in a period of 4 days at room temperature. Survivors that failed to reach L4 stage were considered inviable.

The HT115 RNAi feeding strains (*sex-1* and *sdc-2*) were picked from the *C. elegans* RNAi Collection (Source BioScience). All RNAi experiments were performed as described previously (Kamath et al., 2003). Specifically, synchronized L1 animals were placed on plates spotted with corresponding RNAi strains. Five gravid adults were transferred onto fresh RNAi plates, eggs were counted, and survivors were scored as described above. The percentage of masculinized animals was calculated as the ratio of total number of pseudomale to total number of surviving animals.

Small RNA cloning—Total RNAs were isolated from adult worms using TRI Reagent (Molecular Research Center). Small RNAs were enriched using MirVana Kit (Thermo Fisher Scientific). Samples from wild-type and mutants were pretreated with a homemade 5' Polyphosphatase or RNA 5-Polyphosphatase (Lucigen) and were ligated to the miRNA cloning linker 1 (5' rAppCTGTAGGCACCATCAAT/3ddC/3', IDT). The 5' linkers containing 6 nt barcode were ligated using T4 RNA ligase (NEB). The ligated products were converted to cDNA using SuperScript III (Thermo Fisher Scientific). Libraries were amplified by PCR and sequenced using the HiSeq systems (Illumina) at the UMass Medical School Deep Sequencing facility.

Data analysis—Small RNA sequencing data were analyzed using a reported pipeline (Gu et al., 2012). Briefly, sequencing reads were first sorted according to barcode sequences, and both 5' and 3' adaptor sequences were removed. Reads starting with G/A and 21~23 nt in length were mapped to the reference genome by allowing at most two mismatches. To account for differences in sequencing depth between samples, reads were normalized to library size. Normalized counts were visualized in the UCSC genome browser. All scripts are available upon request.

Quantitative RT-PCR—The reverse transcription (RT) was performed using antisense primers by SuperScript III (Thermo Fisher Scientific). RT reaction was conducted in at least triplicate-reactions. Real-time PCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific). All primers are listed in KEY RESOURCES TABLE. Statistical analysis was performed in Microsoft Excel. Error bars in the graph represent the standard deviation (SD).

Western blotting analysis—Cell lysate were prepared from synchronized population of L4 larvae and gravid adults. 50 µg lysate were loaded onto the precast polyacrylamide gel (Thermo Fisher Scientific), subjected to electrophoresis, and transferred onto the PVDF membrane (Bio-Rad) with Trans-Blot Turbo Transfer System (Bio-Rad). The primary antibodies used were anti-FLAG (M2, Sigma) and anti-alpha-Tubulin (Bio-Rad). The

secondary antibodies were goat-anti-mouse HRP (Abcam) and goat-anti-rat HRP (Abcam) respectively.

Imaging and Microscopy—To collect DIC and epifluorescence images, wild-type and mutant animals were first treated with 0.2 mM levamisole and mounted on the RITE-ON glass slide (Becton, Dickinson and Company). The Axioplan2 Microscope (Zeiss) was used to collect the images and images were processed using AxioVision software (Zeiss).

QUANTIFICATION AND STATISTICAL ANALYSIS

Genetic crosses and qRT-PCR experiments were performed as independent biological replicates and the number of replicates was shown in the figure legends. All statistical analysis was performed with GraphPad Prism 7 or Microsoft Excel. Standard Student's t-test was conducted. p-values < 0.05 were considered to be significant.

DATA AND SOFTWARE AVAILABILITY

HRDE-1/WAGO-9 IP data are available from GEO (Gene Expression Omnibus) under the accession number GSE38724. Small RNA sequencing data from *rde-3* and *mago-12* mutants are available under accession number GSE18215. Small RNA sequencing data from *prg-1* mutants are under the accession number GSE38723. Small RNA sequencing data from wild-type, *21ux-1* mutants, *C. briggsae* wild-type, and piwi mutants are available under PRJNA398958.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- An X-linked piRNA, *2lux-1*, targets *xol-1* and represses its expression in *C. elegans*
- *2lux-1* acts with *sex-1* to promote dosage compensation and sex determination
- The piRNA pathway regulates the expression of the *xol-1* ortholog in *C. briggsae*

control. Data were collected from three independent biological replicates. Error bars represent Standard Deviation.

(C) Representative Differential Interference Contrast (DIC) image and fluorescence micrographs showing GFP::FLAG::XOL-1 expression (green) *from wild-type* and *2lux-1(deletion)* animals. The dashed lines outline the position of the germline. Bright signals detected outside of the germline are from autofluorescence of gut granules.

(D) Western blotting analysis of proteins isolated from *gfp::flag::xol-1* and *2lux-1(deletion)* *gfp::flag::xol-1* strains at late L4 larvae and Adult stages. The anti-FLAG antibody (top) detecting GFP::FLAG::XOL-1 and anti-tubulin antibody (bottom) were used. An asterisk marks a non-specific band.

Figure 2. *21ux-1* acts with *sex-1* to regulate dosage compensation and sex determination
(A and B) Percentage of XX viability and masculinized XX animals in wild-type, *21ux-1(anti-gfp)*, *sex-1(gm41)*, *prg-1; sex-1(gm41)*, *21ux-1(anti-gfp) sex-1(ne4473)* and *21ux-1(anti-gfp) sex-1(ne4474)* mutant strains. n. Total number of animal scored.
(C) Representative DIC imaging of wild-type XX, *21ux-1 sex-1* XX, wild-type XO and surviving *21ux-1 sex-1* masculinized XX animals. An asterisk marks a masculinized tail. Arrows mark the vulva.

Figure 3. Maternal expression of *21ux-1* contributes to dosage compensation and sexual development and piRNAs regulate *xol-1* expression in *C. briggsae*

(A) Viability of *21ux-1(m-/-; z+/-) sex-1(m-/-; z-/-)* was calculated as the ratio of viable hermaphrodites to viable males as equal numbers of males and hermaphrodites are expected. Viability of *21ux-1(m-/-; z-/-) sex-1(m-/-; z-/-)* was calculated as the ratio of viable adults to the total number of embryos. The genetic crosses are illustrated in Figure S2. Data were collected from four independent crosses. Error bars represent Standard Deviation. m, maternal expression; z: zygotic expression. Data were collected from three independent experiments. Error bars represent Standard Deviation. n.s. Not Significant.

(B) Number of *2lux-1(m+/-; z-/-) sex-1(m-/-; z-/-)*, *2lux-1(m+/-; z+/-) sex-1(m-/-; z-/-)*, and *2lux-1(m+/-; z+/+) sex-1(m-/-; z-/-)* progeny produced from *2lux-1+/- sex-1-/-* hermaphrodites detected by sequence-based genotyping. The genetic crosses are illustrated in Figure S2.

(C) 22G-RNAs mapped to *xol-1* locus in isogenic *C. briggsae* wild-type and Piwi mutant strains are illustrated. The histograms show the position and relative abundance of 22G-RNAs.

(D) Relative *xol-1* mRNA level at L4 larvae and Adult stages as measured by qRT-PCR. Expression of actin mRNA (*act-5*) served as the internal control. Data were collected from six independent biological replicates. Error bars represent Standard Deviation.

(E) Model illustrating the function of *2lux-1* and *sex-1* in regulating *xol-1* expression and promoting robust dosage compensation and sex determination in *C. elegans*.

Table 1

The piRNA pathway functions in sex determination and dosage compensation.

Genotype	Hermaphrodite viability (%) (Number of Animal Scored)	Phenotype of Survivors
<i>wild-type</i>	100.4% (1319)	Wild-type
<i>2lux-1(anti-gfp)</i>	98.0% (755)	Slightly Tra
<i>prg-1(tm872)</i>	92.6% (894)	Reduced fertility
<i>rde-3(ne3370)</i>	78.5% (503)	Reduced fertility
<i>sex-1(gm41)</i>	40.1% (764)	Dpy; Tra; Egl
<i>2lux-1(anti-gfp) sex-1(ne4473)</i>	2.6% (936)	Very Dpy; Tra; Egl
<i>2lux-1(anti-gfp) sex-1(ne4474)</i>	2.9% (729)	Very Dpy; Tra; Egl
<i>prg-1(tm872); sex-1(gm41)</i>	2.4% (382)	Very Dpy; Tra; Egl
<i>rde-3(ne3370); sex-1(gm41)</i>	1.4% (147)	Dpy; Tra; Egl; Sterile
<i>sex-1(RNAi)</i>	50.9% (601)	Dpy; Tra; Egl
<i>prg-1(tm872); sex-1(RNAi)</i>	3.2% (755)	Very Dpy; Tra; Egl
<i>2lux-1(anti-gfp) sex-1(RNAi)</i>	5.9% (768)	Very Dpy; Tra; Egl
<i>2lux-1(deletion) sex-1(RNAi)</i>	6.5% (417)	Very Dpy; Tra; Egl
<i>2lux-1(anti-gfp) sex-1 (RNAi) gfp::flag::xol-1</i>	52.9% (223)	Dpy; Tra; Egl
<i>2lux-1(deletion) sex-1 (RNAi) gfp::flag::xol-1</i>	6.2% (350)	Very Dpy; Tra; Egl
<i>sdc-2(RNAi)</i>	78.3% (811)	Dpy; Tra; Egl
<i>2lux-1(deletion) sdc-2(RNAi)</i>	18.1% (341)	Dpy; Tra; Egl
<i>2lux-1(anti-gfp) sdc-2(RNAi)</i>	15.8% (939)	Dpy; Tra; Egl
<i>sex-1(gm41); sdc-2(RNAi)</i>	1.3% (682)	Very Dpy; Tra; Egl

Hermaphrodite viability and phenotype of survivors were scored. RNAi experiments were carried out by feeding worms bacteria expressing dsRNA. Dpy: Dumpy. Tra: Transformer. Egl: Egg-laying defective.

Table 2

2lux-1 acts upstream of *xol-1*.

Genotype	Hermaphrodite viability (%) (Number of Animal Scored)
<i>xol-1(y9)</i>	96.0% (1001)
<i>xol-1(ne4472)</i>	94.6% (835)
<i>2lux-1 sex-1(ne4473)</i> [#]	2.6% (936)
<i>2lux-1 sex-1(ne4474)</i> [#]	2.9% (729)
<i>2lux-1 xol-1(ne4475) sex-1(ne4476)</i>	72.1% (970)
<i>2lux-1 xol-1(ne4475) sex-1(ne4477)</i>	75.8% (959)
<i>sdc-2(RNAi)</i>	77.5% (614)
<i>xol-1(y9) sdc-2(RNAi)</i>	94.3% (703)
<i>2lux-1 sdc-2(RNAi)</i>	15.2% (487)
<i>2lux-1 xol-1(ne4475) sdc-2(RNAi)</i>	91.2% (591)

Viability of progeny from mutant animals was calculated.

[#]The same dataset from Table 1.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Flag	Sigma-Aldrich	Cat#F1804-200UG
Rat polyclonal anti-alpha-Tubulin	Bio-Rad	Cat#VMA00051
Goat Anti-Mouse IgG H&L (HRP)	Abcam	Cat#ab6789
Goat Anti-Rat IgG H&L (HRP)	Abcam	Cat#ab97057
Bacterial and Virus Strains		
Bacteria: OP50	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/OP50
Bacteria: HT115	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/HT115(DE3)
Chemicals, Peptides, and Recombinant Proteins		
CAS9 protein	Integrated DNA Technologies	Cat#1074181
TRI Reagent	Molecular Research Center	Cat#TR118
Critical Commercial Assays		
mirVana miRNA Isolation Kit	Thermo Fisher Scientific	Cat#AM1560
SYBR Green PCR Master Mix	Thermo Fisher Scientific	Cat#4309155
SuperScript IIIReverse Transcriptase	Thermo Fisher Scientific	Cat#18080093
Ex TaqDNA Polymerase	TaKaRa	Cat#RR001C
RNA 5' Polyphosphatase	Lucigen	Cat#RP8092H
Deposited Data		
Small RNA sequencing data from HRDE-1/WAGO-9 IP	Shirayama et al., 2012	GSE38724
Small RNA sequencing data from rde-3 and mago-12 mutants	Gu et al., 2009	GSE18215
Small RNA sequencing data from prg-1 mutants	Lee et al., 2012	GSE38723
Small RNA sequencing data from wild-type and 2 lux-1 mutants	This study	PRJNA398958
Small RNA sequencing data C briggsae wild-type and piwi mutants	This study	PRJNA398958
Experimental Models: Organisms/Strains		
C. elegans: N2 Bristol	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/N2
C. elegans: TY1807	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/TY1807
C. elegans: NG41	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/NG41

REAGENT or RESOURCE	SOURCE	IDENTIFIER
C. elegans:WM161	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/WM161
C. elegans:WM191	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/WM191
C. elegans:CB1489	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/CB1489
C. briggsae:AF16	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/AF16
C. elegans:WM286	Caenorhabditis Genetics Center	https://cgc.umn.edu/strain/WM286
Additional C. elegans strains in this study are listed in Supplemental Table 1	This paper	Supplemental Table 1
Oligonucleotides		
miRNA cloning linker 1: 5' rAppCTGTAGGCACCATCAAT/3ddC/ 3'	Integrated DNA Technologies	N/A
xol-1 (C. elegans)_sense: GGAGCGAAACAGTCCAGTC	Integrated DNA Technologies	N/A
xol-1 (C. elegans)_antisense: ATCATGTGCATGCTTTCGGTA	Integrated DNA Technologies	N/A
act-3 (C. elegans)_sense: GGCCCAATCCAAAGAGAGGTATCC	Integrated DNA Technologies	N/A
act-3 (C. elegans)_antisense: GGGCAACACGAAGCTCATTGTA	Integrated DNA Technologies	N/A
xol-1 (C. briggsae)_sense: GACCACCCATATCGCAAAACG	Integrated DNA Technologies	N/A
xol-1 (C. briggsae)_antisense: TCAACACCCTCGCAAAACCCAG	Integrated DNA Technologies	N/A
act-5 (C. briggsae)_sense: AGCGTGGATACACCTTCACC	Integrated DNA Technologies	N/A
act-5 (C. briggsae)_antisense:AATGAAAAGCTGGCTGGAAGA	Integrated DNA Technologies	N/A
Recombinant DNA		
Ahringer C. elegans RNAi library: RNAi control plasmid: pL4440	Source BioScience	http://www.sourcebioscience.com/products/life-science-research/clones/mai-resources/c-elegans-mai-collection-ahhringer/
Ahringer C. elegans RNAi library: RNAi sex-1 knockdown: pL4440-sex-1	Source BioScience	http://www.sourcebioscience.com/products/life-science-research/clones/mai-resources/c-elegans-mai-collection-ahhringer/
Ahringer C. elegans RNAi library: RNAi sdc-2 knockdown: pL4440-sdc-2	Source BioScience	http://www.sourcebioscience.com/products/life-science-research/clones/mai-resources/c-elegans-mai-collection-ahhringer/
pRF4	Mello CC et al., 1991	https://www.addgene.org/vector-database/7880/
Software and Algorithms		
Bowtie	Langmead et al., 2009	http://bowtie-bio.sourceforge.net/index.shtml
GraphPad Prism 6	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/
Perl	The Perl Foundation	https://www.perl.org/