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Human RNA Methyltransferase BCDIN3D Regulates MicroRNA Processing

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SUMMARY

MicroRNAs (miRNAs) regulate key biological processes and their aberrant expression may lead to cancer. The primary transcript of canonical miRNAs is sequentially cleaved by the RNase III enzymes, Drosha and Dicer, which generate 5′ monophosphate ends that are important for subsequent miRNA functions. In particular, the recognition of the 5′ monophosphate of pre-miRNAs by Dicer is important for precise and effective biogenesis of miRNAs. Here, we identify a RNA-methyltransferase, BCDIN3D, that O-methylates this 5′ monophosphate and negatively regulates miRNA maturation. Specifically, we show that BCDIN3D phospho-dimethylates pre-miR-145 both in vitro and in vivo and that phospho-dimethylated pre-miR-145 displays reduced processing by Dicer in vitro. Consistently, BCDIN3D depletion leads to lower pre-miR-145 and concomitantly increased mature miR-145 levels in breast cancer cells, which suppresses their tumorigenic phenotypes. Together, our results uncover a miRNA methylation pathway potentially involved in cancer that antagonizes the Dicer-dependent processing of miR-145 as well as other miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are short single-stranded RNA molecules (18–24 nucleotides) that posttranscriptionally interfere with gene expression in a variety of eukaryotes (Ghildiyal and Zamore, 2009). miRNAs target the RNA interference effector complex (RISC) to specific messenger RNAs (mRNAs) through partial base-paring to sequences predominantly found in the 3′ untranslated region (3′UTR). This interaction results in decreased translation of the proteins they encode and/or in the degradation of the mRNAs themselves (Fabian et al., 2010). To date, over 1,000 human miRNAs have been identified, and each of them potentially regulates many mRNAs (Friedman et al., 2009; Kozomara and Griffiths-Jones, 2011). Consequently, miRNAs have been involved in numerous cellular processes, including development, differentiation, proliferation, apoptosis, the stress response, and viral defense (Fabian et al., 2010). Importantly, altered expression of miRNAs is a common trait of cancers (Farazi et al., 2011). Indeed, deciphering regulation of miRNA expression may be important not only for diagnostic, but also for therapeutic purposes (Kasinski and Slack, 2011). Due to the way that miRNAs are generated, their expression can be regulated at different levels. The first level is transcriptional as miRNAs are synthesized from larger transcripts by RNA polymerase II or RNA polymerase III complexes. The next level is posttranscriptional, as these primary miRNA precursors (pri-miRNA) undergo at least three steps before the mature single-stranded form. The pri-miRNA is first cleaved by Drosha to release a hairpin-loop-shaped RNA called pre-miRNA (Lee et al., 2003). The loop of this pre-miRNA is further cleaved by Dicer to generate a miRNA duplex (Chendrimada et al., 2005). The miRNA duplex is dissociated, and the passenger strand is discarded, whereas the guide strand is loaded onto the Argonaute (Ago) protein to form an active RISC complex (Kawamata and Tomari, 2010). Each of these steps is potentially subjected to regulation because the rate of transcription of a given pri-miRNA does not always correlate with the levels of its mature miRNA (Thomson et al., 2006). One crucial aspect of this process is related to the 5′ terminal end of these RNA molecules. Both RNase III enzymes, Drosha and Dicer, generate 5′ ends that contain a negatively charged monophosphate group. This 5′ monophosphate is bound by specific positively charged pockets in Dicer and Ago2, and these interactions are necessary for efficient and accurate processing as well as the stability of the mature RISC complex (Frank et al., 2010; Kawamata et al., 2011; Park et al., 2011). Here, we unveil an unexpected posttranscriptional modification of the 5′ monophosphate group. Specifically, we show that the previously uncharacterized human enzyme BCDIN3D O-methylates both in vitro and in vivo the 5′ terminal monophosphate group of the precursor of miR-145, resulting in a complete loss of its negative charge. Consistent with the importance of this charge for interaction with Dicer, methylated pre-miR-145 displays reduced processing by Dicer in vitro. Accordingly, upon BCDIN3D depletion, the association of Dicer with the product of pre-miR-145 processing increases in vivo. As a result, depletion of BCDIN3D in human cells leads to lower levels of pre-miR-145 and a concomitant increase of mature

miR-145. Our results also indicate that this is not limited to miR-145 but may be responsible for the regulation of many other miRNAs. Finally, the interest of our findings is further enhanced by the potential involvement of BCDIN3D in cancer, as BCDIN3D depletion in breast cancer cells abolishes their tumorigenic phenotypes. Altogether our results uncover a human enzyme defining a miRNA methylation pathway that antagonizes the Dicer-dependent processing of miRNAs and that has potential as a therapeutic target in cancer.

RESULTS

BCDIN3D Is a Methyltransferase that Targets the 5′ Monophosphate of Nucleic Acids

Methylation of DNA and specific residues within histones plays a crucial role in the epigenetic regulation of chromatin-based processes, such as transcription and genomic organization (Goldberg et al., 2007; Klose and Bird, 2006; Kouzarides, 2007; Xhemalce et al., 2011). With the aim of identifying novel epigenetic regulators, we set up a screen for previously uncharacterized methyltransferases that target chromatin. Among our candidates were BCDIN3 and BCDIN3D, the human members of the Bin3 family of putative methyltransferases. Members of this family are found from *Schizosaccharomyces pombe* to humans and share homology within their putative S-adenosyl Methionine (SAM) binding motif (Figure 1A). The two paralogs exist from *Drosophila melanogaster* to humans, whereas lower eukaryotes possess only one Bin3 family member (Figure 1B).

We assessed the activity of purified candidate proteins by using in vitro methyltransferase assays with purified histones and nucleosomes as substrates and ^{3}H-radioactive SAM as the methyl group donor. In our assays, BCDIN3D, but not BCDIN3, showed a specific activity in the form of a radioactive band migrating at the level of histone H3 (around 17 kDa) in a SDS-PAGE gel only in the presence of nucleosomes (Figure 1C). In order to test whether BCDIN3D was targeting the tail of histone H3, we repeated the methyltransferase assay with nucleosomes in which histone H3 carried a truncation of its 31 N-terminal amino acids (H3ΔNter). As shown in Figure 1D, the radioactive band did not disappear nor changed its migration in the H3ΔNter nucleosomes, suggesting that the methylated product was not histone H3. As the only material difference between histones and nucleosomes was the DNA-601 used to assemble the nucleosomes (Huynh et al., 2005), it was possible that the methylation product was the DNA-601 itself. Staining of the SDS-PAGE gel with ethidium bromide showed that the 601-DNA does migrate in an SDS PAGE gel forming a band around 17 KDa (Figure 1E). Moreover, BCDIN3D was able to methylate the 601-DNA in the absence of any histone (Figure 1F). However, BCDIN3D did not target the canonical cytosines within CpG sites because the methylation product was still observed when the 601-DNA was premethylated to saturation with the bacterial SspI enzyme that methylates these sites (Figure 1F). Rather, BCDIN3D targeted the 5′ monophosphate generated by the EcoRV digestion used during the 601-DNA purification procedure because the methylation product was no longer observed when the 601-DNA was pretreated with alkaline phosphatase (Figure 1F). Importantly, the methyltransferase activity was intrinsic to BCDIN3D as point mutations in its SAM binding domain abolished its observed activity in our assay (Figures 1A and 1F). While we were performing these experiments, the homolog of BCDIN3D, BCDIN3, was reported to stabilize the nuclear noncoding RNA 7SK by methylating its 5′ γ-phosphate (Jeronimo et al., 2007; Shuman, 2007). In addition, we established that BCDIN3D is localized in the cytoplasm (Figures S1A–S1C available online). Finally, the activity that we observed on the 601-DNA was weak, i.e., the methylation reactions were at the limit of detection by liquid scintillation, and we needed to expose the radioactive gels on film for 2–4 weeks in order to detect a significant signal. Based on these observations, we hypothesized that the bona fide target(s) of BCDIN3D may be RNA(s) that are monophosphorylated at their 5′ end(s) as shown in Figure 1G.

BCDIN3D Affects the Levels of Precursor and Mature Forms of miR-145

We considered that micro RNAs (miRNA) were good candidates for being BCDIN3D targets. Indeed, although most primary precursors of miRNAs (pri-miRNA) are transcribed by RNA polymerase II and are 7-methyl-guanosine-capped, both the precursor miRNAs (pre-miRNA) generated by Drosha and the mature miRNAs generated by Dicer have 5′ monophosphate ends (Sarnow et al., 2006).

We first sought to determine a cellular system in which to identify the targets of BCDIN3D methylation. Because of global mRNA expression data linking BCDIN3D to breast cancer (Liu et al., 2007), we analyzed the role of BCDIN3D in a number of cellular assays relevant to this cancer. Cells from the triple negative breast cancer cell line MDA-MB-231 have the ability to grow in anchorage-independent conditions, a hallmark of cell transformation, and to penetrate through a basement membrane matrix, a key property of cellular invasion. Stable shRNA-mediated depletion of BCDIN3D in these cells reduced their ability to form colonies in soft agar medium (anchorage-independent growth assay, Figure 2A). Moreover, depletion of BCDIN3D by using this shRNA and another siRNA targeting an independent sequence of BCDIN3D (Figure S3A) significantly decreased the invasiveness of the MDA-MB-231 cells (invasion assay, Figure 2D and Figures S2C and S2D) without greatly affecting their growth and migration abilities (growth assay, Figure 2B; MTT assay, Figure S2A and migration assay, Figure 2C and Figure S2B). Importantly, reintroduction of an shResistant BCDIN3D-GFP protein into the cells expressing shBCDIN3D at levels similar to the endogenous BCDIN3D protein (Figure 2E) fully rescued the invasion defect of these cells (Figure 2F), clearly demonstrating that the observed effects are specific.

Having confirmed the relevance of BCDIN3D in breast cancer cells, we depleted BCDIN3D in the MCF-7 breast cancer cell line and monitored its effect on the levels of five mature miRNAs (miR-10b, miR-21, miR-125b, miR-145, and miR-155) that are known to be consistently deregulated in breast cancer (Iorio et al., 2005). As shown in Figure 3A, MCF-7 cells transfected with an siRNA targeting the first exon of BCDIN3D (siBCDIN3D, depletion efficiency shown in Figure S1B and Figure S3B) displayed significantly increased levels of mature miR-145 compared to MCF-7 cells transfected with nontargeting negative