BMB
Reports

BMB Rep. 2019; 52(5): 342-347
www.bmbreports.org

Enhanced anticancer effects of a methylation inhibitor by inhibiting a novel DNMT1 target, CEP 131, in cervical cancer

Dong Hyun Kim[1,5,#], Hye-Min Kim[1,#], Pham Thi Thu Huong[1, #, †], Ho-Jin Han[1,5], Joonsung Hwang[1], Hyunjoo Cha-Molstad[1], Kyung Ho Lee[1], In-Ja Ryoo[1], Kyoon Eon Kim[2], Yang Hoon Huh[3], Jong Seog Ahn[1,5,], Yong Tae Kwon[4,*], Nak-Kyun Soung[1,5,*] & Bo Yeon Kim[1,5,*]*

[1]Anticancer Agent Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju 28116, [2]Department of Biochemistry, College of Natural Science, Chungnam National University, Daejeon 34134, [3]Center for Electron Microscopy Research, Korea Basic Science Institute, Cheongju 28119, [4]Protein Metabolism Medical Research Center, Department of Biomedical Sciences, College of Medicine, Seoul National University, Seoul 03080, [5]Department of Bio-Molecular Science, KRIBB School of Bioscience, Korea University of Science and Technology (UST), Daejeon 34113, Korea

Methylation is a primary epigenetic mechanism regulating gene expression. 5-aza-2′-deoxycytidine is an FDA-approved drug prescribed for treatment of cancer by inhibiting DNA-Methyl-Transferase 1 (DNMT1). Results of this study suggest that prolonged treatment with 5-aza-2′-deoxycytidine could induce centrosome abnormalities in cancer cells and that CEP131, a centrosome protein, is regulated by DNMT1. Interestingly, cancer cell growth was attenuated *in vitro* and *in vivo* by inhibiting the expression of Cep131. Finally, Cep131-deficient cells were more sensitive to treatment with DNMT1 inhibitors. These findings suggest that Cep131 is a potential novel anti-cancer target. Agents that can inhibit this protein may be useful alone or in combination with DNMT1 inhibitors to treat cancer. [BMB Reports 2019; 52(5): 342-347]

INTRODUCTION

DNA methylation involves the addition of a methyl group to the 5′ position of the cytosine pyrimidine ring (1). This modification stably alters expression patterns of mammalian and viral genes as well as other deleterious elements that have been incorporated into the genome of the mammalian host over time (2, 3). Genomic CpG methylation patterns are established and maintained by DNA methyltransferase enzymes (DNMTs). Abnormal DNA methylation may lead to improper silencing of tumor suppressor genes, contributing to the pathogenesis of many cancers (4, 5). DNMT inhibition has been explored as an anticancer strategy under the premise that it may lead to restored expression of tumor suppressor genes. In fact, this approach has been shown to lead to significant death of cancer cells at mitotic stages, suggesting that DNMT1 could be a potential therapeutic target for cancers (6, 7). However, such mitotic cell death in response to inhibition of DNMT1 is accompanied by an increase in the number of cells with abnormal centrosome numbers and morphologies (8-10). Brain tumor cells (MO59J) treated with Zebularine, a potent inhibitor of DNMT, show significant centrosome amplification (11). Centrosomes play an important role in microtubule organization during mitosis for the formation of two daughter cells with an equal amount of DNA. Normally, centrosome duplication occurs once per cell cycle. It is a tightly regulated cellular process for cell survival including the maintenance of genomic fidelity. Aberrant centrosome duplication may result in genetic instability and contribute to tumorigenesis (12, 13). Regulation of centrosomes is complicated, involving a number of proteins. Recent studies have shown that increased expression of CEP131, a centrosomal protein, is associated with the incidence of liver (14) and breast cancers (15).

The relationship between centrosomes and inhibition of methylation has been well established. However, no studies have explained the mechanism involved (16). Our hypothesis is that DNA methylation is involved in the regulation of centrosome formation through CEP131gene regulation.

*Corresponding authors. Jong Seog Ahn, Tel: +82-43-240-6160; Fax: +82-43-240-6169; E-mail: jsahn@kribb.re.kr; Yong Tae Kwon, Tel: +82-2-740-8547; Fax: +82-2-3673-2167; E-mail: yok5@snu-.ac.kr; Nak-Kyun Soung, Tel: +82-43-240-6165; Fax: +82-43-240-6259; E-mail: soungnak@kribb.re.kr; Bo Yeon Kim, Tel: +82-43-240-6100; Fax: +82-43-240-6259; E-mail: bykim@ kribb.re.kr
†Current address: The Key Lab. of Enzyme & Protein Technology (KLEPT), Vietnam National University, 334 Nguyen Trai St., Thanh Xuan District, Hanoi 1000001, Vietnam
#These authors contributed equally to this work.

https://doi.org/10.5483/BMBRep.2019.52.5.055

Received 25 February 2019, Revised 27 March 2019, Accepted 9 April 2019

Keywords: Anti-cancer, Centrosome, CEP131, DNMT1

ISSN: 1976-670X (electronic edition)

RESULTS

DNA methylation impacts centrosome biogenesis via regulation of centrosomal genes

5-aza-2′-deoxycytidine is a well-known anticancer drug which exerts its effect by reducing protein expression following inhibition of DNA methylation. The main target of 5-aza-2′-deoxycytidine is DNMT. Previous studies have shown that DNMT1 inactivation using an inducible knockout system results in mitotic cell death associated with abnormally duplicated centrosomes (17, 18). Results of the present study confirmed that DNA methylation was involved in the regulation of centrosomes and key regulatory proteins. To confirm the relationship between centrosome regulation and changes in methylation, 5-aza-2′-deoxycytidine was used at low concentration to treat cells for 7 days. γ-Tubulin staining was then performed to confirm changes of centrosomes. Results confirmed that the number of centrosomes in surviving cells were increased following treatment with 5-aza-2′-deoxycytidine (Fig. 1A).

To identify genes associated with these changes to the centrosome following methylation inhibition, we determined expression levels of various centrosome genes. To discover genes associated with the increase in the number of centrosomes following methylation inhibition, we examined effects of DNMT1 knockdown on expression levels of 102 centrosome genes. When *DNMT1* was knockdown in HeLa CCL2 cells by shDNMT1 lentiviral shRNA, five of 102 genes showed greater than 2-fold increases in their transcripts assessed by qPCR analysis (Bioneer Ltd, Daejeon, Rep. of Korea) and confirmed by RT-PCR (Fig. 1B). Further assessment showed corresponding increases of their proteins for all five except SHKBP1 (Fig. 1C).

Next, distributions of these candidates at the centrosome were characterized by subcloning them into a GFP-tagged vector (pEGFP-C1; Clontech. Inc. USA), transfecting them into Hela CLL2 cells, and then staining cells with a centrosome-specific γ-tubulin antibody. With the exception of EB1, the GFP signal was largely co-localized with γ-tubulin (Red) to the centrosome, none more strongly than Cep131 (Fig. 1D). To confirm the effect of methylation on Cep131, changes in endogenous levels of Cep131 were observed in cells treated with shDNMT1. In these cells, intracellular distribution of Cep131 was concentrated in the centrosome and the number of centrosomes was abnormally increased (Fig. 1E). Under the condition of DNMT1 knockdown by shRNA, the accumulation of Cep131 protein at centrosomes was significantly increased compared to the control (Fig. 1F).

Cep131 is regulated by the activity of DNMTs

To characterize the role of DNMT1 in the transcriptional regulation of *CEP131*, we analyzed the promoter region of *CEP131*. A region from nucleotide (nt) −893 relative to the transcriptional start site (TSS) to the 5′ UTR was enriched with CpG islets. This might lead to higher level methylation. A luciferase reporter assay of a series of *CEP131* promoter fragments revealed that the region from nt −893 to nt +1 was essential for the basal expression of *CEP131* (Fig. 2A). The effect of methylation on Cep131 expression was determined by treating cells with 5-aza-2′-deoxycytidine. After the

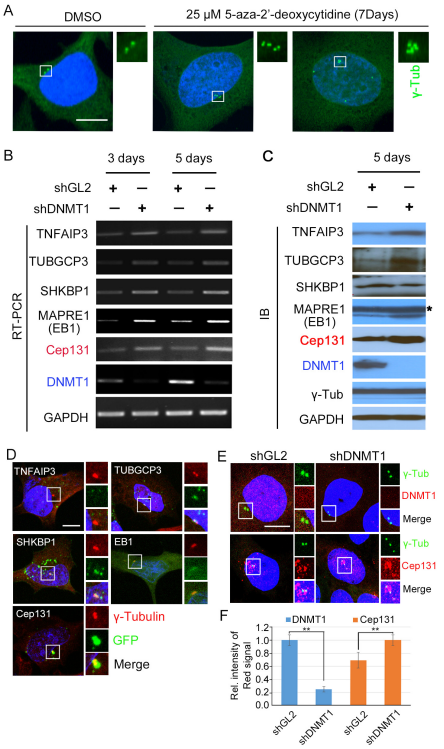

Fig. 1. Treatment with 5-aza-2′-deoxycytidine or DNMT inhibition both lead to an increase in the number of centrosomes via regulation of centrosomal genes. (A) HeLa CCL2 cells were treated with DMSO or 25 μM 5-aza-2′-deoxycytidine for 7 days followed by staining with centrosome marker protein γ-tubulin (green signal) and Heochst33342 (blue signal). (B) DNMT1-depleted HeLa CCL2 cells and shGL2 control cells were incubated for 3 or 5 days under puromycin (2 μg/ml) selection. Total RNAs were extracted and gene expression levels of the listed centrosome-related genes were compared between the two groups by qPCR. Primer sequences were designed and data were analyzed by Bioneer Ltd (Daejeon, Republic of Korea). Among tested genes, five genes showing the greatest differences in expression between treatment and control were selected as target genes regulated by DNMT1. (C) Candidates were identified by immunoblotting with indicated antibodies. (D) In addition, intracellular distributions of candidates were confirmed using a GFP-tagged form in HeLa CCL2 cells. "*" means non-specific bands. (E and F) After DNMT1 depletion by shRNA, cells were stained with anti-DNMT1 (red signal, upper panel) or anti-Cep131 antibodies (red signal, bottom panel) with γ-tubulin antibody (green signal). The relative intensity of DNMT1 or Cep131 is presented in a graph. All *t*-tests resulted in a P-value below the adjusted significance threshold. **P < 0.01. Scale bar is 10 μm.

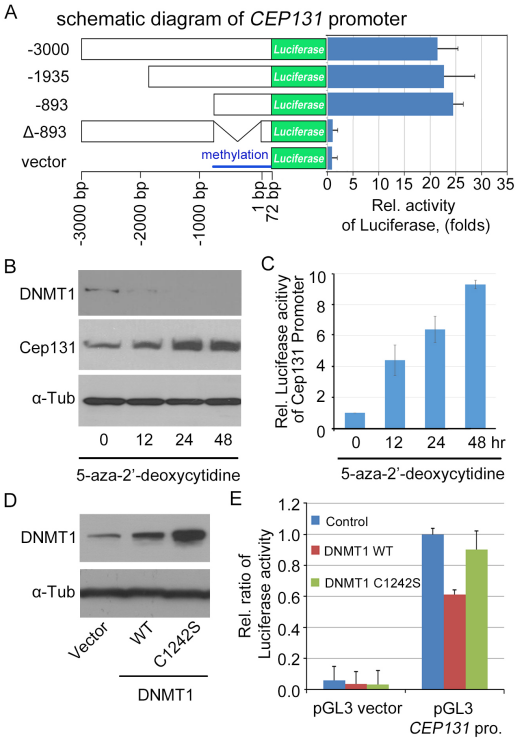

Fig. 2. Expression of Cep131 is regulated by DNMT activities. (A) *CEP131* promoter was cloned into a reporter assay vector as illustrated in the schematic diagram. Sequential truncations of the *CEP131* promoter were tested for their activities using luciferase reporter gene assay. Activities are summarized as relative luciferase activity, with vector plasmid activity set as 1.0. (B) In HeLa CCL2 cells, after treatment with 25 μM 5-aza-2′-deoxycytidine for the indicated time points, each sample was identified using indicated antibodies. (C) CEP131 promoter reporter gene activity in a luciferase assay using 5-aza-2'-deoxycytidine in a time-dependent manner is presented. (D) HeLa CCL2 cells were infected with lentiviral vectors containing wild type DNMT1 or the Cys1242Ser mutant that lacked methyltransferase activity (methyltransferase dead mutant). A lentivirus-only expressing vector was used as a negative control. Established cells were confirmed with indicated antibodies and α-tubulin blotting served as a loading control. (E) Each cell line was transfected with pGL3 vector plasmid and pGL3 *CEP131* promoter (−893 bp) plasmid. Luciferase activity was measured after 48 hours. The graph summarizes relative luciferase activity. pGL3 CEP131 promoter (−893 bp) plasmid activity in wild type DNMT1-expressing cells was set as 1.0.

treatment, Cep131 protein expression was increased along with a time-dependent decrease in the expression of DNMT1 (Fig. 2B). In addition, *CEP131* promoter activity was increased following treatment with 5-aza-2′-deoxycytidine (Fig. 2C). To further determine whether the transcription of *CEP131* was normally repressed by DNMT1-mediated methylation, we established HeLa cell lines stably expressing wild type DNMT1 and compared its expression with cells transfected with a C1242S mutant lacking enzymatic activity (19, 20). Cells expressing wild-type and C1242S DNMT1 displayed 2- and 4-fold increases in protein levels, respectively, compared with cells stably transfected with an empty vector (Fig. 2D). When luciferase construct containing the −893 bp *CEP131* promoter fragment was used for transfection, the luciferase activity was selectively inhibited by wild-type DNMT1, but not by the C1242S mutant (Fig. 2E).

DNMT1 methylates *CEP131* promoter and such methylation enhances SP1 recruitment and *CEP131* gene expression

In mammalian cells, the DNMT family consists of three methyltransferases: DNMT1, DNMT3A, and DNMT3B (21). To determine if the latter two could also affect *CEP131* expression, each DNMT protein in HeLa cells was depleted using siRNAs. Results revealed that the expression of *CEP131* was only marginally induced by knockdown of either DNMT3A or DNMT3B. The degree of induction was much smaller compared to that with DNMT1. Together, these results suggest that DNMT1 is the major methyltransferase responsible for *CEP131* regulation. The relative importance and compensatory role of DNMT3A and DNMT3B need to be further investigated (Fig. 3A).

Next, to determine whether DNMT1 could bind to the *CEP131* promoter, ChIP assays were performed using an anti-DNMT1 antibody. Results revealed that DNMT1 could directly bind to the *CEP131* promoter under the tested condition with DNMT1 binding to the positive marker gene, $p16^{INK4}$ (Fig. 3B). Results of bisulfite sequencing also revealed that approximately 34% of CpG islets located between nt −137 and nt +11 in the *CEP131* promoter were methylated. Most of these methylated CpG islets disappeared in *DNMT1*-knockdown cells. In contrast, CpG islands from nt −541 to −391 were rarely methylated (Figs. 3C and 3D). These results suggest that the region spanning nt −137 and +11 is crucial for the silencing of *CEP131* by DNMT1 in a sequence-specific manner (22, 23). Our previous study has shown that transcription factor SP1 can regulate *CEP131* expression by binding to its promoter region (24). SP1 has a strong affinity for the GC-rich motif, a potential target of methylation. Therefore, the methylation status could influence SP1 binding to target genes. Indeed, demethylation-dependent binding of SP1 to promoters of tumor suppressors (eg, p21, BIK Foxp3) has been reported (25, 26). To understand the relationship between DNMT1 and SP1 in regulating *CEP131* expression, we explored the binding of SP1 to *CEP131* promoter following knockdown of *DNMT1* in HeLa cells. Our results confirmed that the protein level of SP1 was not significantly affected by depletion of *DMNT1* (Fig. 3E). Results of ChIP analysis revealed that the binding force was increased 5-fold at the promoter region of Cep131 of SP1 under DNMT1 knockdown condition (Fig. 3E). These results suggest that DNMT1-mediated methylation can inhibit the accumulation of transcription factor SP1 at the CEP131 promoter. Through this process, we found that the expression of Cep131 protein was increased when methylation was suppressed.

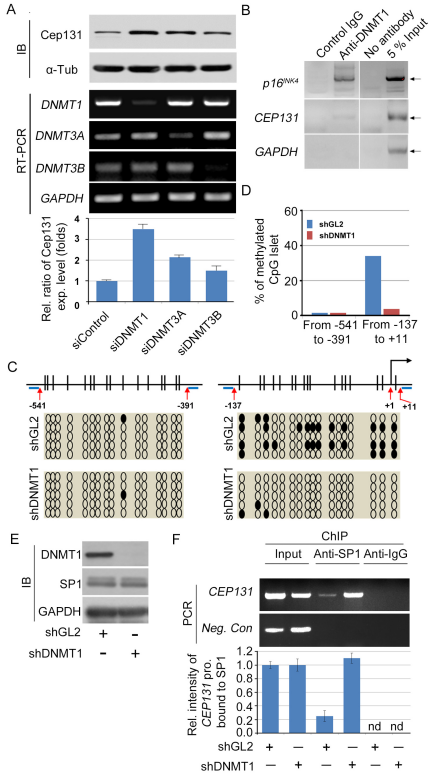

Fig. 3. DNMT1-mediated methylation of *CEP131* promoter region enhances SP1 recruitment for *CEP131* gene expression. (A) siRNA-mediated inhibition of DNMTs was confirmed by RT-PCR. The amount of Cep131 protein expression was indicated by IB and α-tubulin blot was used as loading control. Band intensities were analyzed using image J software. The graph was expressed as a percentage of Cep131 expression (bottom panel). (B) ChIP assay was performed using an anti-DNMT1 antibody in 2 mg of HeLa CCL2 cell lysate. Detection of *CEP131*, $p16^{INK4}$ or *GAPDH*, promoter regions was performed by PCR with specific primer sets. (C) Genomic DNA was purified from shGL2- or shDNMT1-infected HeLa CCL2 and frequencies of CpG islands with and without methylation were measured by sequencing after bisulfate treatment. Five clones were selected from each sample. Methylation statuses of CpG dinucleotides are marked as non-methylated (open circle) and methylated (closed circle). Among marked regions, the methylation level was measured as a ratio of methylated CpG to total CpG. (D) Results of C are summarized in a graph. (E) Each sample was blotted with indicated antibodies, (F) In the same cell lines as in E, ChIP assays were performed using an anti-SP1 antibody and the promoter region was detected using specific primer sets. Band intensities were analyzed using image J software. The graph showed the relative intensity of interaction between the *CEP131* promoter region and SP1. *Neg. Con*, negative control; *nd*, not detecte (bottom panel).

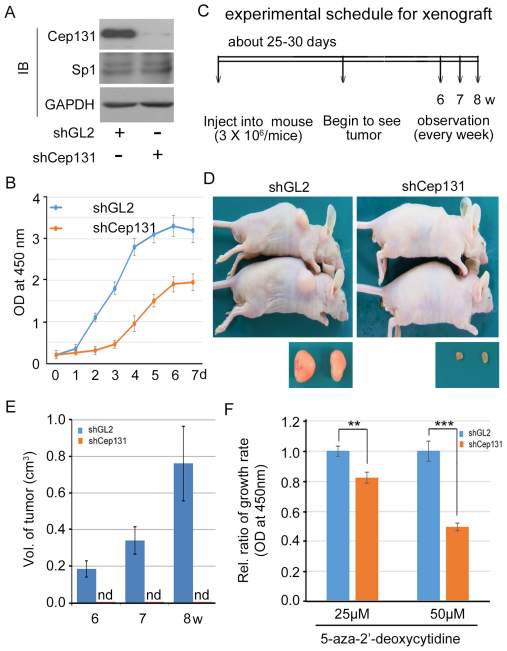

Fig. 4. Inhibition of Cep131 results in attenuation of tumor growth. Both shGL2- and shCep131-generating lentiviruses were infected into HeLa CCL2 cells. After selection with puromycin for 2 days, (A) cells were harvested and IB was performed with indicated antibodies. (B) Growth rates of cells were assessed using MTT assay for 7 days. Values represent absorbance at 450 nm. Cells were also examined *in vivo* using a mouse xenograft model. (C) Experimental schedule. (D) Mouse image revealing the result of xenograft assay. (E) Tumor volume is presented in a graph (n = 7 mice per group). (F) After treatment with 5-aza-2′-deoxycytidine (25 μM or 50 μM) for 2 days in both types of cells, cell growth rates were determined using an MTT assay. All *t*-tests resulted in a P-value below the adjusted significance threshold. **P < 0.01, ***P < 0.001.

Inhibition of Cep131 expression influences both cell proliferation and tumorigenesis

Centrosome-associated proteins play an important role in cell proliferation and tumorigenesis (13, 27-29). Indeed, it has been shown that acute inhibition of Cep131 can cause cilia degeneration and genomic instability (30). In addition, Cep131-knockout mice were found to be defective in spermatogenesis, with no other null phenotypes observed in other tissues (31). We therefore established a *CEP131* knockdown cell line using lentivirus-based shRNA (Fig. 4A) and determined the role of Cep131 in cell growth. MTT assay results revealed that Cep131 deficiency retarded the growth of human cancer cells *in vitro* (Fig. 4B). To determine the *in vivo* role of Cep131, we injected Cep131-depleted HeLa CCL2 cells under the skin of BalB/c male mouse and measured tumor sizes at different time points (Fig. 4C). Xenograft assay results revealed that growth of Cep131-depleted cancer in mice was significantly reduced, showing decreased tumor size and tumor volume compared to shGL2 control (Figs. 4D and 4E). When we co-treated cells with 5-aza-2′-deoxycytidine, we noted a dose-dependent synergistic effect. After treatment with 50 uM of 5-aza-2′-deoxycytidine, Cep131-depleted cells revealed sensitivity to 5-aza-2′-deoxycytidine treatment (Fig. 4F). Taken together, these results reveal that Cep131 could be

a crucial target to prevent tumor cell proliferation.

DISCUSSION

Epigenetic regulation of centrosomal protein CEP131

In this study, we noted that inhibiting DNMT1 resulted in an increase in the number of cells with abnormal number of centrosomes, a condition associated with tumorigenesis (Fig. 1). Although there have been numerous reports concerning genes regulated by DNMT1 (32, 33), little is known about centrosome genes regulated by the activity of DNMT1. To further characterize DNMT1-mediated dysregulation of centrosome numbers, we characterized centrosome-associated genes in DNMT1-depleted cells. Among 102 centrosome-related genes, *CEP131* was identified as a novel direct target of DNMT1. Interestingly, genes regulated by DNMT1 usually have GC-rich motifs in their promoter regions (26). CpG islets are scarce in promoter regions of *TNFAIP3*, *TUBGCP3*, and *MAPRE1*. In contrast, *CEP131* contained a relatively high number of CpG islets in its promoter region spanning from between −1 kb of the 5′ untranslated region (UTR). Consistent with these results, the expression of *CEP131* mRNA was markedly upregulated following silencing of *DNMT1* by shRNA or after treatment with a chemical inhibitor (i.e., 5-aza-2′-deoxycytidine). Results of bisulfite sequencing further revealed that the CEP131 promoter was methylated in the presence of DNMT1 (Figs. 2 and 3). As is common for genes with DNMT1-mediated hyper-methylated promoters, levels Cep131 expression were increased when DNMT1 was inhibited by 5-aza-2'-deoxycytidine treatment or shDNMT1 virus infection (Figs. 1 and 2). In other words, CEP131 is regulated by methylation. To better understand mechanisms involved, we investigated the previously described relationship of DNMT1-mediated binding of SP1 at the CEP131 promoter (24). As expected, the interaction of SP1 to the CEP131 promoter region could be competitively interrupted by DNMT1 binding. As shown in Fig. 3F, the regulatory mechanism of *CEP131* expression was similar to that of p21 and BIK reported previously (25).

Attenuation of tumor cell growth in Cep131-deficient cells

Previous reports have shown that the accumulation of Cep131 is associated with the development of cancer cells (15). In the present study, we further tested the tumor-suppressive effect of Cep131. Our results confirmed that inhibition of Cep131 decreased the growth of cancer cells, a potentially critical observation for those seeking novel targets to treat cancer. This is particularly relevant given that previous studies have revealed no significant deleterious impact is observed in CEP131-knockout mice other than sperm production (31). However, in the case of other siRNAs or shRNAs, various problems (e.g., growth retardation, cilia degeneration) were observed (30, 34). These results suggest that, although Cep131 plays a very important role for centrosome, other proteins might be capable of compensating for its function (31).

Of note, 5-aza-2′-deoxycytidine as a DNMT1 inhibitor has been approved for use in treatment for acute myeloid leukemia (35). There have also been reports of relatively improved efficacy in patients with lung cancer co-treated with 5-aza-2′-deoxycytidine and nivolumab, a negative regulator of T-cell activation by checkpoint inhibition (36). Although recent attempts have been made to extend the range of its application to other solid tumors, reports suggest that patients with lung cancer treated with epigenetic inhibitors 5-aza-2′-deoxycytidine and entinostat show no significant anti-cancer benefits (37). However, in our study, treatment with 5-aza-2′-deoxycytidine resulted in favorable effects in Cep131-depleted cells (Fig. 4F). Our results suggest that a combination of DNMT1 with a substance inhibiting Cep131 might have potential to display a synergistic effect. Furthermore, Cep131 may be a useful target for proper diagnosis and treatment of cancer, particularly when considering a combination therapy using epigenetic regulators (e.g., DNMT1 inhibitor).

MATERIALS AND METHODS

See supplementary information for Materials and Methods.

ACKNOWLEDGEMENTS

This work was supported by the R&D Convergence Program (CAP-16-03-KRIBB) and the International Cooperation Research Program of National Research Council of Science & Technology (NST) of Republic of Korea, the Bio and the Basic Science Research Program (NRF-2016R1A2B3011389) funded by the Ministry of Science and ICT (MSIT) of Korea, a KBSI Grant #T36416 (to Yang Hoon Huh), and KRIBB Research Initiative Program. We thank Dr. Yangrae Cho for proofreading the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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