DOT1L suppresses nuclear RNAi originating from enhancer elements in *Caenorhabditis elegans*

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Summary

Methylation of histone H3 lysine 79 (H3K79) by DOT1L is associated with actively transcribed genes. We have previously demonstrated that DOT-1.1, the *Caenorhabditis elegans* DOT1L homologue, cooperates with the chromatin-binding protein ZFP-1 (homologue of the mammalian AF10) to negatively modulate transcription of highly and widely expressed target genes. Also, *zfp-1* deficiency has been associated with reduced RNA interference (RNAi) response. In addition, in MLL-rearranged leukemia, H3K79 methylation at homeobox genes was shown to antagonize repressive chromatin marks. Here, we connect these various manifestations of DOT1L function. We demonstrate that DOT1L suppresses transcription at enhancers, thereby preventing double-stranded RNA (dsRNA) formation and ectopic histone H3 lysine 9 (H3K9) methylation. Moreover, we show that lethality of *dot-1.1* mutant worms is suppressed by mutations in the RNAi pathway, suggesting ectopic activation of endogenous RNAi in the absence of DOT1L. Since DOT1L is conserved and similarly prevents H3K9 methylation in *C. elegans* and

mammals, our observations point to a possible link between nuclear RNAi and H3K9 methylation in higher organisms.

Introduction

Screens for chromatin-binding factors involved in RNAi in *C. elegans* have identified the zinc finger protein ZFP-1 as a putative mediator of dsRNA-induced silencing in the nucleus (Dudley et al., 2002; Grishok et al., 2005; Kim et al., 2005). Interestingly, its mammalian homologue, AF10, is a frequent fusion partner of MLL in chimeric proteins causing leukemia (Meyer et al., 2018), and its interaction with H3K79 methyltransferase DOT1L is critical for DOT1L recruitment to the *HOXA9* and *MEIS* genes and their subsequent hyperactivation (Okada et al., 2005). In the attempt to identify proteins interacting with ZFP-1 that could explain its role in RNAi, we have previously identified a *C. elegans* homologue of DOT1L, which we named DOT-1.1, as the major ZFP-1 binding partner (Cecere et al., 2013).

Although DOT1L is the only H3K79 methyltransferase in mammals and H3K79 methylation is present on all actively transcribed genes, inhibition of DOT1L methyltransferase activity does not result in dramatic changes in gene expression in cultured cells (Zhu et al., 2018). In addition, expression of specific genes, such as *HOXA9* and *MEIS*, is strongly dependent on DOT1L, especially in leukemias induced by MLL-fusion proteins (Okada et al., 2005). The importance of H3K79 methylation in antagonizing heterochromatin formation at the *HOXA* cluster has recently been established (Chen et al., 2015), resembling pivotal studies in yeast showing that Dot1 prevents the spreading of factors associated with heterochromatin into active regions

(Katan-Khaykovich and Struhl, 2005; Ng et al., 2002). Importantly, knockout of DOT1L in mice results in embryonic lethality, underscoring its important developmental function. In *Drosophila*, both DOT1L (*Grappa*) and AF10 (*Alhambra*) mutants showed developmental defects (Mohan et al., 2010; Shanower et al., 2005). In *C. elegans*, we observed specific developmental abnormalities in a *zfp-1* loss-of-function mutant, such as defects in neuronal migration (Kennedy and Grishok, 2014), and implicated ZFP-1 in lifespan control (Mansisidor et al., 2011). Whether or not the anti-silencing effect of DOT1L contributes to its developmental role is presently not known.

We previously established that, in *C. elegans*, ZFP-1 and DOT-1.1 co-localize to promoters of highly and widely expressed genes and negatively modulate their expression during development (Cecere et al., 2013). This mechanism is likely conserved and applicable to the majority of ubiquitously expressed genes in other systems. Here, we demonstrate the role of the ZFP-1/DOT-1.1 complex in enhancer regulation. Known epigenetic signatures typical of enhancers include histone H3 lysine 27 acetylation (H3K27ac) and histone H3 lysine 4 monomethylation (H3K4me1) on active enhancers, whereas high level of H3K27me3 and absence of H3K27ac are found on enhancers poised for later tissue-specific expression (Bonn et al., 2012). Enhancer elements are themselves transcription units, generating non-coding enhancer RNAs (eRNAs) (Li et al., 2016), and, most recently, regulation of enhancer transcription was shown to be very similar to that of protein-coding genes (Henriques et al., 2018). Indeed, we show that DOT-1.1 negatively modulates enhancer RNA transcription, similarly to the negative modulation of active genes described earlier (Cecere et al., 2013). Moreover, we find an antagonistic relationship between H3K79 methylation and

H3K9 methylation at enhancers, a mechanism consistent with studies in leukemic cells (Chen et al., 2015). Finally, we demonstrate that suppression of anti-sense transcription by ZFP-1/DOT-1.1 prevents dsRNA formation and induction of nuclear RNAi via the Dicer/RDE-4/RDE-1 pathway, which likely promotes H3K9 methylation at enhancers in the absence of DOT-1.1. The elevation of endogenous dsRNA level is most likely responsible for the apparent deficiency of *zfp-1* mutant worms in responding to exogenous dsRNA. Based on these compelling observations, we propose that negative modulation of bidirectional transcription at enhancers by DOT1L does not allow dsRNA levels to reach the threshold required for inducing heterochromatin formation.

Results

ZFP-1 and DOT-1.1 Are Enriched at Enhancers

Recently, the *C. elegans* genome was organized into 20 domains of different structure and activity based on chromatin modification signatures (Evans et al., 2016). We intersected coordinates of such domains with ZFP-1 ChIP-seq (third larval stage, L3) and DOT-1.1 ChIP-chip (embryo) peaks to interrogate where the ZFP-1/DOT-1.1 complex is most dominant in the genome. More than 40% of promoter regions (domains 1 and 8 in embryos and domain 1 at L3) are enriched in ZFP-1/DOT-1.1 (Figure 1A), in line with our previous observations of promoter-proximal binding of the complex (Cecere et al., 2013). More interestingly, similar levels of enrichment are observed for domains corresponding to predicted intronic (domain 8 at L3) and intergenic enhancers (domain 9 in both embryos and L3 animals) (Figures 1A). These domains have chromatin signatures characteristic of enhancers, such as H3K4me1 and H3K27ac. In addition,

both promoters and enhancers are often devoid of nucleosomes and hence amenable to be identified by techniques assessing open chromatin regions. A recent study has identified such regions in *C. elegans* embryos and L3 animals by ATAC-seq. In this study, approximately 5000 distal non-coding regions displayed dynamic changes in chromatin accessibility between the two developmental stages. Also, several of these putative enhancers revealed enhancer activity in transgenic reporter assays (Daugherty et al., 2017). Interestingly, overlap analysis shows that both intragenic and distal ATAC-seq peaks are enriched in DOT-1.1 and ZFP-1 in embryos and L3 animals, respectively (Figure 1B). These observations raise the exciting possibility that, in addition to the previously disclosed role of ZFP-1/DOT-1.1 in the negative modulation of transcription via promoter-proximal binding, this complex may also control transcription through enhancers.

The ZFP-1/DOT-1.1 Complex Regulates Distal and Intragenic Enhancer Transcription

In light of the observation that enhancer elements in *C. elegans* are enriched in ZFP-1/DOT-1.1, we have hypothesized that this complex could control enhancer transcription. Distal enhancers often produce non-coding transcripts (eRNAs) which are typically unstable and not represented in the steady-state transcript population (Li et al., 2016). This technical limitation may be circumvented by using techniques designed to assess nascent transcription, such as the global run-on sequencing (GRO-seq) method (Hah et al., 2013; Lam et al., 2013). GRO-seq data previously obtained in our lab were instrumental in providing insight into the modulatory role of ZFP-1/DOT-1.1 in transcription of genes targeted by the complex at promoter-proximal regions (Cecere et

al., 2013). We re-analyzed our published data to gain a comprehensive understanding of the effect of ZFP-1/DOT1L on the regulatory genomic regions and chromatin domains revealed by recent studies (Daugherty et al., 2017; Evans et al., 2016). We quantified GRO-seq coverage at distal enhancers, defined by chromatin signatures (Evans et al., 2016), in wild-type (WT) and *zfp-1* mutant (*zfp-1(ok554)*) L3 animals. In this mutant, ZFP-1 is C-terminally truncated and does not interact with DOT-1.1, leading to decreased abundance of DOT-1.1 at chromatin (Cecere et al., 2013). Interestingly, both intragenic and distal eRNAs were globally increased in *zfp-1* mutant animals in comparison with WT larvae (Figure 1C). Analysis of GRO-seq data at distal ATAC-seq peaks also revealed a global increase in transcription *zfp-1* mutant animals compared with WT larvae (Figure 1D). These observations indicate that, in addition to negatively modulating target gene transcription via promoter-proximal binding, the ZFP-1/DOT-1.1 complex also regulates production of RNAs derived from both intragenic and distal enhancers.

Genes with Enhancer Signature Bound by ZFP-1/DOT-1.1 Display Bidirectional Transcription upon Loss of ZFP-1

Intragenic enhancers have been suggested to interfere with host gene transcription (Cinghu et al., 2017). In light of the elevation of intragenic enhancer transcription observed in the *zfp-1* mutant, we have analyzed cumulative GRO-seq coverage along gene bodies. Genes bound by ZFP-1/DOT-1.1 at the promoter were not considered, since we have previously observed that these show increased GRO-seq coverage in the *zfp-1* mutant compared with WT (Cecere et al., 2013). The remaining genes in the assembly were called either bound or not bound by ZFP-1 at the coding region. Both

sense transcription and anti-sense transcription were analyzed. Interestingly, transcriptional changes in the *zfp-1* mutant compared with WT at target genes depended on the presence of enhancer chromatin signature. Genes targeted by ZFP-1 and lacking enhancer signature displayed an overall decrease in sense transcription (Figure 2A, top panel) and no significant change in anti-sense transcription (Figure 2A, middle panel). However, those with enhancer signature showed a significant increase in anti-sense transcription (Figure 2A, top panel), whereas sense transcription did not change significantly (Figure 2A, middle panel).

We then compared changes in sense and anti-sense transcription assessed by GRO-seq with mRNA expression assessed by microarray and, interestingly, genes targeted by ZFP-1 at the coding region did not display an overall change in mRNA levels between *zfp-1* mutant worms and WT animals (Figure 2A, bottom panel). These observations suggest a role for the ZFP-1/DOT-1.1 complex in preventing anti-sense transcription at target genes with intragenic enhancer signatures.

Pervasive bidirectional transcriptional may result in double-stranded RNA (dsRNA) formation from complementary base pairing of sense and anti-sense transcripts (Piatek et al., 2016). We hypothesized that the increase in anti-sense transcription observed in the *zfp-1* mutant could result in formation of dsRNA. To address this hypothesis, we looked directly at the amount of dsRNA in *zfp-1* mutant worms by immunostaining with a dsRNA-specific antibody previously employed to visualize dsRNA *in vivo* (Kaneko et al., 2011; Saldi et al., 2014). In agreement with our hypothesis, we have observed an accumulation of nuclear dsRNA foci in *zfp-1* mutant worms, when compared with WT (Figure 2B).

Lethality of *dot-1.1* Knockout Is Suppressed by Dicer/RDE-4/RDE-1 Pathway Mutations

Our attempts to generate *dot-1.1* knockout mutants in the WT background by CRISPR/Cas9 technology were not successful. Motivated by the implication of DOT1L in apoptotic cell death (Feng et al., 2010; Nguyen et al., 2011), we have resorted to the *ced-3(n1286)* mutant strain, which is defective in the pro-apoptotic caspase CED-3 (homologue of mammalian caspase-3) (Xue et al., 1996), and have successfully generated a viable *dot-1.1* null mutant in this mutant background. Upon outcrossing the *ced-3(n1286)* allele, we found that a few surviving adult *dot-1.1* null animals produced only developmentally arrested embryos and larvae. The arrested larvae displayed numerous vacuoles typically seen in worms undergoing necrotic cell death (Xu et al., 2001). Notably, the most pronounced effect was seen in the intestinal cells that do not normally undergo programmed cell death (Figure 3B). Therefore, DOT-1.1 does not simply suppress the initiation of programmed cell death in specific cells that are destined to die.

We then hypothesized that the *dot-1.1(-)* lethality phenotype could be related to the increase in dsRNA observed in *zfp-1* mutant worms. In *C. elegans* and other organisms, exogenous dsRNA is subject to processing by the Dicer complex, which cleaves it into small interfering RNAs (siRNAs) (Grishok, 2005). In *C. elegans*, the first discovered *R*NAi-*de*ficient mutants included *rde-1* and *rde-4* (Tabara et al., 1999). The product of the *rde-1* gene is the Argonaute protein RDE-1, which binds primary siRNAs produced by Dicer cleavage (Yigit et al., 2006), and the RDE-4 protein binds long dsRNA and assists Dicer in the processing reaction (Parker, 2006; Tabara et al., 2002). The RDE-4

and RDE-1 proteins are also important for the anti-viral response (Wilkins et al., 2005) and thought to have a limited role in endogenous RNAi. To determine if the RDE-4/RDE-1 pathway responds to the ectopic dsRNA accumulation seen in the *zfp-1* and *dot-1.1* mutants, we crossed the double *dot-1.1; ced-3* mutant strain with null mutants for *rde-4* and *rde-1*. Excitingly, the double *dot-1.1(-); rde-4(-)* and *dot-1.1(-); rde-1(-)* mutant worms were homozygous viable and did not require the presence of the *ced-3* mutant allele for the viability. In a control experiment, we crossed the double *dot-1.1; ced-3* mutant strain with a mutant unrelated to the Dicer/RDE-4/RDE-1 pathway and the lethality of *dot-1.1(-)* was not rescued (Figure 3C). These clear genetic results indicate that the lethality phenotype associated with *dot-1.1* deletion is due to generation of ectopic small RNAs from excessive nuclear dsRNA.

Excessive DsRNA Resulting from Loss of ZFP-1/DOT-1.1 Competes for the Dicer Complex

Genetic analyzes of RNAi in *C. elegans* initially identified genes regulating silencing in response to exogenous dsRNA (exo-dsRNA), some of which were later found to participate in endogenous RNAi as well (Grishok, 2005; Lee, 2006). Importantly, other mutants in the endogenous RNAi ERI (Enhancer RNAi) pathway were found in screens selecting for the increased response to exo-dsRNA (Duchaine et al., 2006; Kennedy et al., 2004; Simmer et al., 2002; Yigit et al., 2006). In the ERI pathway, the RNAi-initiating dsRNA is generated by the RNA-dependent RNA polymerase RRF-3 at the select endogenous loci, mostly representing duplicated genes (Fischer et al., 2013; Gent et al., 2010). This dsRNA is subject to Dicer processing and results in the generation of primary siRNAs bound by the ERGO-1 Argonaute (Vasale et al., 2010; Yigit et al.,

2006). The ERI-1 protein and RRF-3 both interact with Dicer and act upstream of ERGO-1 in the ERI pathway. Thus, the ERI/ERGO-1 and the exo-RNAi/RDE-1 pathways compete for the Dicer complex. We hypothesized that, upon loss of DOT-1.1 or ZFP-1, another pool of endogenous dsRNA (in addition to the RRF-3-produced) is competing with exo-RNAi, and efficiency of the latter is reduced as a result of this competition. To test this hypothesis, we used the *elt-2::*GFP*/lacZ* transgenic strain (Fukushige et al., 1999), which contains a repetitive transgenic array prone to silencing through the Dicer/RDE-4/RDE-1 pathway (Grishok et al., 2005). This transgene is exclusively expressed in the nuclei of intestinal cells and shows partial silencing at 16°C in the WT background and complete silencing in ERI pathway mutants (Grishok et al., 2005), such as *eri-1(mg366)* (Figure 4A). On the contrary, in *zfp-1(ok554)* (not shown) or *dot-1.1(gk520244)* partial loss-of-function mutant backgrounds, it is readily apparent that transgenic expression is increased compared to WT (Figure 4A). Importantly, the level of GFP expression in the double mutant *dot-1.1; eri-1* remains low, similar to that of *eri-1* alone (Figure 4A). This result indicates that the effect of *dot-1.1* mutation (i.e. transgene de-silencing) is suppressed by the loss of ERI-1 working together with Dicer. In other words: DOT-1.1 acts upstream of ERI-1/Dicer. This result is not consistent with the long-standing belief that ZFP-1 induces RNAi in the nucleus in response to siRNAs produced by Dicer. However, it completely supports our hypothesis about the mechanism of the apparent RNAi-promoting function of ZFP-1 and DOT-1.1. In summary, our results demonstrate that, upon loss of ZFP-1/DOT-1.1, in addition to the dsRNA generated through the ERI pathway and the dsRNA produced from transgenes, the dsRNA produced from endogenous loci enters in the competition for the Dicer

complex (Figure 4B). Our data strongly suggest that this competition occurs in the nucleus.

Loss of DOT-1.1 Results in Ectopic Heterochromatin at Distal and Intragenic Enhancers

DOT1L activity has been shown to restrict the spreading of factors associated with heterochromatin in yeast and leukemia cell lines (Chen et al., 2015; Katan-Khaykovich and Struhl, 2005; Ng et al., 2002). On the other hand, siRNAs direct Argonaute proteins to complementary nascent transcripts to initiate H3K9 methylation, resulting in heterochromatin formation (Grewal and Elgin, 2007; Ni et al., 2014). We reasoned that these two distinct phenomena could be put together in the context of the regulation of enhancer transcription by ZFP-1/DOT-1.1. Interestingly, histone H3 lysine 9 dimethylation (H3K9me2), a hallmark of heterochromatin, is increased in both intragenic and distal enhancers in *dot-1.1; ced-3* animals compared to *ced-3* animals (Figures 5A and 5B). In addition, genes displaying chromatin enhancer signatures and ZFP-1/DOT-1.1 binding have increased H3K9me2 in *dot-1.1; ced-3* animals (Figure 5C). These observations show that DOT1L activity prevents the encroachment of heterochromatin to enhancer elements.

Discussion

In the past few years, genomic and transcriptomic data from high-throughput sequencing studies have been shedding light on the importance of non-coding genomic regulatory regions, including enhancers. These are typically demarcated by certain characteristics, such as prevalence of the H3K4me1 and H3K27ac marks and open

chromatin configuration, and these features have been extensively used to predict their coordinates in a variety of organisms. Importantly, exciting new observations point to the remarkable similarity between enhancers and promoters in terms of chromatin architecture and transcriptional profile, challenging traditional binary views of enhancers and promoters (Henriques et al., 2018; Mikhaylichenko et al., 2018; Rennie et al., 2017). As these notions become dimmer, an interesting view gaining momentum is that some enhancers can act as weak promoters, while, conversely, bidirectional promoters often act as strong enhancers. This plasticity of enhancer and promoter activities is manifested in the levels and directionality of transcription (Mikhaylichenko et al., 2018). In the face of this shifting paradigm, it is reasonable to speculate that the commonalities between enhancers and promoters may extend to how their transcriptional output is controlled by the epigenetic machinery of the cell, including molecular writers such as histone methyltransferases and histone acetyltransferases. We had previously demonstrated that ZFP-1/DOT-1.1 exert a negative modulatory role on transcription of genes with promoter-proximal binding of the complex (Cecere et al., 2013). These are mostly highly and widely expressed genes not subject to spatiotemporal regulation. Here, we present tantalizing evidence that the DOT1L complex also modulates enhancer-directed transcription in a mechanism that likely partakes on the regulation of genes subject to tissue and time-specific control of expression, ensuring the fidelity of developmental programs. Of note, DOT1L has been implicated in processes as diverse as embryonic and postnatal hematopoiesis, proliferation of mouse embryonic stem cells, induced and natural reprogramming, cardiac development and chondrogenesis (McLean et al., 2014), and the regulation of these processes is greatly contributed by

enhancer activity. Therefore, it is possible that DOT1L assists promoter-enhancer cooperativity at key developmental transitions. Also, perturbation of enhancer activity is increasingly being recognized as an important player in malignant transformation (Sur and Taipale, 2016). Recently, chimeric oncoproteins MLL-AF9 and MLL-AF4 were found to bind specific subsets of non-overlapping active distal enhancers in acute myeloid leukemia cell lines. Interestingly, in this study, MLL-AF9-bound enhancers displayed higher levels of H3K79me2 than enhancers bound by the MLL protein alone (Prange et al., 2017). This observation can be explained by the fact that AF9, similarly to AF10, is a frequent binding partner of DOT1L and drives its localization to chromatin (Kuntimaddi et al., 2015). Therefore, it is possible that, in MLL-driven leukemias, inappropriate recruitment of DOT1L to distal regulatory elements perturbs their transcriptional output. In addition, homeobox loci with an indisputable role in malignant transformation, such as *HOXA9* and *MEIS*, display both coding and non-coding transcription, including anti-sense transcripts (Popovic et al., 2008; Sessa et al., 2006), and DOT1L activity may play a yet unappreciated role in the control of such transcripts. In fact, our observations suggest that DOT1L controls anti-sense transcription, particularly at genes characterized by presence of enhancer signature. Interestingly, a sequence conserved in vertebrate *Hox* gene introns was reported to function as an enhancer element in *Drosophila* (Haerry and Gehring, 1996; Keegan et al., 1997). In addition, the c-*myc* (Nepveu and Marcu, 1986) and N-*myc* (Krystal et al., 1990) oncogenic loci display anti-sense transcription, and exciting new findings point to the implication of DOT1L in cancers underlain by c-Myc and N-Myc activation, particularly breast cancer (Cho et al., 2015) and neuroblastoma (Wong et al., 2017). Also, the

contribution of anti-sense transcription to malignant transformation has been gaining attention in the past few years (Balbin et al., 2015; Wenric et al., 2017). Therefore, the enticing implications of our observations on the *C. elegans* DOT1L homologue call for the role of anti-sense and non-coding transcription in cancer to be appreciated in the context of DOT1L activity.

Our analysis of GRO-seq data revealed an increase in anti-sense transcription upon loss of ZFP-1/DOT-1.1, particularly at genes with enhancer signatures, implying overlapping bidirectional transcription and formation of sense-antisense dsRNA structures. This inference was reinforced by the clear observation of dsRNA foci in the nucleus of *zfp-1* mutant worms, indicating accumulation of dsRNA. Formation of dsRNA is a common feature of multiple gene suppression phenomena known collectively as RNAi, which may be endogenous or exogenous. Importantly, production of both endogenous (endo-siRNAs) and exogenous (exo-siRNAs) small interfering RNAs requires the activity of DCR-1/Dicer. Our observations demonstrate that endogenous dsRNA resulting from loss of ZFP-1/DOT-1.1 competes with exogenously introduced dsRNA for DCR-1/RDE-4/RDE-1-mediated processing into small RNAs. This competition can explain the implication of ZFP-1 in RNAi (Dudley et al., 2002; Grishok et al., 2005; Kim et al., 2005). Also, lethality of *dot-1.1* deletion is suppressed by Dicer/RDE-4/RDE-1 pathway mutations, showing that endogenous small RNA generation is involved in the lethality phenotype. It is well known that exo-dsRNA induces apoptosis in a variety of mammalian systems (Estornes et al., 2012; Kalai et al., 2002; Yamane et al., 2006). Our observations uncover an interesting phenomenon whereby ectopically formed endogenous dsRNA, and not exo-dsRNA, causes cell

death. To the best of our knowledge, this finding is unprecedented and lays ground for further research.

A further implication of our observations lies at the crosstalk between H3K79 methylation and H3K9 methylation. Knowledge of this crosstalk was pioneered by work in yeast (Katan-Khaykovich and Struhl, 2005; Ng et al., 2002) and later placed in the context of MLL-driven leukemias (Chen et al., 2015). We have observed that H3K9me2, a hallmark of heterochromatin, is elevated at enhancer regions upon loss of ZFP-1/DOT-1.1. Therefore, DOT1L may prevent the encroachment of heterochromatin to enhancers.

In summary, the compelling observations disclosed here bring together various aspects of DOT1L function (Figure 6). In addition to controlling transcription through promoters, as previously described (Cecere et al., 2013), DOT1L activity controls enhancer-directed transcription. This finding may attest to the novel concept of the fluidity of enhancer/promoter states. Furthermore, we have linked DOT1L-mediated transcriptional control to dsRNA formation and small RNA generation, as well as to heterochromatin formation, specifically at enhancer regions.

Experimental Procedures

Strains

Strains were maintained at 20°C under standard conditions (Brenner, 1974). Bristol N2 was the WT strain used. The following mutant strains were obtained from the Caenorhabditis Genetics Center (CGC): VC40220, containing *dot-1.1(gk520244)* I, RB774 – *zfp-1(ok554)* III, WM49 – *rde-4(ne301)* III, VC20787, containing *mml-*

1(gk402844) III, GR1373 – *eri-1(mg366)* IV, MT3002 – *ced-3(n1286)* IV, WM27 – *rde-1(ne219)* V. The RB and VC strains were provided by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation and by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, respectively, which were part of the International *C. elegans* Gene Knockout Consortium. Mutations of interest were outcrossed four times and the following derivative strains were obtained: AGK760 – *mml-1(gk402844)* III, AGK768 – *dot-1.1(gk520244)* I. The *rtIs11 (elt-2P::GFP/LacZ)* X transgenic strain was a gift from Dr. Anne Hart. The *dot-1.1* null mutant (strain COP1302 – *dot-1.1 [knu339 - (pNU1092 - KO loxP::hygR::loxP)]* I; *ced-3 (n1286)* IV), in which exons one to four in the *dot1.1* gene locus were deleted, was generated using a proprietary protocol by Knudra Transgenics. The following derivative strains were produced in this study:

AGK779 – *dot-1.1(gk520244)* I; *rtIs11 (elt-2P::GFP/LacZ)* X

AGK780 – *eri-1(mg366)* IV; *rtIs11 (elt-2P::GFP/LacZ)* X

AGK781 – *dot-1.1(gk520244)* I; *eri-1(mg366)* IV; *rtIs11 (elt-2P::GFP/LacZ)* X

AGK782 – *dot-1.1 [knu339 - (pNU1092 - KO loxP::hygR::loxP)]* I; *rde-4(ne301)* III

AGK783 – *dot-1.1 [knu339 - (pNU1092 - KO loxP::hygR::loxP)]* I; *rde-1(ne219)* V

Microscopy and Immunofluorescence

The *elt-2P::GFP/LacZ* transgenic strains were grown at 16°C for at least two generations without starvation before imaging. Live animals were mounted on 2% agarose pads and examined using the Zeiss AxioImager Z1 instrument. The same exposure times were used for capturing images and comparing GFP expression in different strains.

For immunofluorescence, young adult worms were handpicked and placed in M9 solution supplemented with 0.01% Tween and 25 mM sodium azide on a slide. Worms were dissected using scalpel and fixed with 2% paraformaldehyde for 1 h at room temperature and post-fixed in ice-cold methanol for 5 minutes. Control worms were treated with RNase A prior to fixation at 37°C for 30 minutes to confirm the antibody specificity, and then washed 3 times with M9. After fixation, worms were transferred to glass tubes and blocked with 1% bovin serum albumin (BSA) in M9 supplemented with 0.05% Tween-20. Blocked samples were then incubated with the J2 antibody (1:1000) (a generous gift of Dr. Elke Mühlberger, National Emerging Infectious Diseases Laboratories) overnight at 4°C, washed 3 times in washing buffer (BSA 0.25%, Tween-20 0.05% in M9) and then incubated with secondary fluorophore-conjugated (AF 568) antibody (1:500) for 2 h in the dark. After 3 washes, the samples were transferred to a slide and mounted with DAPI-containing solution (Invitrogen, Prolong Diamond Antifade Mountant with DAPI). The images were taken at 63X on a Zeiss AxioImager Z1 instrument and processed with ImageJ (version 1.51k).

Chromatin Immunoprecipitation and Library Preparation

Hypochlorite-synchronized L1 stage WT or mutant animals were grown for 36 hours post-hatching at 20ºC on standard NGM plates with OP-50 *E. coli*. Synchronized L3 larvae were then washed off the plates using isotonic M9 solution. Fixation was performed for 30 min at 20°C in crosslinking solution (2% paraformaldehyde in M9 buffer), and excess formaldehyde was quenched with 0.1 M Tris-HCl pH 7.5. The pellet was then washed twice with M9 buffer at 4°C. The worm pellet was resuspended in 1 mL ice-cold RIPA buffer (10 mM Tris-HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 0.1%

sodium deoxycholate, 1 mM EDTA, 0.15 M NaCl) supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). The suspension was then transferred to a Covaris milliTUBE 1 mL with AFA® fiber and shearing was performed using the following settings in SonoLab7: Peak Incident Power (PIP) = 240W; Duty Factor = 20%; Cycles per burst (cpb) = 200; time = 480 seconds. After shearing, the crude extract was spun for 10 min at 10,000 x *g* and the supernatant was collected. Protein concentration was determined by the Bradford method. Extract corresponding to 1 mg protein was diluted to 500 µl with ice-cold RIPA buffer supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). Antibody specific for H3K9me2 (Abcam, ab1220, 5 µg) was added and, after incubation at 4°C for 1 h, 50 µl of Dynabeads™ Protein G suspension (Thermo Fisher Scientific) were added and the mixture was incubated at 4°C for 1 h on rotation. Beads were recovered with a Dynal® Magnetic Particle Concentrator (Invitrogen) and washed at 4°C with: 0.5 mL buffer TSE-150 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), 2 X 0.5 mL buffer TSE-500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), 0.5 mL buffer TSE-1M (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 1 M NaCl), 0.5 mL buffer LiCl (250 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), 2 X 0.5 mL buffer TE (10 mM Tris pH 8.0, 1 mM EDTA). Antibody-bound chromatin was recovered in 2 X 0.2 mL elution buffer (1% SDS, 0.1 M NaHCO3) by shaking at room temperature for 15 min. Elution buffer was added to input samples (5%) to a total volume of 0.4 mL. Input and ChIP chromatin samples were subsequently processed in parallel. After 20 µl of 5 M NaCl was added to each sample, cross-links were reversed overnight at 65°C. 10 µl of 0.5 M

EDTA, 20 µl of 1 M Tris-HCl pH 6.5 and 0.5 µl of 20 mg/mL Proteinase K (Thermo Fisher Scientific) were added and each sample was incubated for 1 h at 42°C. DNA was recovered using the QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. The immunoprecipitated DNA was either quantified by qPCR to calculate the percentage of immunoprecipitation relative to the input, or subjected to library preparation and high-throughput sequencing. Immunoprecipitated DNA and input libraries were prepared using TruSeq ChIP Library Preparation kit following the manufacturer's instructions (Illumina) and checked for concentration and quality on DNA chips with a Bioanalyzer (Agilent). 75-bp single read sequences were generated on the NextSeq 500 sequencer according to manufacturer’s instructions (Illumina).

RNA Extraction and Expression Profiling

Synchronized L3 larvae were washed off the plates using isotonic M9 solution. RNA was isolated in triplicate for each mutant strain using TRIzol (Invitrogen) followed by miRNeasy mini column (Qiagen). RNA integrity was confirmed using a 2100 Bioanalyzer (Agilent Technologies). Samples were labeled and hybridized to Affymetrix GeneChip™ *C. elegans* Gene 1.0 ST arrays. Raw Affymetrix CEL files were normalized to produce gene-level expression values using the Robust Multiarray Average (RMA) (Irizarry et al., 2012) in Affymetrix Expression Console (version 1.4.1.46). The default probesets defined by Affymetrix were used to assess array quality using the Area Under the [Receiver Operating Characteristics] Curve (AUC) metric. Principal Component Analysis (PCA) was performed using the *prcomp* R function with expression values that had been normalized across all samples to a mean of zero and a standard deviation of

one. Differential expression was assessed using the moderated (empirical Bayesian) ANOVA and *t* test implemented in the *limma* package (version 3.14.4) (i.e., creating simple linear models with *lmFit*, followed by empirical Bayesian adjustment with *eBayes*). Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995). All microarray analyses were performed using the R environment for statistical computing (version 2.15.1).

Analysis of Chromatin Domains and ATAC-Seq Peaks

Coordinates of chromatin domains were obtained from Evans et al., 2016. DOT-1.1 ChIP-chip and ZFP-1 ChIP-seq peak coordinates were obtained from modENCODE (modENCODE_2970 and modENCODE_6213, respectively). A chromatin domain region was called bound by DOT-1.1 or by ZFP-1 if the center base pair of at least one peak was located within the coordinates of the region. Putative enhancer regions were obtained by combining coordinates of domains 8, 9 and 10. Enhancer domains at least 1500 bp distal to any annotated transcription start site or transcription termination site were considered distal enhancer domains. Enhancer domains intersecting coordinates of genes < 15kb by at least 50 bp were considered intragenic enhancer domains. ATAC-seq peak coordinates (Daugherty et al., 2017) were downloaded from the NCBI GEO database (GSE89608). Distal and intragenic ATAC-seq peaks were obtained as for enhancer domains. Intersections of genomic intervals were performed in R using the valr package (A. Riemondy et al., 2017).

Analysis of GRO-Seq Data

GRO-seq data was obtained from the NCBI GEO database (GSE47132) and the reads were mapped to the *C. elegans* genome (ce10 assembly) using ChIPdig, a software application to analyze ChIP-seq data (Esse and Grishok, 2017). Then, reads matching ribosomal RNA loci were removed, as described before (Cecere et al., 2013). Read counting in regions (either genes, regions or genomic bins) was performed with package GenomicAlignments (Lawrence et al., 2013); only reads with mapping quality 20 or higher were included in subsequent analyses. Regions without reads across the sample set were removed. Counts were then normalized using the TMM method, which takes RNA composition bias into account (Robinson and Oshlack, 2010), using the edgeR package (Robinson et al., 2010). Coverage was expressed as RPKM (reads per kilobase per million mapped) and log2-transformed.

Analysis of ChIP-Seq Data

Analysis of ChIP-seq data was performed as described for the analysis of GRO-seq data, with the exception that, after normalization of read counts, coverage was expressed as the log2-transformed ratio of the RPKM value in the immunoprecipitated DNA sample divided by the RPKM value in the non-immunoprecipitated (input) DNA. Only regions in which the normalized count value in the immunoprecipitated DNA sample was higher than that in the corresponding input DNA in at least one sample in the set were considered.

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Figures Legends

Figure 1. ZFP-1/DOT-1.1 Negatively Modulate Enhancer-Directed Transcription

(A) Overlap analysis showing that promoters and enhancer chromatin domains are enriched in ZFP-1/DOT-1.1. DOT-1.1 ChIP-chip and ZFP-1 ChIP-seq peak coordinates were obtained from modENCODE (modENCODE_2970 and modENCODE_6213, respectively). Chromatin domain coordinates were obtained by hidden Markov models (Evans et al., 2016). Data are presented as percentage of regions enriched in DOT-1.1 or ZFP-1 in each of the 20 chromatin domains described for embryos and for third larval stage animals, respectively. A chromatin domain region was called bound by DOT-1.1 or ZFP-1 if the center base pair of at least one peak was located within the coordinates of the region. Domains 1 and 8 at the embryonic stage, as well as domain 1 at the third larval stage (L3), were annotated as promoter regions. Domain 8 at L3 and domain 9 in both embryos and L3 correspond to predicted intronic and intergenic enhancers, respectively (Evans et al., 2016).

(B) Intragenic and distal ATAC-seq peaks in embryos and L3 animals are enriched in DOT-1.1 and ZFP-1, respectively. *C. elegans* gene coordinates (WS220/ce10) were extracted from the UCSC genome browser and intersected with ATAC-seq peak coordinates (Daugherty et al., 2017). Peaks at least 1500 bp distal to any annotated transcription start site or transcription termination site were considered distal ATAC-seq peaks. Peaks intersecting coordinates of genes < 15kb by at least 50 bp were considered intragenic ATAC-seq peaks.

(C) Transcription originating from intragenic and distal enhancers predicted by chromatin modification signatures (Evans et al., 2016) is elevated in *zfp-1* mutant animals compared with WT larvae. GRO-seq coverage was normalized using 250 bp bins spanning the genome. The P-value was determined by Mann-Whitney U test (enhancers versus genomic bins).

(D) Transcription originating from distal enhancers corresponding to open chromatin regions (Daugherty et al., 2017) is elevated in *zfp-1* mutant animals compared with WT larvae. GRO-seq coverage normalization and statistical tests were performed as in (C).

Figure 2. ZFP-1/DOT-1.1 Prevent Double-Stranded RNA Formation at Target Genes with Intragenic Enhancers

(A) Anti-sense transcription at ZFP-1 target genes with intragenic enhancer signature is increased in *zfp-1* mutant animals compared with WT larvae, whereas sense transcription does not change. The corresponding steady-state mRNA population does not overall change significantly between *zfp-1* mutant worms and WT animals. ** and *** denote P-values < 0.05 and < 0.001, respectively, as determined by Mann-Whitney U test (each category of genes versus all genes).

(B) Double-stranded RNA (dsRNA) accumulates in *zfp-1* mutant animals. Fixed, isolated intestinal tissue was probed with anti-dsRNA antibody (J2) recognizing stretches of 40 bp or more in a sequence-independent manner. Stained inclusions (red) were detected in intestinal nuclei (blue, DAPI counterstain) and were increased in *zfp-1* mutant animals compared with WT larvae. Scale bar: 50 µm.

Figure 3. Lethality of *dot-1.1* Knockout Is Suppressed by Dicer/RDE-4/RDE-1 Pathway Mutations

(A) The *dot-1.1; ced-3* mutant displays global loss of H3K79me2 as assessed by Western blotting, in agreement with the function of DOT-1.1 in catalyzing H3K79 methylation.

(B) Outcrossing the *ced-3(n1286)* allele from the *dot-1.1; ced-3* mutant results in arrested development, cell death. Note the numerous vacuoles typical of necrotic cell death in *C. elegans*.

(C) Mutations in *rde-4* and *rde-1*, both affecting primary siRNA production and function, rescue the lethality phenotype associated with knockout of the *dot-1.1* locus.

Figure 4. Ectopic Endogenous DsRNA Formed upon Loss of ZFP-1/DOT-1.1 Titrates the Dicer Complex away from Exogenous dsRNA

(A) In *dot-1.1(gk520244)* partial loss-of-function mutant worms, expression of the *elt-2::*GFP*/lacZ* transgene is increased compared with WT animals, indicating reduced efficiency of silencing of exogenously introduced dsRNA. The level of GFP expression in *dot-1.1; eri-1* mutant worms is similar to that observed in *eri-1* mutant worms, showing that DOT-1.1 acts upstream of ERI-1/Dicer. Scale bar: 200 µm.

(B) Different pools of dsRNA compete for the Dicer/RDE-4/RDE-1 pathway, including ectopic dsRNA produced endogenously. The latter is exacerbated upon loss of ZFP-1/DOT-1.1.

Figure 5. Distal and Intragenic Enhancers Show Ectopic Heterochromatin upon Loss of DOT-1.1

(A and B) Distal and intragenic enhancers predicted by chromatin enhancer signatures (A) or by ATAC-seq peaks (B) display ectopic H3K9me2 in the *dot-1.1; ced-3* mutant

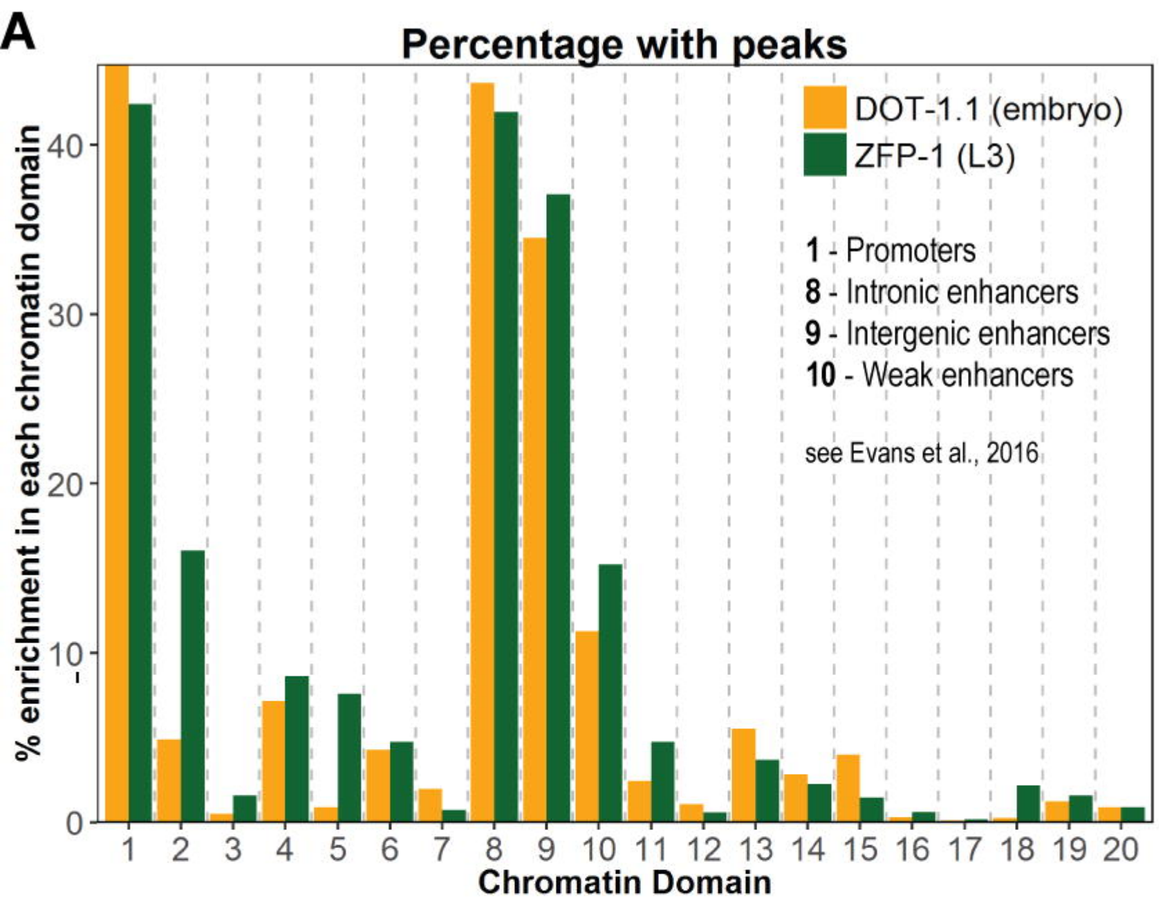
compared with *ced-3* animals. The P-value was determined by Mann-Whitney U test (enhancers versus genomic bins).

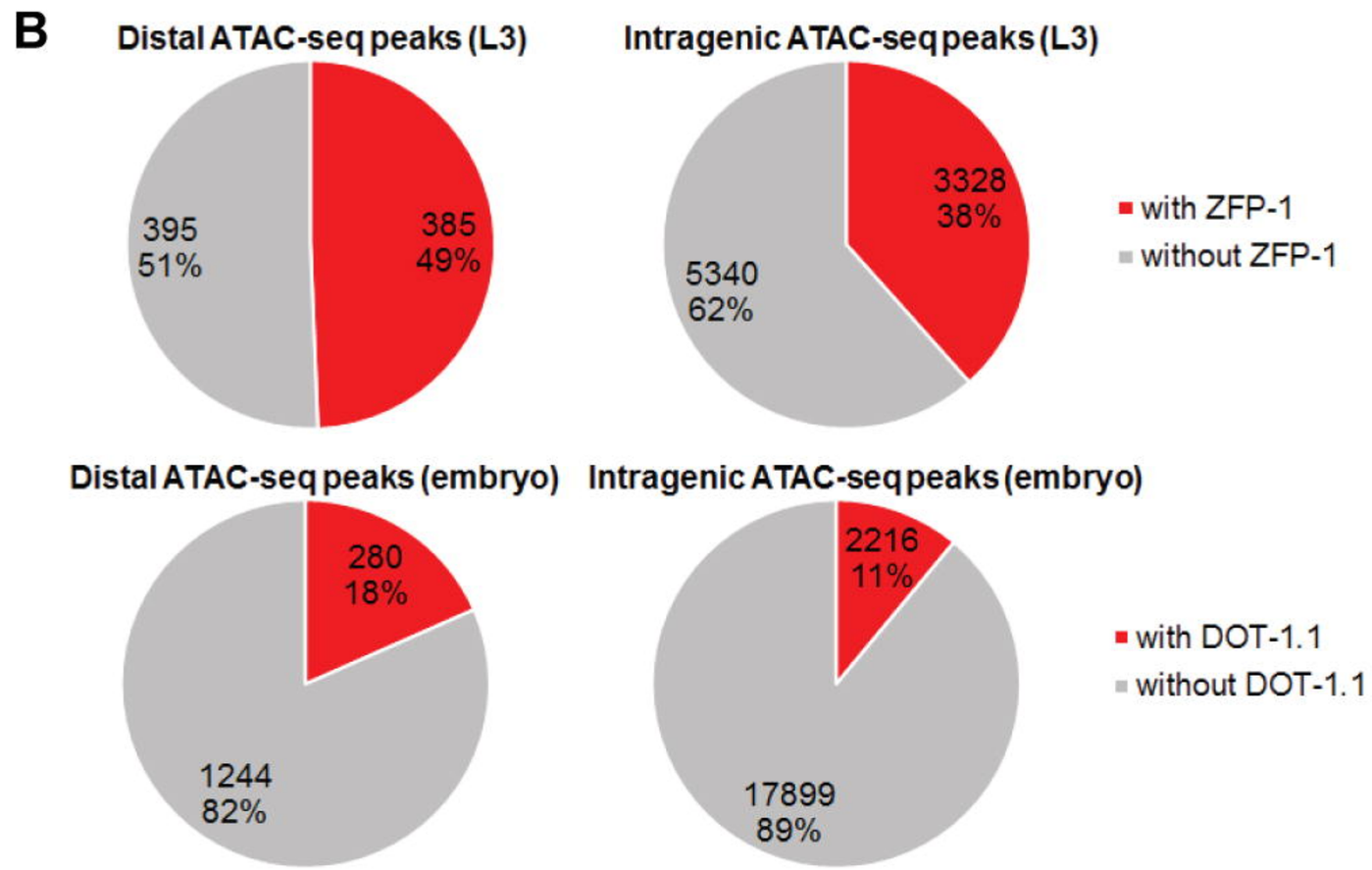
(C) H3K9me2 is elevated at ZFP-1 target genes with enhancer signature in the *dot-1.1; ced-3* mutant compared with *ced-3* animals. *** denote a P-value < 0.001 as determined by Mann-Whitney U test (*dot-1.1; ced-3* versus *ced-3*).

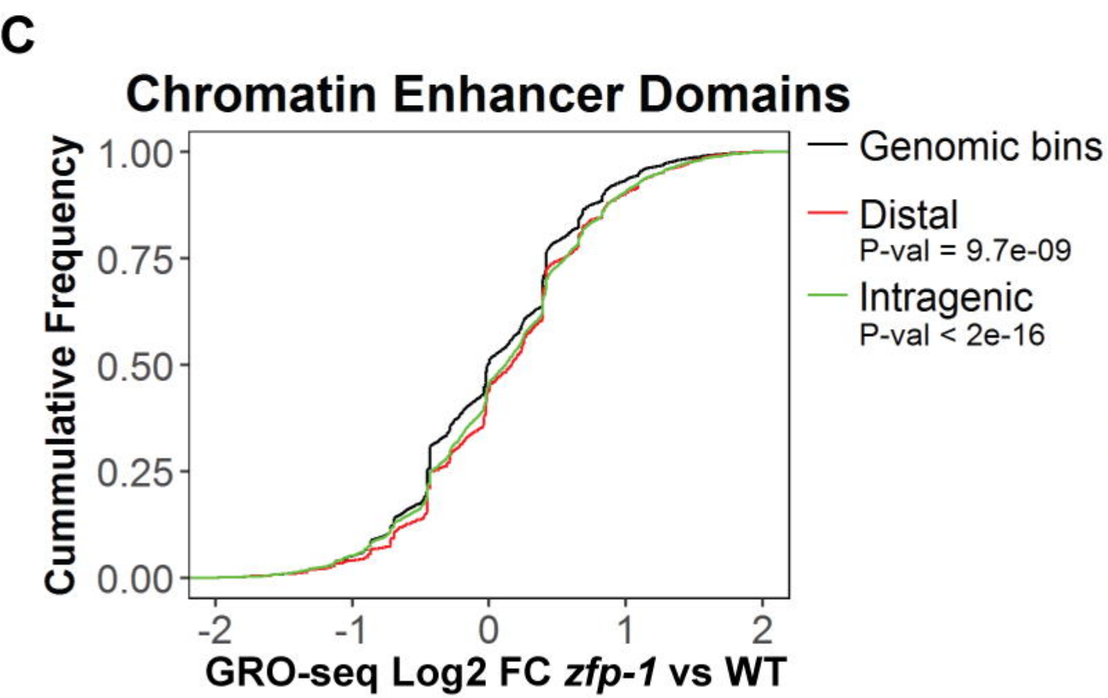
Figure 6. Mechanism of Initiation of Nuclear RNAi upon Loss of DOT1L

Loss of ZFP-1/DOT1L leads to increase in anti-sense transcription, resulting in accumulation of endogenous dsRNA molecules. These are processed by the Dicer complex into endo-siRNAs, ultimately leading to H3K9 methylation and heterochromatin formation. Generation of this new pool of dsRNA attracts the dsRNA-binding RNAi pathway protein RDE-4 and Dicer, thus limiting the efficiency of exogenous dsRNA-induced silencing.

Figure 1


A
Percentage with peaks
DOT-1.1 (embryo)
ZFP-1 (L3)
1 - Promoters
8 - Intronic enhancers
9 - Intergenic enhancers
10 - Weak enhancers
see Evans et al., 2016
% enrichment in each chromatin domain
Chromatin Domain


B
Distal ATAC-seq peaks (L3)
395
51%
385
49%
Intragenic ATAC-seq peaks (L3)
3328
38%
5340
62%
with ZFP-1
without ZFP-1
Distal ATAC-seq peaks (embryo)
280
18%
1244
82%
Intragenic ATAC-seq peaks (embryo)
2216
11%
17899
89%
with DOT-1.1
without DOT-1.1


C
Chromatin Enhancer Domains
Cummulative Frequency
GRO-seq Log2 FC zfp-1 vs WT
Genomic bins
Distal
P-val = 9.7e-09
Intragenic
P-val < 2e-16

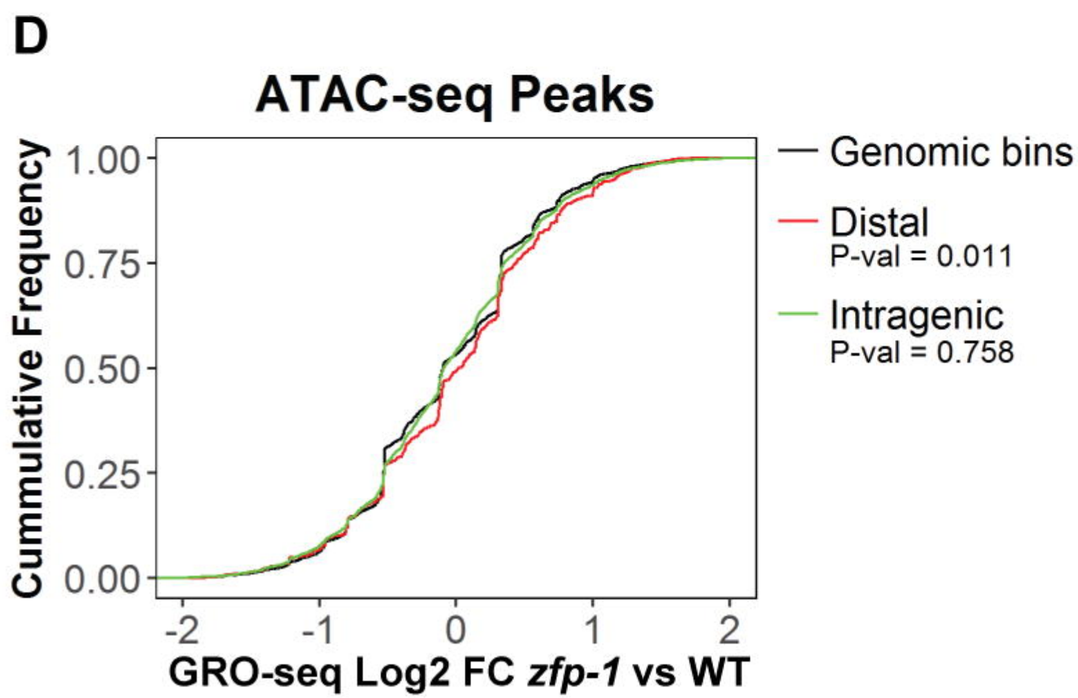
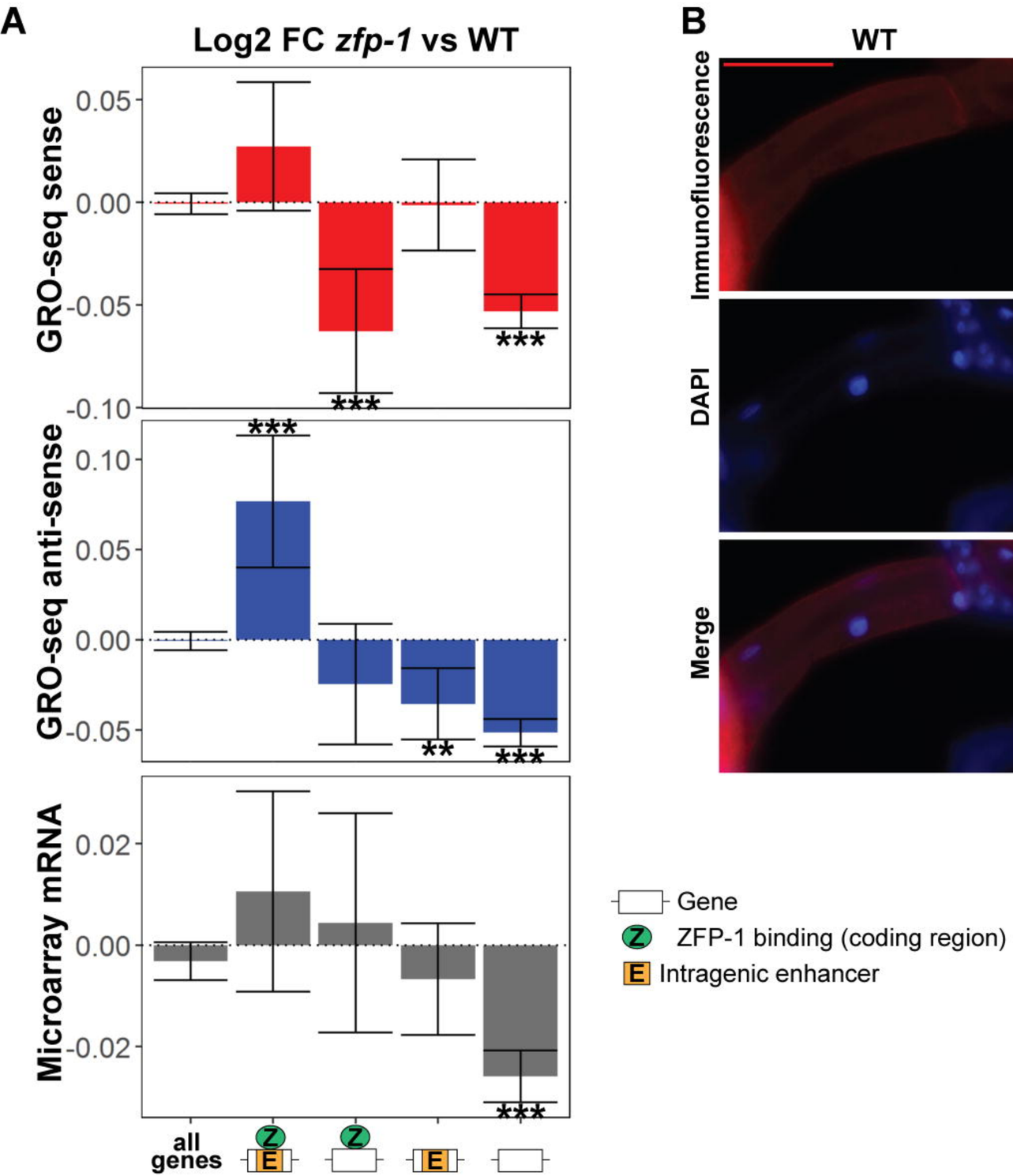

D
ATAC-seq Peaks
Cummulative Frequency
GRO-seq Log2 FC zfp-1 vs WT
Genomic bins
Distal
P-val = 0.011
Intragenic
P-val = 0.758

Figure 2


A
Log2 FC zfp-1 vs WT
GRO-seq sense
0.05
0.00
-0.05
-0.10
***
***
GRO-seq anti-sense
0.10
0.05
0.00
-0.05
***
**
***
Microarray mRNA
0.02
0.00
-0.02
***
all genes
Gene
ZFP-1 binding (coding region)
Intragenic enhancer

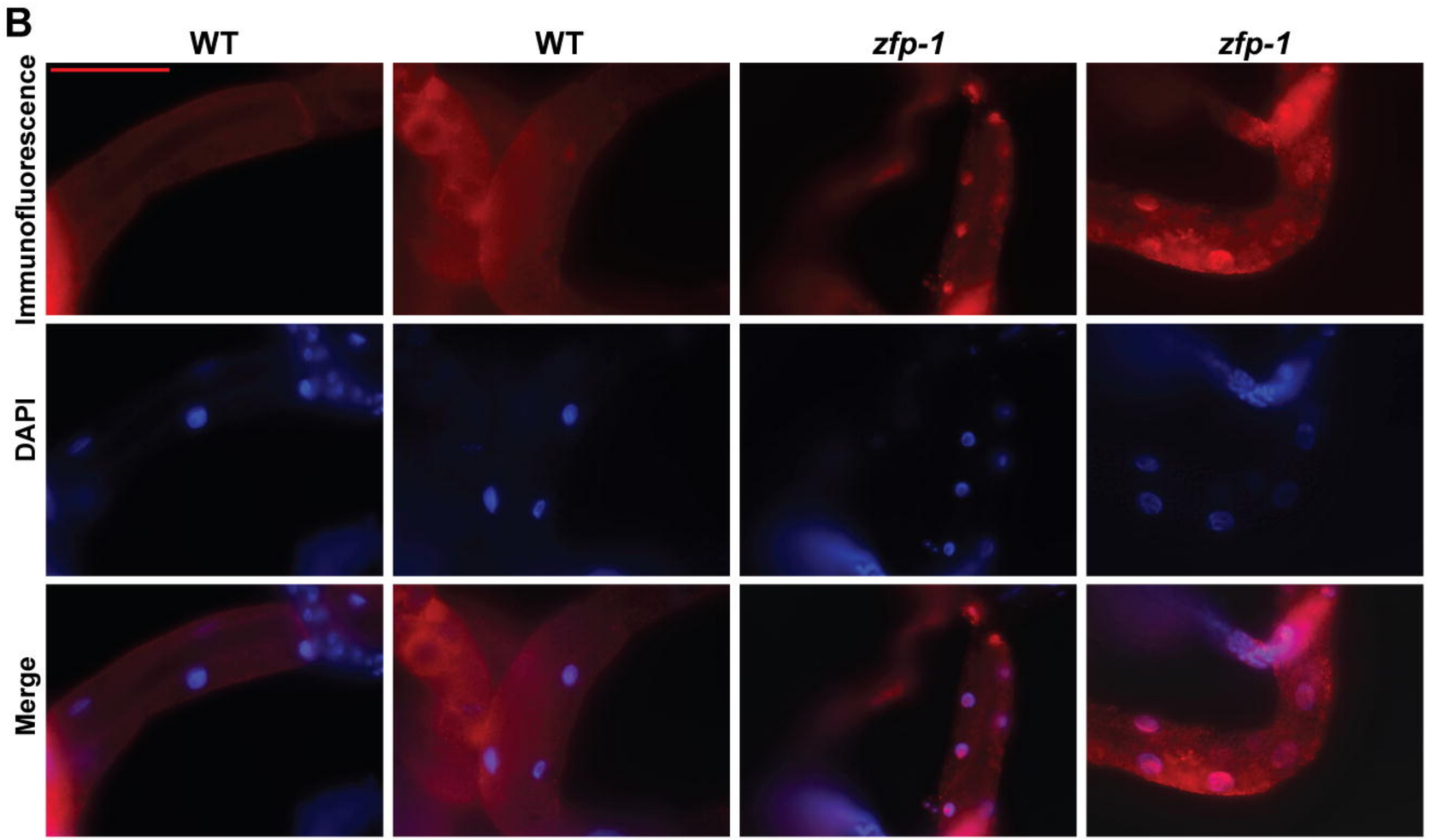
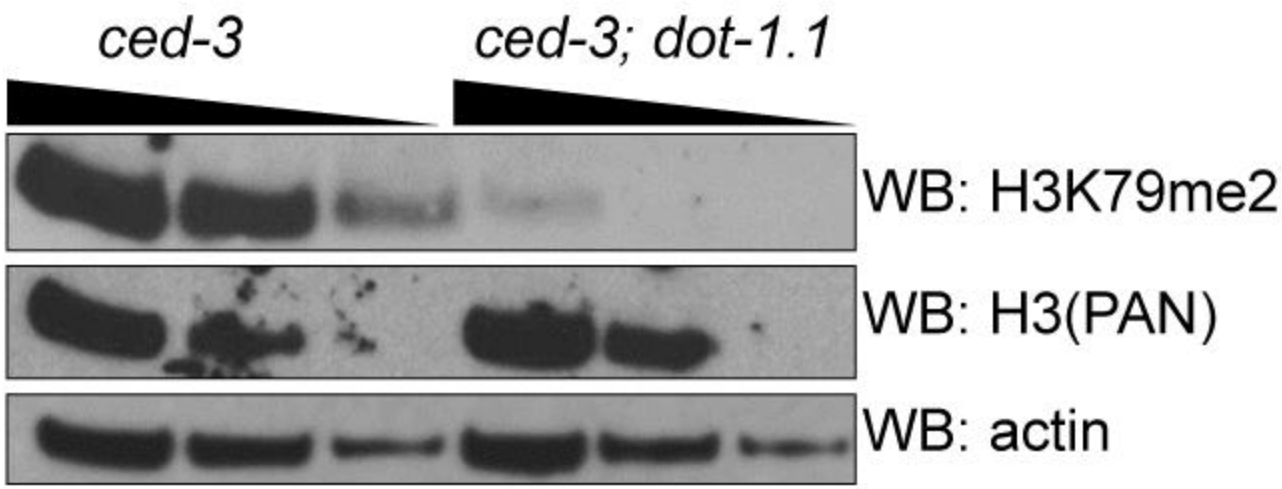
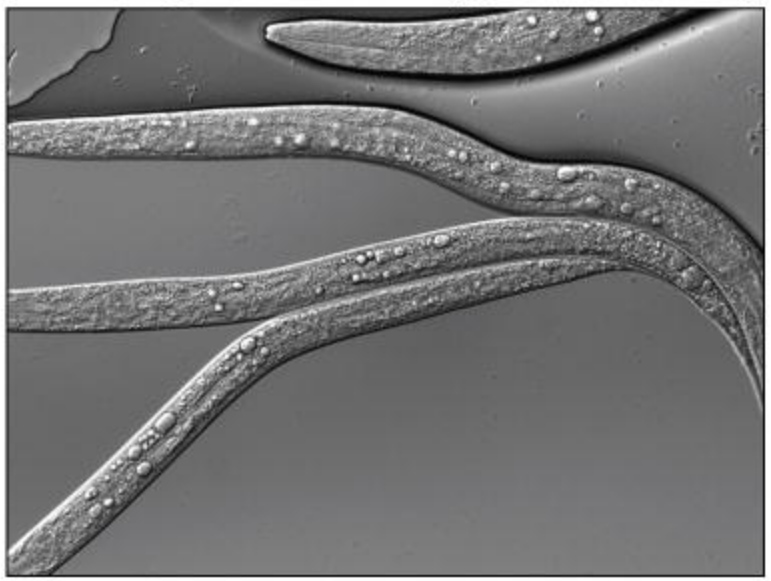

B
WT
WT
zfp-1
zfp-1
Immunofluorescence
DAPI
Merge

Figure 3

A



B *dot-1.1(-); ced-3(+)* arrested larva resulting from *ced-3(-)* outcrossing



C

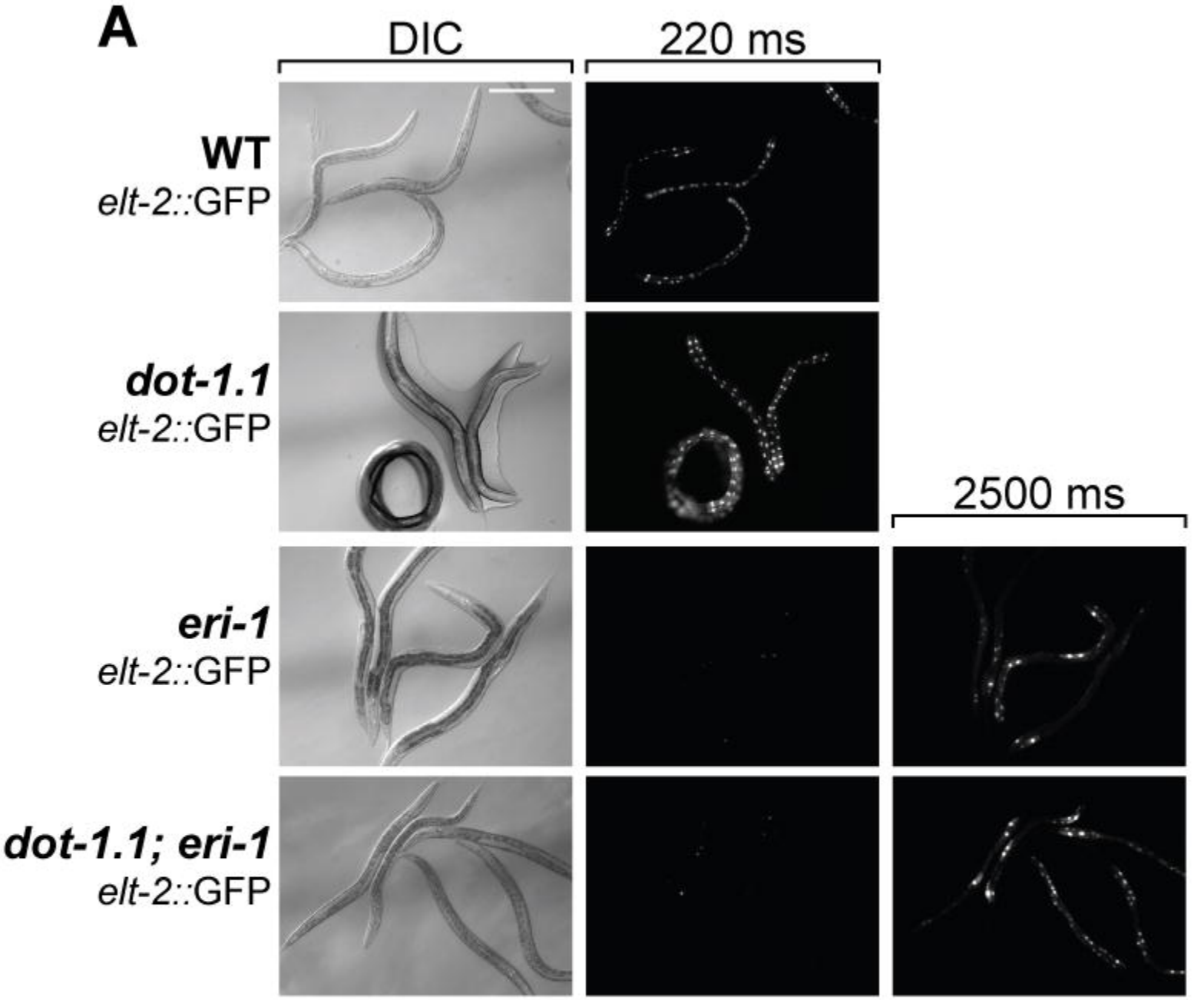
Results of *dot-1.1* outcrosses from *ced-3* background

Mutant crossed	F2 picked (N)	Homozygous viable *dot-1.1* F2 lines			
		WT background	*ced-3* status	Mutant background	*ced-3* status
mml-1(gk402844)	21	2	2/2 (-)	3	3/3 (-)
rde-4(ne301)	20	3	3/3 (-)	2	2/2 (+)
*rde-1(ne219)**	10	n/a	n/a	4	1/4 (-) 3/4 (+)

* Mutant F2 animals were pre-selected

Figure 4

A



B

***elt-2::* GFP background**	**Competing dsRNA pools**		
	Repetitive transgenes	ERI pathway-produced	Ectopic endogenous
WT	+	+	-
eri-1	+	-	-
dot-1.1	+	+	+
eri-1; dot-1.1	+	-	+

Figure 5

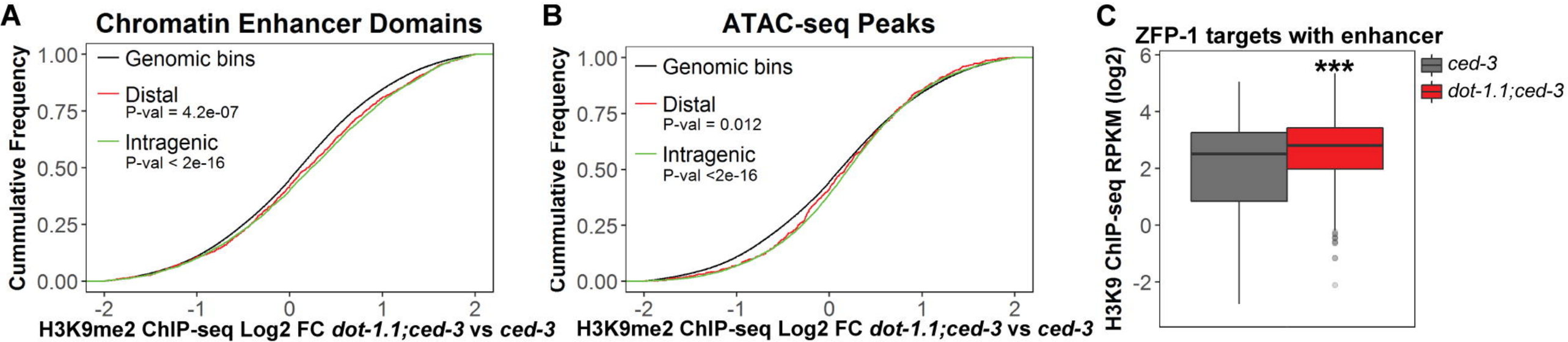
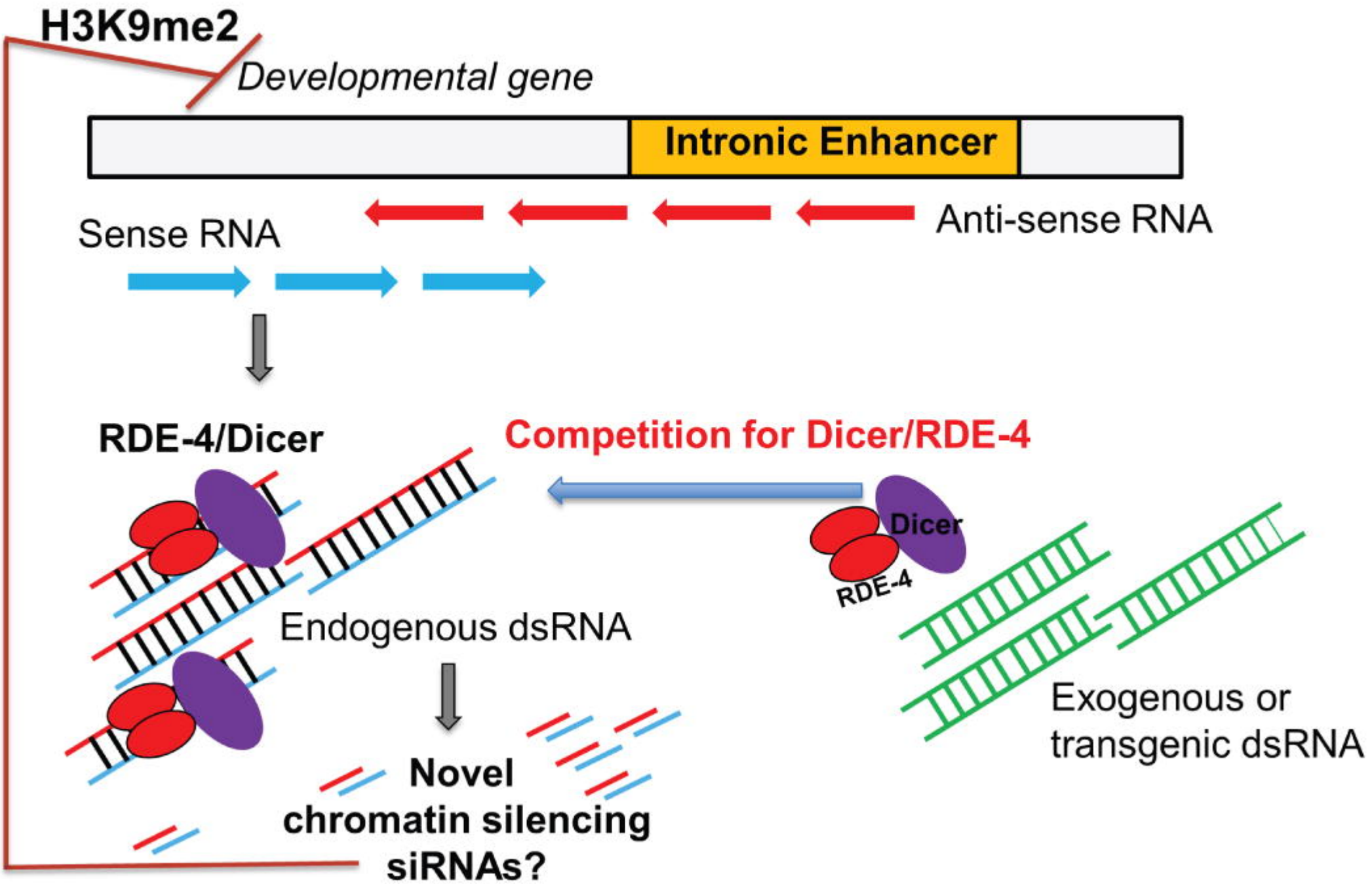

A
Chromatin Enhancer Domains
Cummulative Frequency
1.00
0.75
0.50
0.25
0.00
Genomic bins
Distal
P-val = 4.2e-07
Intragenic
P-val < 2e-16
-2
-1
0
1
2
H3K9me2 ChIP-seq Log2 FC dot-1.1;ced-3 vs ced-3
B
ATAC-seq Peaks
Cummulative Frequency
1.00
0.75
0.50
0.25
0.00
Genomic bins
Distal
P-val = 0.012
Intragenic
P-val <2e-16
-2
-1
0
1
2
H3K9me2 ChIP-seq Log2 FC dot-1.1;ced-3 vs ced-3
C
ZFP-1 targets with enhancer
H3K9 ChIP-seq RPKM (log2)
6
4
2
0
-2
***
ced-3
dot-1.1;ced-3

Figure 6


H3K9me2
Developmental gene
Intronic Enhancer
Sense RNA
Anti-sense RNA
RDE-4/Dicer
Competition for Dicer/RDE-4
Dicer
RDE-4
Endogenous dsRNA
Exogenous or
transgenic dsRNA
Novel
chromatin silencing
siRNAs?