DOT1L complex suppresses transcription from enhancer elements and ectopic RNAi in Caenorhabditis elegans

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ABSTRACT

Methylation of histone H3 on lysine 79 (H3K79) by DOT1L is associated with actively transcribed genes. Earlier, we described that DOT-1.1, the Caenorhabditis elegans homolog of mammalian DOT1L, cooperates with the chromatin-binding protein ZFP-1 (AF10 homolog) to negatively modulate transcription of highly and widely expressed target genes. Also, the reduction of ZFP-1 levels has consistently been associated with lower efficiency of RNA interference (RNAi) triggered by exogenous double-stranded RNA (dsRNA), but the reason for this is not clear. Here, we demonstrate that the DOT1L complex suppresses transcription originating from enhancer elements and antisense transcription, thus potentiating the expression of enhancer-regulated genes. We also show that worms lacking H3K79 methylation do not survive, and this lethality is suppressed by a loss of caspase-3 or Dicer complex components that initiate gene silencing response to exogenous dsRNA. Our results suggest that ectopic elevation of endogenous dsRNA directly or indirectly resulting from global misregulation of transcription in DOT1L complex mutants may engage the Dicer complex and, therefore, limit the efficiency of exogenous RNAi.

Keywords: DOT1L; ZFP-1/AF10; H3K79; enhancers; antisense RNA; RNA interference

INTRODUCTION

Screens for chromatin-binding factors involved in RNAi in Caenorhabditis 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; Cui et al. 2006; Lehner et al. 2006). In our attempt to identify proteins interacting with ZFP-1 that could explain its role in RNAi, we previously identified the C. elegans homolog of the mammalian H3K79 methyltransferase DOT1L (Dot1 (yeast) -Like), which we named DOT-1.1 (Cecere et al. 2013). The mammalian homolog of ZFP-1, AF10, is a frequent fusion partner of MLL in chimeric proteins causing leukemia (Meyer et al. 2018), and its interaction with DOT1L is critical for recruitment of the latter to the oncogenes HOXA9 and MEIS1 and their subsequent activation (Okada et al. 2005).

Although DOT1L is the only H3K79 methyltransferase in mammals and H3K79 methylation is present on actively transcribed genes, inhibition of DOT1L methyltransferase activity does not result in dramatic changes in gene expression in cultured cells (Zhu et al. 2018). However, expression of specific genes, such as HOXA9 and MEIS1, 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 (Ng et al. 2002; Katan-Khaykovich and Struhl 2005). Importantly, knockout of DOT1L in mice results in embryonic lethality, underscoring its important developmental function (Nguyen et al. 2011). In Drosophila, both DOT1L (Grappa) and AF10 (Alhambra) mutants show developmental defects (Shanower et al. 2005; Mohan et al. 2010). In C. elegans, we observed specific developmental abnormalities in a zfp-1 reduction-of-function

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mutant, such as defects in neuronal migration (Kennedy and Grishok 2014), and implicated zfp-1 in lifespan control (Mansisidor et al. 2011). Whether the antisilencing effect of DOT1L contributes to its developmental role is presently unknown.

We previously established that ZFP-1 and DOT-1.1 colocalize to promoters of highly and widely expressed genes and negatively modulate their expression during C. elegans development (Cecere et al. 2013). Here, we demonstrate the role of the ZFP-1/DOT-1.1 complex in enhancer regulation. Enhancer elements are themselves transcription units, generating noncoding 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 find that ZFP-1 negatively modulates transcripts that originate from enhancer elements, similarly to the negative modulation of active genes described earlier (Cecere et al. 2013). Moreover, we demonstrate that antisense transcription is especially apparent at tissue-specific and developmental genes that contain intragenic enhancers, and many of them rely on the DOT1L complex for transcriptional activation, similarly to HOX9 and MEIS1 in mammals. Importantly, we found that complete lack of H3K79 methylation is incompatible with viability, and that loss-of-function of the RNAi pathway genes, rde-4, encoding a dsRNA-binding protein member of the Dicer complex (Tabara et al. 2002), and rde-1, encoding an Argonaute that binds small interfering RNAs (siRNAs) produced by Dicer-mediated cleavage (Tabara et al. 1999, 2002; Yigit et al. 2006), fully rescue the dot-1.1 deletion mutant lethality. Taken together, our results suggest that DOT-1.1 loss leads to elevation of endogenous dsRNA and siRNA levels, which is detrimental for C. elegans development. Also, the apparent RNAi deficiency at zfp-1 reduction-of-function conditions (Dudley et al. 2002; Grishok et al. 2005; Kim et al. 2005; Cui et al. 2006; Lehner et al. 2006) might be explained by the excess endogenous dsRNA substrates competing for the Dicer complex.

RESULTS
ZFP-1 and DOT-1.1 localize to predicted and validated 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 these domains with ZFP-1 and DOT-1.1 chromatin localization peaks to determine in which genomic regions the ZFP-1/DOT-1.1 complex is most dominant. 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 (Fig. 1A; Supplemental Fig. S1A), in line with our previous observation of preferential binding of the complex to promoter regions (Cecere et al. 2013). Interestingly, similar levels of ZFP-1/DOT-1.1 enrichment are also observed for domains corresponding to predicted enhancers (Fig. 1A,B; Supplemental Fig. S1A,B; see “Analysis of chromatin domains and ATAC-seq peaks” in Materials and Methods for our definitions of “distal” and “intragenic” enhancer elements). These domains have chromatin signatures characteristic of enhancers, such as enrichment of H3K4me1 and H3K27ac (Bonn et al. 2012). In addition, both promoters and enhancers are often devoid of nucleosomes and hence amenable to be identified by techniques assessing open chromatin regions, such as DNase I hypersensitivity mapping (DNase-seq) (Ho et al. 2017), and ATAC-seq (Daugherty et al. 2017). The latter study has identified open chromatin regions in C. elegans embryos and L3 animals. In this study, approximately 5000 distal noncoding regions displayed dynamic changes in chromatin accessibility between the two developmental stages. Many of the identified DNase-seq (Ho et al. 2017) and ATAC-seq (Daugherty et al. 2017) peaks matched enhancers that were earlier found through genetic mutations and/or reporter assays (Gaudet and McGhee 2010). These include, for example, hlh-1 enhancers (Lei et al. 2009), elt-2 enhancers (Wiesenfahrt et al. 2016), and lir-1/lin-26 operon enhancers (Fig. 1B, top left; Landmann et al. 2004). Also, several of the newly annotated putative enhancers, such as those controlling mtl-8, nhr-25, and swip-10 genes (Fig. 1B), were shown to be functional in transgenic reporter assays (Daugherty et al. 2017). Importantly, we compared the locations of enhancers, either predicted through a chromatin signatures (Evans et al. 2016) or based on ATAC-seq (Daugherty et al. 2017), and found significant overlaps (Fig. 1C). Interestingly, overlap analysis has shown that ATAC-seq peaks are enriched in DOT-1.1 (Fig. 1D, left) and ZFP-1 (Fig. 1D, right) in embryos and L3 animals, respectively. Moreover, we observe ZFP-1 peaks at experimentally proven enhancers (see Fig. 1B for examples). 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.

ZFP-1/DOT-1.1 control enhancer-directed 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 controls enhancer transcription. Distal enhancers often produce 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 laboratory were instrumental in providing insight into the modulatory role of ZFP-1/DOT-1.1 in the transcription of genes targeted by the complex at promoter-proximal regions (Cecere et al. 2013). For this, we utilized a zfp-1(ok554) reduction-of-function deletion mutant, which produces a carboxy-terminally truncated ZFP-1 protein that does not interact with DOT-1.1, leading to decreased abundance of DOT-1.1 at chromatin (Cecere et al. 2013). We reanalyzed 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 (Evans et al. 2016; Daugherty et al. 2017). Nascent transcription is not limited to gene-directed transcription and is observed also at noncoding regions of the genome, including enhancers. We sought to establish if there is an increase in enhancer transcription (i.e., GRO-seq reads at enhancer elements) in zfp-1(ok554) compared to wild-type (WT) L3 animals. For this, we computed the log2-transformed fold change (FC) in GRO-seq coverage between zfp-1(ok554) and WT in all 20 chromatin domains spanning the genome (Fig. 2A, blue line; Evans et al. 2016), and also at chromatin domains corresponding to intragenic (Fig. 2A, green line) and intergenic (Fig. 2A, red line) enhancers. Importantly, the cumulative GRO-seq data frequencies for both intragenic and intergenic predicted enhancers are shifted to the right compared to “All domains” cumulative frequencies, indicating that enhancer transcription in zfp-1 mutant animals compared to WT increases more significantly at these domains than overall in the genome (Fig. 2A). Analysis of GRO-seq data with respect to enhancers defined by ATAC-seq peaks (Fig. 2B, green and red lines) also reveals a greater increase in transcription in zfp-1 mutant animals at ATAC-seq peak regions compared to all 2.5 kb-sized regions spanning the entire C. elegans genome (Fig. 2B, blue line, “All regions”). Similar results were obtained when cumulative GRO-seq data at distal and intragenic enhancers, expressed in reads per kilobase per million mapped (RPKM), were compared between zfp-1 mutant and WT animals (Supplemental Fig. S2A,B); specific examples are shown in Figure 2C,D. These observations indicate that, in addition to negatively modulating target gene transcription via promoter-proximal binding,
the ZFP-1/DOT-1.1 complex regulates the production of RNAs originating from both intragenic and distal enhancers.

**Reduction of zfp-1 function elevates expression of genes regulated by promoter-proximal binding of ZFP-1 and reduces expression of genes with ZFP-1 binding exclusively at their bodies**

Intragenic enhancer transcription has 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 sought to analyze cumulative GRO-seq coverage along gene bodies of enhancer-hosting genes. To do this, we had to take into account the fact that genes hosting enhancers could also be bound by ZFP-1/DOT-1.1 at their promoters. Therefore, both sense transcription and antisense transcription were analyzed and each gene was considered either bound or not bound by ZFP-1, both at the promoter region and at the coding region. The results of GRO-seq analyses of genes with promoter-bound ZFP-1 are presented in Figure 3A (top and middle panels), whereas Figure 3B (top and middle panels) shows GRO-seq data for genes not containing ZFP-1 ChIP-seq peaks at their promoters. To compare changes in the levels of nascent transcription with those of mature transcripts, we performed a microarray analysis of mRNA expression in WT and zfp-1(ok554) L3 larvae (Fig. 3A,B, bottom panels). Overall, all genes bound by ZFP-1/DOT-1.1 at the promoters displayed a significant increase of expression in zfp-1(ok554) compared with WT (Fig. 3A, top and bottom panels), which is consistent with our earlier analyses of the GRO-seq data (Cecere et al. 2013), and now confirmed by the microarray. In genes with ZFP-1 also binding within gene bodies, both sense and antisense transcription were increased in zfp-1(ok554) compared with WT, and this effect was unaffected by the presence of chromatin enhancer signatures (Fig. 3A, top and middle panels, compare third and fourth bars). Noteworthy, the elevation of mRNA expression in the mutant was still significant for these genes (Fig. 3A, bottom panel, third and fourth bars). On the contrary, GRO-seq changes in the zfp-1 mutant compared with WT at genes targeted by ZFP-1 intragenically, and not at the promoter, were most prominent in the subgroup with enhancer chromatin signatures (Fig. 3B, top and middle panels, compare fourth bar to the rest). In this case, both sense and antisense transcription were significantly elevated, but, interestingly, the mRNA levels of these genes were not overall affected (Fig. 3B, bottom panel, fourth bar). ZFP-1 target genes lacking enhancer signature displayed no significant change in GRO-seq reads (Fig. 3B, top and middle panels, third bar). Overall, genes not targeted by ZFP-1/DOT-1.1 at the promoter tend to decrease in expression in zfp-1(ok554) compared with WT (Fig. 3B, bottom, all bars; Cecere et al. 2013). The described observations suggest that a negative modulation of transcription by the ZFP-1/DOT-1.1 complex results in both negative and positive effects on gene expression (mRNA levels). Earlier, we described the role of promoter-bound ZFP-1/DOT-1.1 complex in suppressing growth-related and other widely expressed genes (Cecere et al. 2013) that largely correspond to genes analyzed in Figure 3A here. Now, we find that ZFP-1 binding at the gene bodies is beneficial for their expression and suggest that ZFP-1 facilitates gene expression by attenuating antisense.
transcription at these loci (Fig. 3B). Moreover, the observed increase in bidirectional transcription at genes harboring enhancers suggests endogenous dsRNA formation, which may affect tissue-specific mRNA levels. Of note, mRNA expression profiling was performed on whole-body RNA extracts, and therefore tissue-specific changes in transcriptional output resulting from compromised enhancer activity may be masked by mRNA species contributed by other tissues. Also, in light of a previous study in which transcription at intragenic enhancers in mouse embryonic stem cells was found to interfere with host gene transcription (Cinghu et al. 2017), we assume that, also in *C. elegans*, intragenic enhancers regulate the expression of genes in which they are embedded. However, it is possible that some enhancers located within genes serve as distal enhancers for other transcription units.

To gain more experimental evidence in support of the idea that ZFP-1 and DOT-1.1 regulate genes through enhancer-binding, we selected eight genes that: (i) harbor predicted enhancer elements bound by ZFP-1 within their bodies, (ii) show elevation in antisense GRO-seq reads in *zfp-1(ok554)* compared with WT, and (iii) whose mRNA levels decrease in *zfp-1(ok554)* according to the microarray data (Fig. 4A; Supplemental Table 1). We analyzed the expression of these genes by RT-qPCR using new RNA samples from L3 stage WT, *zfp-1(ok554)*, *dot-1.1(gk520244)*, and *dot-1.1(gk105059)* larvae (see Materials and Methods for *dot-1.1* reduction-of-function alleles description). Five out of eight genes that we selected showed reduced expression in all three mutants (Fig. 4B), and the rest were down-regulated in at least one mutant background. Notably, the F49E12.12 gene, which shows the highest level of ZFP-1 enrichment at its intronic enhancer (Fig. 3A, top left), exhibited the most prominent dependence on ZFP-1 and DOT-1.1 for its expression (Fig. 4B, left bars).

We conclude that binding of the DOT-1.1/ZFP-1 complex is important for expression of genes harboring enhancers (most notably intronic) in their bodies.

**FIGURE 2.** ZFP-1/DOT-1.1 negatively modulate transcription from enhancers. (A) Cumulative distribution of log2-transformed fold changes of GRO-seq RPKM (reads per kb per million reads) values at chromatin domains (either “all domains” [1–20], or “enhancer domains” [8–10]) between *zfp-1(ok554)* and WT larvae. Raw GRO-seq reads were downloaded from NCBI GEO database (GSE47132), aligned to genome assembly WS220/ce10, normalized, and counted in chromatin domains (1–20 or 8–10). The “Intragenic” and “Distal” enhancer domains derived from domains 8–10 were defined as described in Materials and Methods. The P-values were determined by two-sided two-sample Kolmogorov–Smirnov tests (enhancer domains compared with all chromatin domains). (B) Cumulative distribution of log2-transformed fold changes of GRO-seq RPKM values at genome-wide regions (all regions) and ATAC-seq peaks (Daugherty et al. 2017) between *zfp-1(ok554)* and WT larvae. Genome-wide regions were defined by dividing the genome into windows of 2.5 kb. The P-values were determined by two-sided two-sample Kolmogorov–Smirnov tests (ATAC-seq peaks compared with all regions). (C) UCSC genome browser snapshots show representative examples of increased GRO-seq coverage in *zfp-1(ok554)* compared with WT at a distal enhancer region (compare gray, WT, with yellow, *zfp-1(ok554)*, [−] strand transcription peaks, and black, WT, with red, *zfp-1(ok554)*, [+] strand transcription peaks). (D) Same as C for a genic region.
ZFP-1/DOT-1.1 control enhancer-containing neuronal genes

The types of genes containing ZFP-1-bound enhancers (Fig. 4) are distinct from widely and highly expressed genes regulated by promoter-bound ZFP-1/DOT-1.1 (Cecere et al. 2013). We hypothesized that enhancer-controlled genes may exhibit tissue-specific or temporally regulated expression. Such genes are often switched on and off in time and space, and enhancer elements play an important role in their regulation. Therefore, we performed pathway overrepresentation analysis of C. elegans genes characterized by the presence of enhancer chromatin signature and found them to be enriched in GPCR signaling, ion channel transport, and genes with neuronal function, such as neurotransmitter release (Fig. 5). Indeed, genes included in the RT-qPCR analyses described in Figure 4 largely belong to these groups (Supplemental Table 1). For example, F49E12.12 encodes an orthologue of human PGAP2 (Post-GPI Attachment to Proteins 2) that is highly expressed in the human brain and has been implicated in intellectual disability, when mutated (Perez et al. 2017). Moreover, our previous findings connected zfp-1 function with neuronal migration (Kennedy and Grishok 2014).

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 proapoptotic caspase CED-3 (homolog of mammalian caspase-3) (Xue et al. 1996), and have successfully generated a viable dot-1.1(knu339) null mutant in this mutant background (Fig. 6A,B). Upon outcrossing the ced-3(n1286) allele, we found that dot-1.1 null animals were not viable (Fig. 7).

We then hypothesized that the dot-1.1(knu339) lethality phenotype could be related to the increase in global transcription in zfp-1 mutant worms, which may lead to ectopic dsRNA and small RNA formation. In C. elegans and other organisms, exogenous dsRNA is subject to processing by the Dicer complex, which cleaves it into siRNAs (Grishok 2005). In C. elegans, the first discovered RNAi-deficient mutants included rde-1 and rde-4 (Tabara et al. 1999).

FIGURE 3. Reduction of zfp-1 function elevates expression of ZFP-1/DOT-1.1 promoter-bound genes and reduces expression of genes with ZFP-1 binding in their bodies. (A) Global changes in sense (red bars) and antisense (blue bars) transcription (GRO-seq data from Cecere et al. 2013), as well as steady-state mRNA levels (gray bars, microarray data) between zfp-1(ok554) and WT L3 larvae (log2-transformed fold change is shown on y-axis, values represent mean and 95% confidence interval), for genes bound by ZFP-1 at the promoter (green circle with “Z” next to the gene body cartoon). The ZFP-1 promoter-bound genes are further separated according to two additional criteria: (i) no ZFP-1 binding (cartoons 1 and 2) or ZFP-1 binding (cartoons 3 and 4) at the coding region, and (ii) enhancer signature (yellow box with “E”) present (cartoons 2 and 4) or absent (cartoons 1 and 3). The number of genes in each group is indicated below the cartoons. The “all genome” group was defined by dividing the genome into windows of 2.5 kb. (B) Similar analyses for genes that do not contain ZFP-1 peaks at their promoters. The differences between each group of genes and the group denoted as “all genome” were determined by Wilcoxon rank-sum tests ($\text{[\*]}$, $\text{[\*\*]}$, and $\text{[\*\*\*]}$ denote P-values <0.05, <0.01, and <0.001, respectively).
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). The RDE-4 protein binds long dsRNA and assists Dicer in the processing reaction (Tabara et al. 2002; Parker et al. 2006). The RDE-4 and RDE-1 proteins are also important for the antiviral response (Wilkins et al. 2005) and thought to have a limited role in endogenous RNAi. To determine if RDE-4/RDE-1 pathway mutants suppress the lethality of dot-1.1 (knu339), we crossed the double dot-1.1;ced-3 mutant strain with loss-of-function mutants for rde-4 (Fig. 7A) and rde-1 (Fig. 7B). Excitingly, the double dot-1.1 (knu339);rde-4(ne301) and dot-1.1(knu339);rde-1(ne219) mutant worms were homozygous viable and did not require the presence of the ced-3 mutant allele for viability (Fig. 7A,B). 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 (knu339) was not rescued (Fig. 7C). These results strongly suggest that the lethality phenotype associated with dot-1.1 deletion is due to generation of small RNAs from some ectopic dsRNA.

Our genetic data might explain the apparent RNAi deficiency observed under zfp-1 reduction-of-function conditions (Dudley et al. 2002; Grishok et al. 2005; Kim et al. 2005; Cui et al. 2006; Lehner et al. 2006) when Dicer is likely engaged with ectopic dsRNA produced from the genome and therefore is not capable of efficient processing of externally provided dsRNA meant for RNAi initiation (Fig. 8A,B). Remarkably, a recently described increase in influenza virus production after DOT1L inhibition in mammalian cells (Marcos-Villar et al. 2018) is consistent with our data and suggests that the role of DOT1L in endogenous dsRNA control is conserved.

**DISCUSSION**

In summary, we find that, in addition to controlling transcription through promoters, as previously described (Cecere et al. 2013), the ZFP-1/DOT-1.1 complex controls enhancer-directed and antisense transcription. This finding supports the novel concept of the fluidity of enhancer/promoter states. Furthermore, we link DOT1L-mediated transcriptional control to suppression of dsRNA formation and small RNA generation. Notably, we have recently described enhancer-matching small RNAs likely produced through dsRNA and/or eRNA degradation in *C. elegans* and correlated their production with H3K9me3 deposition at enhancers (Gushchanskaia et al. 2019). We hypothesize that the ZFP-1/DOT-1.1 complex controls generation of such small RNAs, and it will be important to directly test this in the future.

**Commonality between promoters and enhancers**

In recent years, genomic and transcriptomic data from high-throughput sequencing studies have been shedding light on the importance of noncoding genomic regulatory regions, including enhancers. These are typically demarcated by certain histone modifications, such as H3K4me1 and H3K27ac, and open chromatin configuration. Both these features have been extensively used to predict enhancer coordinates in a variety of organisms. Importantly, 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. 2018). Now, some enhancers are thought to act as weak promoters, while, conversely, bidirectional promoters are often viewed as strong enhancers (Mikhaylichenko et al. 2018). Thus, the commonalities between enhancers and promoters may extend to how their transcriptional output is controlled by the epigenetic machinery of the cell, including chromatin writers, such as histone methyltransferases and histone acetyltransferases.

We had previously demonstrated that ZFP-1/DOT-1.1 exert a negative modulatory role on the transcription of genes with the 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 evidence that the C. elegans DOT1L complex also modulates the enhancer-directed expression of genes subject to tissue and time-specific control. Of note, the mammalian 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). Since enhancers play important roles in the regulation of these processes, it is possible that DOT1L assists promoter–enhancer cooperativity at key developmental transitions in diverse species.

Enhancer transcription control and oncogenic function of DOT1L

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 nonoverlapping active distal enhancers in acute myeloid leukemia cell lines, and 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.

Homeobox loci with an indisputable role in malignant transformation, such as HOXA9 and MEIS1, display both coding and noncoding transcription, including antisense transcripts (Sessa et al. 2007; Popovic et al. 2008), and DOT1L activity may play a yet unappreciated role in the control of such transcripts, similarly to our findings in C. elegans. Interestingly, a sequence conserved in vertebrate Hox gene introns was reported to function as enhancer element in Drosophila (Haerry and Gehring 1996; Keegan FIGURE 5. ZFP-1/DOT-1.1 control neurotransmitter receptor genes proximal to or harboring enhancer elements. Pathway overrepresentation analysis of C. elegans genes (WS220/ce10 assembly) with enhancer chromatin domains (8–10) using the ReactomePA Bioconductor R package (Yu and He 2016). The x-axis shows the ratio of the number of genes proximal to or harboring enhancer domains (8–10) compared to the number of genes in each pathway. Dot sizes correspond to the number of genes in each pathway. Dot colors represent the P-values corresponding to each pathway. Some pathways include overlapping sets of genes.

FIGURE 6. Generation of a viable dot-1.1 deletion strain in an apoptosis-deficient background. (A) Western blotting images show the absence of the DOT-1.1 protein in a mutant strain in which the dot-1.1 locus was deleted by CRISPR/Cas9 (see Materials and Methods for deletion description). (B) H3K79me2 depletion in dot-1.1 deletion mutant compared with the background ced-3(n1286) strain, western blots with antibodies specific to H3K79 dimethylation or specific to histone H3 regardless of its modification status are shown.
et al. 1997), further supporting the conservation of enhancer-control mechanisms. Moreover, the c-myc (Nepveu and Marcu 1986) and N-myc (Krystal et al. 1990) oncogenic loci display antisense transcription, and exciting new findings implicate DOT1L in cancers underlain by c-Myc and N-Myc activation, particularly breast cancer (Cho et al. 2015) and neuroblastoma (Wong et al. 2017). Overall, the contribution of antisense transcription to malignant transformation has been gaining attention in the past few years (Balbin et al. 2015; Wenric et al. 2017). Therefore, our C. elegans findings call for the role of antisense and noncoding transcription in cancer to be appreciated in the context of DOT1L activity.

**DOT1L and control of neural genes**

In mammals, a set of enhancer elements characterized by the presence of the H3K4me1 mark and binding of the coactivator CBP mediate activity-dependent transcription in neurons, and this transcription is bidirectional (Kim et al. 2010; Malik et al. 2014). Also, DOT1L targets genes expressed in the cerebellum and primes neuronal layer identity in the developing cerebral cortex (Bovio et al. 2018; Franz et al. 2019). We observed that C. elegans genes characterized by the presence of enhancer chromatin signatures are enriched in neuronal gene categories. Therefore, DOT1L likely operates at enhancers involved in neuronal development and activity from nematodes to humans.

**DOT1L, RNAi, and cell death control**

We find that dot-1.1 deletion mutants are not viable, which is in line with the embryonic lethality observed upon DOT1L knockout in mice (Nguyen et al. 2011). In addition, DOT1L is required to maintain genomic and chromosomal stability, thereby preventing cell death (Giannattasio et al. 2005; Lazzaro et al. 2008; Tatum and Li 2011; Zhu et al. 2018). Interestingly,
death are connected and involved in the lethality phenotype elicited by dot-1.1 knockout.

Formation of dsRNA is a common feature of multiple gene suppression phenomena known collectively as RNAi, which may be endogenous or exogenous. Importantly, the production of both endogenous (endo-siRNAs) and exogenous (exo-siRNAs) small interfering RNAs requires the activity of DCR-1/Dicer (Grishok 2013). Our results suggest 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 might explain the implication of zfp-1 in RNAi (Dudley et al. 2002; Grishok et al. 2005; Kim et al. 2005; Cui et al. 2006; Lehner et al. 2006). What could be the source of ectopic dsRNA accumulating in worms lacking DOT-1.1? One possibility is that elevated bidirectional transcription may lead to dsRNA accumulation in the nucleus (Fig. 8A). Another is that gene expression misregulation might cause an increase in cellular dsRNA formation indirectly. For example, a massive increase in dsRNA derived from mitochondrial DNA upon depletion of factors involved in its degradation has recently been described in mammalian cells (Dhir et al. 2018). Notably, this triggered type I interferon response.

dsRNA control by ADARs and DOT1L

It is well established that C. elegans ADARs (double-stranded RNA-specific adenosine deaminases) compete for dsRNA with the DCR-1/RDE-4/RDE-1 pathway, and that rde-4 and rde-1 mutants suppress developmental phenotypes associated with ADAR null animals (Tonkin and Bass 2003; Warf et al. 2012; Reich et al. 2018). Here, we demonstrate that the dot-1.1 mutant lethality is also suppressed by rde-1(−) and rde-4(−), although the mechanistic details of presumed dsRNA accumulation in dot-1.1(−) remain to be explored. Regardless of whether ectopic dsRNA is a direct or indirect consequence of dot-1.1 loss, a similar phenomenon might occur in mammalian cells (Marcos-Villar et al. 2018). However, in this case, the ectopic dsRNA accumulation might activate the interferon response rather than the RNAi pathway.

Overall, our findings open numerous new directions for mechanistic research of nuclear dsRNA and RNAi controlled by DOT1L in connection with enhancer function in C. elegans and other species.

MATERIALS AND METHODS

Strains
Strains were maintained at 20°C under standard conditions (Brenner 1974). Bristol N2 was the WT strain used. The following strains were obtained from the Caenorhabditis Genetics Center (CGC):
Bristol N2 (WT), VC40220, containing dot-1.1(gk520244) I (Thompson et al. 2013), VC20674, containing dot-1.1(gk105059) I (Thompson et al. 2013), RB774 - zfp-1(gk554) III, WM49 - rde-4 (ne301) III, VC20787, containing mmi-1(gk402844) III (Thompson et al. 2013), MT3002 - ced-3(n1286) IV, WM27 - rde-1(ne219) V. The dot-1.1 null mutant strain COP1302, dot-1.1 [knu339 - (pNU1092 - KO loxP::hygR::loxP)] I; AGK782: dot-1.1 [knu339 - (pNU1092 - KO loxP::hygR::loxP)] I; AGK784: - dot-1.1(gk105059) I, outcrossed four times from VC40220; gk520244 (II185T) is a point mutation in the histone methyltransferase domain of DOT-1.1.

agt-1 (modEncode, USQ00110, 1:5000), rabbit anti-DOT-1.1 (modEncode, SDQ3964, 1:5000), rabbit anti-H3 (Millipore, 05-928, 1:5000), and rabbit anti-3K79me2 (Millipore, 04-835, 1:5000).

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 by the Trizol reagent protocol (Invitrogen) followed by miRNAeasy mini column (Qiagen). The RNA integrity was confirmed using a 2100 Bioanalyzer (Agilent Technologies). Samples were submitted to the BU Microarray and Sequencing Resource Core Facility for labeling and hybridization 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 multivariate average (RMA) (Irizarry et al. 2003) 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. All samples had similar quality metrics, including mean relative log expression (RLE) (0.14–0.23 for all samples), and AUC values >0.8. Fold change values were computed and log2-transformed. The results were submitted to the NCBI GEO database (GSE115677).

For RT-qPCR experiments, cDNA was generated from 1–2 μg of total RNA using random hexamers and Maxima Reverse Transcriptase (Thermo Fisher Scientific). Quantitative PCR was performed on the Viia 7 Real-Time PCR System (Life Technologies) using the Quantifast SYBR Green PCR Kit (Qiagen). Thermocycling was done for 40 cycles in a two-step cycling in accordance with the manufacturer’s instructions and each membrane was blocked with blocking buffer (5% nonfat dry milk in TBS-T buffer) at room temperature for 1 h. Subsequently, it was incubated with an appropriate primary antibody overnight at 4°C and with a secondary antibody for 2 h at room temperature. Three washes with TBS-T buffer were made between and after the incubation with the antibodies. The membrane was developed with the SuperSignal West Femto kit (Thermo Fisher) and scanned with a KwikQuant Imager (Kindle Biosciences). The antibodies used are as follows: mouse anti-actin (Millipore, MAB1501R, 1:5000), rabbit anti-DOT-1.1 (modEncode, SDQ3964, 1:5000), rabbit anti-H3 (Millipore, 05-928, 1:5000), and rabbit anti-3K79me2 (Millipore, 04-835, 1:5000).

TABLE 1. Sequences of primers used to genotype the dot-1.1(knu339) allele

| Primer | Sequence | Description |
|--------|----------|-------------|
| CEH5154 | Tcggcttttgccgcccattttt | F dot-1.1 knu339 |
| CEH4350 | CTCGTCCGAGGGCAAGGAATA | R hygR |
| Left arm PCR, 1265 bp band in dot-1.1(knu339) mutant (no amplification in WT) | | |
| CEH4929 | GGTGCTGACACACAGATACGCT | F dot-1.1 WT |
| CEH5105 | ttcattaaaaacagggattttctggg | R dot-1.1 |
| Left arm PCR, 796 bp band in WT [no amplification in dot-1.1(knu339) mutant] | | |
| CEH4672 | CTTTTCAATCCGGAATGAAGgtaatgg | F linker intron |
| CEH5105 | ttcattaaaaacagggattttctggg | R dot-1.1 |
| Right arm PCR, 895 bp band in dot-1.1(knu339) mutant (no amplification in WT) | | |
| CEH4672 | GGTGCTGACACACAGATACGCT | F linker intron |
| CEH3896 | ttcattaaaaacagggattttctggg | R backbone |
| Right arm PCR, 721 bp band in WT [no amplification in dot-1.1(knu339) mutant] | | |
PCR reaction was performed in triplicate. Changes in mRNA expression were quantified using act-3 mRNA as reference. The primers used for RT-qPCR shown in Figure 4 are listed in Supplemental Table 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 (mixed-stage embryo), ZFP-1 ChIP-chip (mixed-stage embryo) and ZFP-1 ChIP-seq (third larval stage, L3) peak coordinates were obtained from modENCODE (modENCODE_2970, modENCODE_3561, 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 DOT-1.1/ ZFP-1 peak was located within the coordinates of the region. Putative enhancer regions at L3 were obtained by combining coordinates of domains 8, 9, and 10, annotated as intronic, intergenic and weak enhancers, respectively (Evans et al. 2016). Enhancer domains located 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 <15 kb 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 (Riemondy et al. 2017). Genomic intervals obtained by the analysis were uploaded to the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgTracks?hgS_doOtherUser=submit&hgS_otherUserName=ruben.esse&hgS_otherUserSessionName=Esse_et_al_DOT1L_enhancers_manuscript).

**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 2019). 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. Coverage tracks were uploaded to the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgTracks?hgS_doOtherUser=submit&hgS_otherUserName=ruben.esse&hgS_otherUserSessionName=Esse_et_al_DOT1L_enhancers_manuscript).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

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