The enemy within: intronic miR-26b represses its host gene, ctdsp2, to regulate neurogenesis

Jinju Han, Ahmet M. Denli, and Fred H. Gage
Laboratory of Genetics, Salk Institute for Biological Studies, La Jolla, California 92037, USA

Differentiation of multipotent stem cells occurs through the highly coordinated control of gene expression. Repressor element 1 (RE1) silencing transcription factor (REST), a master transcriptional regulator in neuronal stem cells, restricts neuronal gene expression. REST activity is context-dependent and is modified by its cofactors, such as Ctdsp2. In this issue of *Genes & Development*, Dill and colleagues (pp. 25–30) report on the microRNA-mediated regulation of neural differentiation. Interestingly, this microRNA is post-transcriptionally regulated and modulates expression of its host gene, *ctdsp2*.

REST recognizes 21- to 23-base-pair (bp) consensus RE1 sites that are located at regulatory regions of neural genes and directly binds to DNA through its zinc finger domains. It also recruits chromatin remodeling factors to form heterochromatin and interacts with C-terminal domain small phosphatases (CTDSPs) to inhibit RNA polymerase II [Pol II] activity for the suppression of neural gene expression [Ooi and Wood 2007].

CTDSPs are C-class phosphatases that act on Ser 5 of the Pol II C-terminal domain (CTD) to decrease transcription [Yeo et al. 2005]. The zebrafish and mammalian genomes encode at least three *ctdsp* genes. CTDSPs are expressed in diverse organs [except the CNS] and interact with the REST complex. CTDSP enzymatic activity is required for REST function, and knockdown of CTDSP induces the neuronal differentiation of mouse embryonic carcinoma P19 cells. CTDSP2 has been shown to enhance TGF-β signaling by the dephosphorylation of SMAD proteins and attenuates androgen receptor-mediated transcription [Thompson et al. 2006; Wrighton et al. 2006]. In neuronal cells, expression of CTDSP is decreased at least in part by a neuron-specific small noncoding RNA: miR-124 [Visvanathan et al. 2007].

In this issue of *Genes & Development*, Dill et al. (2012) report that an additional microRNA (miRNA), miR-26b, regulates Ctdsp2 expression during neuronal differentiation. Interestingly, miR-26b is located in an intronic region of the *ctdsp2* gene, and the miRNA is transcribed together with its host gene. Dill et al. (2012) demonstrate that post-transcriptional regulation of miR-26b expression allows for the cell type-specific repression of *ctdsp2*, providing the first experimental evidence of host gene regulation by an intronic miRNA.

*ctdsp2* regulation by its intronic miRNA, miR-26b

miRNAs are small noncoding RNAs of ~22 nucleotides [nt] that control gene expression at the post-transcriptional level. miRNAs are expressed in a spatiotemporally specific manner and induce decay of target transcripts or translational suppression by binding to the 3’ untranslated region [UTR] of target transcripts. Maturation of most miRNAs takes place through a two-step process. When a miRNA gene is transcribed, the primary transcript, called primary miRNA (pri-miRNA), forms a local hairpin structure. The nuclear ribonuclease III (RNase III) Drosha cuts the hairpin structure and releases a short hairpin-shaped RNA of ~70 nt, called pre-miRNA. The pre-miRNA is exported to the cytoplasm and converted into functional, mature miRNA by the cytoplasmic RNase III Dicer [Kim et al. 2009].
Most miRNAs are located in intragenic regions of the genome, especially in introns (Rodriguez et al. 2004). Usually, intragenic miRNAs share promoters with their host genes, so that transcription of miRNAs occurs together with the host genes (Baskerville and Bartel 2005). From one RNA transcript, two different products are generated: One is pre-miRNA cropped by Drosha during transcription, and the other one is mature host gene messenger RNA (mRNA) (Kim and Kim 2007). Since these RNAs are coexpressed both spatially and temporally, there could be a functional relationship between an intronic miRNA and its host gene (Lutter et al. 2010). Indeed, there are some experimental reports about cooperation of intronic miRNAs with their host genes for cellular function. For example, apoptosis-associated tyrosine kinase (AATK), an activator of neuronal differentiation, encodes miR-338 in its eighth intron. miR-338 mediates AATK function by suppressing antagonistic genes of neuronal differentiation, such as Nova (Barik 2008). The direct targeting of a host gene by its own intronic miRNA was predicted in the domestic dog, Canis familiaris. The zinc finger protein 265 harbors miR-186 in an intron and a predicted miR-186-binding site in its 3' UTR (Zhou et al. 2008). However, experimental proof of such regulation has been lacking.

In this issue of Genes & Development, Dill et al. (2012) illustrate the targeting of the ctdsp2 transcript by its intronic miRNA, miR-26b, during neuronal differentiation in early zebrafish development (Fig. 1). In their study, injection of synthetic miR-26b into one-cell stage zebrafish embryos reduced endogenous Ctdsp2 protein levels. When miR-26b was coinjected with reporter transcripts that encoded GFP and the ctdsp2 3' UTR, expression of GFP protein was suppressed, whereas the control reporter was not. This finding demonstrated that ctdsp2 mRNA was directly targeted by miR-26b. Functional depletion of miR-26b with morpholino antisense oligonucleotides (MO) in the gata2:GFP embryo resulted in the reduction of motor neurons, as evidenced by the decreased expression of GFP and the endogenous neuronal marker zinc.

![Image](https://example.com/image.png)

**Figure 1.** Decrease of host gene expression by intronic miRNA during neuronal differentiation. miR-26b (red), which targets ctdsp2 mRNA (green), is located in an intronic region of ctdsp2 and is cotranscribed with its host gene (black lines denote introns). The shared RNA transcript ctdsp2 pre-mRNA/pri-miR-26b generates ctdsp2 mRNA and pre-miR-26b concurrently in the nucleus. (A) In neural stem cells, ctdsp2 mRNA is translated into the Ctdsp2 protein, which contributes to the inhibition of neuronal gene expression by REST via suppression of Pol II activity on RE1 sites (denoted as a blue stretch of double helix). Translation of ctdsp2 is possible because pre-miR-26b is not processed into functional miR-26b. Stem cell-specific RNA-binding proteins and/or -modifying enzymes (denoted as modifier X in the nucleus or Y in the cytoplasm) may block pre-miR-26b processing. (B) In differentiated neuronal cells, miR-26b is processed from pre-miR-26b and prevents ctdsp2 translation. (A,B) Top portions (brown) represent a general model for host gene inhibition by intronic miRNAs.
transporter 8. Furthermore, attenuation of the neuronal differentiation markers Tuj1 and tubb5 was detected in miR-26b MO-treated fish. However, expression of the neuronal stem cell marker olig2 was not affected by miR-26b knockdown. These results demonstrate that miR-26b acts during neural differentiation rather than neural stem cell maintenance. Since a single miRNA can repress hundreds of genes, it is hard to explain phenotypes that are generated by a miRNA based on just one target gene. However, it is possible to validate which gene is a major target of the miRNA and is responsible for the phenotypes. Dill et al. (2012) performed double-knockdown experiments using the gata2:GFP animals to confirm that the phenotypes were due to miR-26b MO-mediated ctdsp2 mRNA derepression. Coinjection of ctdsp2 MO rescued the decreased GFP expression caused by miR-26b MO. Dill et al. (2012) were thus able to show that suppression of ctdsp2 by miR-26b is critical for neuronal differentiation.

Since miRNAs work pleiotropically, it cannot be ruled out that there are other targets of miR-26b involved in neural differentiation. Furthermore, it is possible that miR-26b targets other REST cofactors in addition to ctdsp2. For example, one of the predicted targets for miR-26 is CoREST, an essential cofactor for REST function. The verification of additional target genes that are suppressed by miR-26 during neural differentiation will improve our understanding of miRNA-mediated regulation of neurogenesis.

Post-transcriptional regulation of miRNA maturation controls host gene expression

How can an intronic miRNA that is generated with its host transcript by a single transcription event suppress the expression of its host gene? If this suppression occurs all the time, the host gene would never be expressed. To delineate the repression mechanism, Dill et al. (2012) analyzed the expression pattern of miR-26b and its host gene, ctdsp2. As shown in their Northern blot analysis, mature miR-26b production began with the process of neurulation at 24 h post-fertilization (hpf), coincident with production of miR-124, the neural cell-specific miRNA and suppressor of ctdsp mRNA. In contrast, pre-miR-26b was detected before 24 hpf— as early as in one-cell stage embryos. These data suggested that there was post-transcriptional regulation of miR-26b expression.

The spatiotemporal expression of miRNAs is determined at both the transcriptional and post-transcriptional levels. Diverse regulatory elements in the genome, including promoters, control transcription of miRNA genes. Post-transcriptional regulation of miRNA expression can occur at several stages during maturation (Siomi and Siomi 2010). General miRNA expression can be affected by changes in the activity levels of essential miRNA biogenesis components. Cell-type-specific proteins can regulate the expression of particular miRNA families by the recognition of unique motifs on precursors and mature miRNAs. The let-7 family is the best example of the post-transcriptional control of miRNA expression. In embryonic stem cells, mature let-7 is not expressed, although pri-let-7 is very abundant (Suh et al. 2004). Lin28, a stem cell-specific protein, binds to pri-let-7 and pre-let-7 through recognition of specific motifs on the loop and inhibits DROSHA and DICER processing (Heo et al. 2008; Newman et al. 2008; Viswanathan et al. 2008). In addition, the end of pre-let-7 is modified by a terminal uridylyl transferase 4 (TUT4) [Heo et al. 2009]. This modification reduces DICER processing efficiency and results in the decreased generation of mature miRNAs. Conversely, KH-type splicing regulatory protein (KSRP) enhances pri-let-7 and pre-let-7 processing by binding to the terminal loop structure of the miRNA [Trabucchi et al. 2009].

Previous studies done in different experimental systems showed miR-26b to be a substrate of KSRP and TUT4. Knockdown of KSRP reduced miR-26b expression in HeLa and NIH-3T3 cell lines [Trabucchi et al. 2009]. TUT4 added extra uridines to mature miR-26b in the A549 cell line (Jones et al. 2009), and the uridylated miR-26b could not suppress expression of its target gene, IL6. However, it is important to note that since the regulation of miRNAs is context-dependent, the effects of KSRP and TUT4 on miR-26b expression should be investigated in neural stem cells. In addition to KSRP and TUT4, other modifiers can affect miR-26b biogenesis at different steps, leading to the observations made in the Dill et al. (2012) study (Fig. 1). For instance, nuclear modifiers that recognize unique motifs on pre-miR-26b can regulate the export of pre-miR-26b. If pre-miR-26b cannot be exported into cytoplasm and is retained in the nucleus, mature miR-26b cannot be produced. Alternatively, Dicer processing can be blocked by cytoplasmic modifiers that bind to pre-miR-26b, or, as in the case of pre-let-7 regulation, Dicer processing efficiency can be decreased by TUT-mediated uridylation [Heo et al. 2008]. The discrepancy between pre-miR-26b and mature miR-26b levels may also result from the turnover rate of mature miR-26b. Mature miRNAs have been thought to be very stable. However, recent studies have shown that extracellular signals can induce the rapid degradation of specific miRNAs [Hwang et al. 2007; Krol et al. 2010]. In human and mouse liver cell lines, the adenylation of mature miR-122 by GLD2 cytoplasmic poly(A) polymerase leads to its stabilization [Katoh et al. 2009]. Similarly, unknown modifiers may work on mature miR-26b to affect its stability. Finding such modifications of miR-26b will be instrumental in understanding the nature of miR-26b regulation. Further experimentation in cell culture systems may be useful to identify miR-26b targets and post-transcriptional regulators.

Perspective

Expression correlations between mature miRNA and miRNA precursors have been made in cancer cell lines [Thomson et al. 2006; Lee et al. 2008]. While miRNA precursor expression status is fairly stable, mature miRNA expression patterns do change depending on cellular status. These widespread expression changes suggest that
there may be other miRNA candidates that can be regulated at the post-transcriptional level during differentiation. The post-transcriptional regulation of miRNA maturation has been observed in stem cell populations. It is possible that the candidate miRNAs may be expressed in stem cells and decrease with differentiation (Fig. 2). These kinds of regulation can occur for intronic miRNAs as well as intergenic miRNAs.

As discussed, the intronic miRNA of ctdsp2, miR-26b, contributes to the down-regulation of host gene expression in differentiated neurons. In neural stem cells, miR-26 is repressed, and Ctdsp2 collaborates with REST to inhibit neural gene expression. However, what triggers pre-miR-26b processing to generate mature miR-26b remains to be determined.

The miR-26 family is composed of miR-26a and miR-26b, which are generated from four different precursors: pre-miR-26a-1, pre-miR-26a-2, pre-miR-26a-3, and pre-miR-26b. All of the miR-26 precursors are located in intronic regions of ctdsp genes, and pre-miR-26b are coexpressed during neural differentiation. Expression of ctdsp genes is diminished in neuronal lineages during development, and their functions may be partially redundant [Zohn and Brivanlou 2001; Yeo et al. 2005]. In fact, it is highly probable that each ctdsp gene can be regulated by its own intronic miR-26. However, there may be functional differences between these family members that are too subtle to be detected by current experimental designs. In glioblastomas, human miR-26 expression correlates with CTDSPL but not with CTDSP2 [Kim et al. 2010]. Analyses of mRNA expression patterns of ctdsp genes and miR-26-binding sites in different systems may provide clues about the conservation of this regulatory mechanism between intronic miRNAs and the host gene family.

As shown by Dill et al. (2012), ctdsp2 is expressed in the presence of mature miR-26b in nonneuronal tissues. Therefore, one can speculate that different regulatory interactions may exist between ctdsp2 and miR-26b. In nonneuronal tissues, miR-26b may be responsible for the fine-tuning of ctdsp2 expression. Also, it is possible that ctdsp2 mRNA can escape from miR-26b-mediated suppression. For instance, miRNA-binding sites on a transcript can be hidden by changing tertiary structures of the 3’ UTR [Ameres et al. 2007; Kertesz et al. 2007]. Ultimately, while a regulatory relationship between miRNAs and miRNAs that are cotranscribed is a very interesting possibility, it remains to be seen how widespread this mode of regulation is.

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