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DNA Methyltransferase Inhibitor Zebularine Inhibits Human Hepatic Carcinoma Cells Proliferation and Induces Apoptosis

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Abstract

Hepatocellular carcinoma is one of the most common cancers worldwide. During tumorigenesis, tumor suppressor and cancer-related genes are commonly silenced by aberrant DNA methylation in their promoter regions. Zebularine (1-(β-$_D$-ribofuranosyl)-1,2-dihydropyrimidin-2-one) acts as an inhibitor of DNA methylation and exhibits chemical stability and minimal cytotoxicity both *in vitro* and *in vivo*. In this study, we explore the effect and possible mechanism of action of zebularine on hepatocellular carcinoma cell line HepG2. We demonstrate that zebularine exhibits antitumor activity on HepG2 cells by inhibiting cell proliferation and inducing apoptosis, however, it has little effect on DNA methylation in HepG2 cells. On the other hand, zebularine treatment downregulated CDK2 and the phosphorylation of retinoblastoma protein (Rb), and upregulated $p21^{WAF/CIP1}$ and p53. We also found that zebularine treatment upregulated the phosphorylation of p44/42 mitogen-activated protein kinase (MAPK). These results suggest that the p44/42 MAPK pathway plays a role in zebularine-induced cell-cycle arrest by regulating the activity of $p21^{WAF/CIP1}$ and Rb. Furthermore, although the proapoptotic protein Bax levels were not affected, the antiapoptotic protein Bcl-2 level was downregulated with zebularine treatment. In addition, the data in the present study indicate that inhibition of the double-stranded RNA-dependent protein kinase (PKR) is involved in inducing apoptosis with zebularine. These results suggest a novel mechanism of zebularine-induced cell growth arrest and apoptosis via a DNA methylation-independent pathway in hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common newly diagnosed cancer and the third most common cause of cancer mortality worldwide. Its treatment outcome is far from satisfactory and the five-year survival rate is dismal (approximately 10%) [1]. Liver transplantation is currently considered to be the only curative therapy. Unfortunately, however, a majority (>80%) of patients with advanced and unresectable HCC are not suitable candidates for transplantation or surgical resection [2,3]. Chemotherapy using conventional cytotoxic drugs, such as doxorubicin, cisplatin, and fluorouracil, is a common treatment option, especially for patients with unresectable tumors. However, because of poor response rates, severe toxicities, and high recurrence rates, the mean survival time is approximately six months [3,4]. Thus, there is a very high demand for more effective agents to better combat this malignancy.

It has been considered that hypermethylation of CpG islands in tumor suppressor genes represents one of the hallmarks in human cancer development [5,6]. It has been reported that the analysis of gene expression and promoter CpG island hypermethylation in HCC revealed that both genetic and epigenetic changes contribute to the initiation and progression of liver cancer and are correlated with poor survival [7]. Epigenetic changes such as DNA methylation are pharmacologically reversible, and this offers a promising multi-target translational strategy against cancer in which the expression of a variety of silenced genes could be reactivated. DNA methylation is specifically mediated by the action of DNA methyltransferase (DNMT) enzymes [8], which includes DNMT1, DNMT2, DNMT3a, and DNMT3b [9]. DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3a and DNMT3b are potent de novo methyltransferase [10]. Overexpression of DNMT has been reported to be involved in tumorigenesis [11] and has been suggested as a prognostic factor in large B cell lymphomas [12]. Therefore, it has been proposed that the inhibition of DNMT activity can strongly reduce the formation of tumors [13]. Thus far, three DNMT-inhibiting cytosine nucleoside analogs (i.e., 5′-azacitidine, decitabine, and zebularine) have been studied as potential anti-cancer drugs [14–16]. Decitabine and its prodrug 5′-azacitidine are two widely used DNMT inhibitors for the

treatment of patients with various cancers, such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) [17,18]. Although Decitabine and its prodrug 5′-azacitidine are effective in treating various cancers [17,18], the formation of irreversible covalent adducts with DNA may cause long-term side effects, including DNA mutagenesis, a potential cause of tumor recurrence.

Zebularine is a second-generation, highly stable hydrophilic inhibitor of DNA methylation with oral bioavailability that preferentially targets cancer cells [19], as demonstrated in bladder, prostate, lung, colon, and pancreatic carcinoma cell lines [20]. It acts primarily as a trap for DNMT protein by forming tight covalent complexes between DNMT protein and zebularine-substitute DNA [21]. Zebularine is also a cytidine analog that was originally developed as a cytidine deaminase inhibitor. It exhibits low toxicity in mice, even after prolonged administration [22–24]. Given that aberrant methylation is a major event in the early and late stages of tumorigenesis [25,26], including hepatocarcinogenesis [7], this process may represent a critical target for cancer risk assessment, treatment, and chemoprevention [19]. In the previous study, a zebularine signature that classified liver cancer cell lines into two major subtypes with different drug response was identified. In drug-sensitive cell lines, zebularine caused inhibition of proliferation coupled with increased apoptosis, whereas drug-resistant cell lines were associated with the upregulation of oncogenic networks (e.g., E2F1, MYC, and TNF) [19]. However, little is known about the anti-cancer effect and possible mechanism of action of zebularine on HCC.

In the present study, we investigated the molecular mechanism of zebularine against HCC. We demonstrated that zebularine exhibited antitumor activity by inhibiting cell proliferation and inducing apoptosis. This effect was independent of DNA methylation, and characterized by the downregulation of CDK2 and the phosphorylation of retinoblastoma protein (Rb) as well as the upregulation of $p21^{WAF/CIP1}$ and p53. We also found that zebularine induced apoptosis though the intrinsic and extrinsic apoptosis pathways. In addition, the data in the present study suggest that the inhibition of the double-stranded RNA-dependent protein kinase (PKR) is involved in inducing apoptosis with zebularine.

Materials and Methods

Cell culture

HepG2 cells (JCRB1054) and HeLa cells (JCRB9004) were purchased from the Health Science Research Resources Bank (Japan Health Sciences Foundation, Osaka, Japan), and were maintained at 37°C under an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were immersed in a culture medium containing the indicated zebularine concentrations. Zebularine (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in distilled water as a stock solution.

Cell viability assay

The cell viabilities after exposure to zebularine were determined using WST assay. The assay was performed using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Cell cultures exposed to 0 μM zebularine were considered to be 100% viable. The cell viability of each drug-treated sample was presented as a percentage of the viability of cultures treated with 0 μM zebularine. All samples were run five times in the same assay.

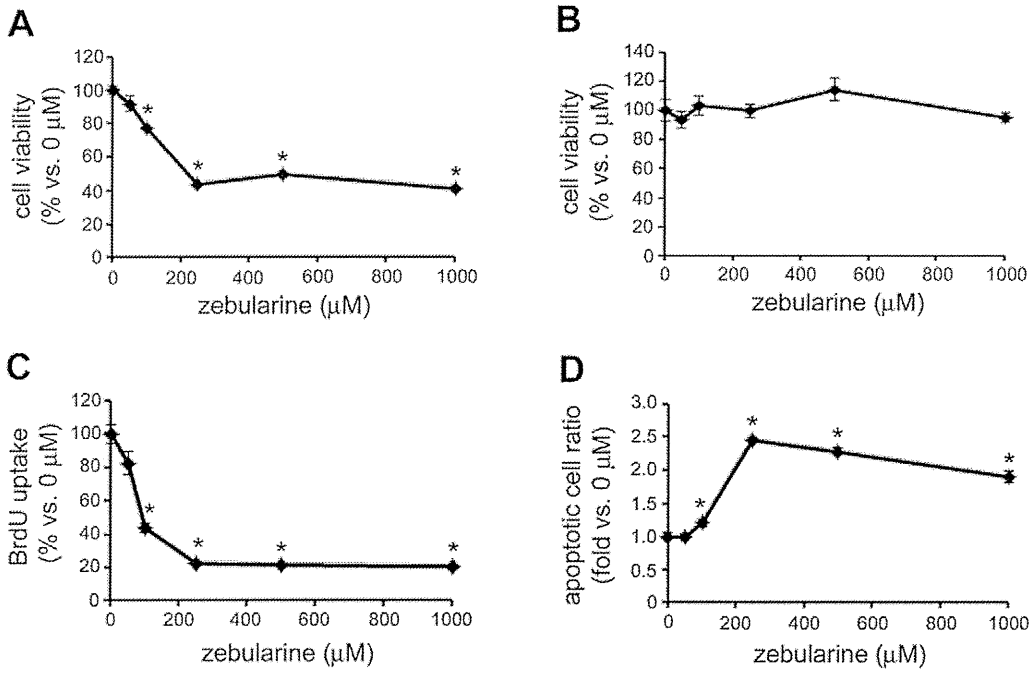

Figure 1. The effect of zebularine on HepG2 cell viability. HepG2 cells were treated with zebularine at indicated concentrations for 72 h (A) and 24 h (B). Cell growth was measured by WST assay. (C) HepG2 cells were treated with zebularine at indicated concentrations for 24 h. Uptake of BrdU was measured by ELISA. (D) HepG2 cells were treated with zebularine at indicated concentrations for 72 h. Apoptosis was measured by TUNEL assay. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g001

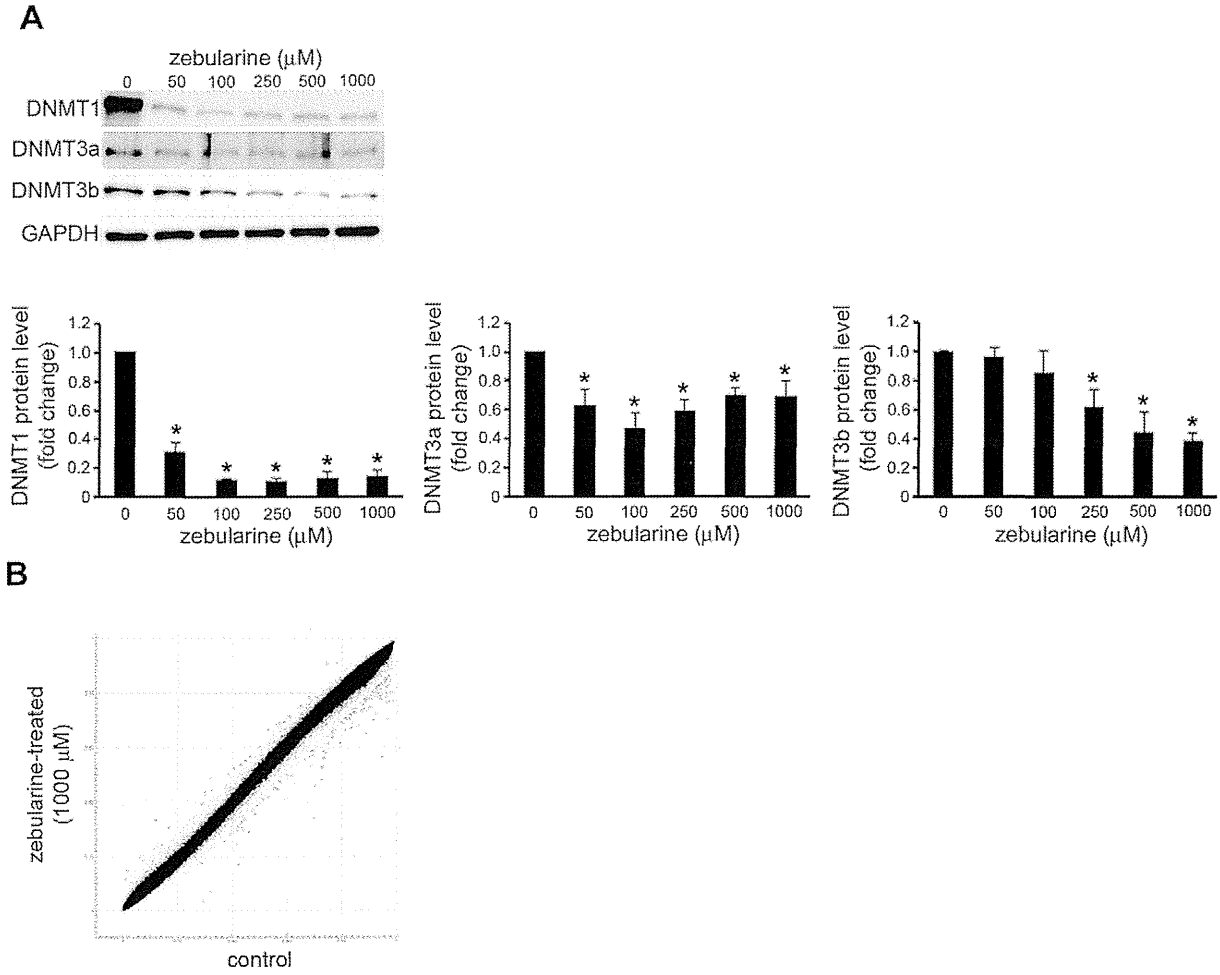

Figure 2. Effect of zebularine on the DNMTs expression and DNA methylation in HepG2 cells. (A) The protein level of DNMT1, DNMT3a, and DNMT3b after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the protein level of DNMT1, DNMT3a, and DNMT3b. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM. (B) Scatter plot of the average beta values at 485,415 CpG sites for zebularine-treated (y-axis) and control (x-axis) HepG2 cells (n = 3 for each group). Dots for CpG sites whose delta-beta value is >0.1 or <−0.1 are shown in green (35 [0.0072%] hypermethylated and 162 [0.033%] hypomethylated CpG sites).
doi:10.1371/journal.pone.0054036.g002

Apoptosis analysis

Quantification of apoptotic cells was performed using a Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Tokyo, Japan). After 72 h of incubation with zebularine, cells were lysed with a lysis buffer (included in the kit). The assay was performed according to the manufacturer's instructions. Absorbance values were measured at 405 nm using a microplate reader (ARVO, PerkinElmer Japan, Kanagawa, Japan). The apoptotic ratio of each drug-treated sample was presented as a fold-change of the apoptosis of cultures treated with 0 μM zebularine. All samples were run five times in the same assay.

5-bromo-2′-deoxy-uridine (BrdU) incorporation assay

Cellular DNA synthesis rates were determined by measuring BrdU incorporation with the commercial Cell Proliferation ELISA System (Roche Diagnostics). After 24 h of incubation with zebularine, cells were incubated for 3 h with a BrdU labeling solution (included in the kit) containing 10 μM BrdU. The assay was performed according to the manufacturer's instructions. Absorbance values were measured at 405 nm using a microplate reader. The BrdU incorporation of each drug-treated sample was presented as a percentage of the BrdU incorporation of cultures treated with 0 μM zebularine. All samples were run five times in the same assay.

Illumina Infinium HumanMethylation450 BeadChip analysis

Genomic DNA was extracted from three independent cell culture batches for zebularine (1000 μM)-treated and control HepG2 cells. Genomic DNA (1000 ng) was bisulfite-treated and purified using the EpiTect Bisulfite Plus Kit (QIAGEN K.K., Tokyo, Japan). Three hundred nanograms of bisulfite-treated DNA were hybridized to the Illumina Infinium HumanMethylation450 BeadChip using Illumina-supplied reagents and protocols. Both the CpG loci included on this array and the technologies behind the platform have been described previously [27]. GenomeStudio software (Illumina) was used to calculate the methylation level at each CpG site as beta value (β = intensity of the methylated allele [M]/[intensity of the unmethylated allele (U)

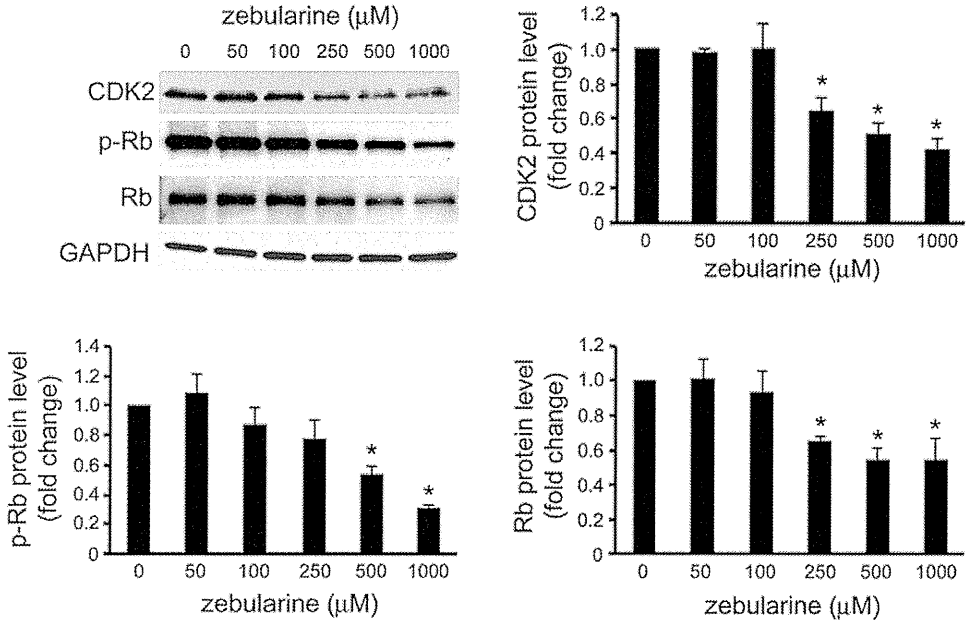

Figure 3. Effects of zebularine on the protein expression of cell-cycle regulator. The protein level of CDK2, p-Rb, and Rb after zebularine treatment for 24 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the protein level of CDK2, p-Rb, and Rb. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
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+ intensity of the methylated allele (M) + 100]) [27]. Region-level methylation analysis was conducted using the IMA package [28].

Caspase assays

Caspase-3/7, -8, and -9 activities were assayed with Caspase-Glo Assays (Promega KK, Tokyo, Japan) according to the respective manufacturer's standard cell-based assay protocol. The luminescence of each sample was measure using a plate-reading luminometer. Comparison of the luminescence from a treated sample with a control sample enables determination of the relative increase in caspase activity. All samples were run five times in the same assay.

Overexpression of PKR and forward transfection

The PKR plasmid, pFN21A-hPKR (pFN21AE2332), and empty vector, HaloTag control vector, were purchased from Promega. Transient transfection in HepG2 cells was performed according to the Lipofectamine 2000 (Invitrogen, LifeTechnologies Japan, Tokyo, Japan) methods. Cells cultured in a six-well culture plate were washed twice with phosphate-buffered saline and the medium was replaced with 2 ml of Opti-MEM (Invitrogen) with 1% FBS. Two micrograms per well of pFN21A-hPKR or the empty vector (HaloTag control vector) were then mixed with 10 μl/well of Lipofectamine 2000 in Opti-MEM and the mixture was added to the wells 20 min later. After 6 h of transfection, the cells were then cultured in regular medium for 48 h and subsequently treated with zebularine for 72 h.

Immunoblotting

Cells were lysed in lysis buffer (20 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 1% NP-40, 1.5 mM MgCl2, 1 mM EGTA, 1 μg/ml leupeptin, 1 mM PMSF, and 1 mM Na_3VO_4) and stored at −80°C until use. After centrifugation, aliquots of the supernatants underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretically separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes, blocked, and immunoblotted with anti-CDK2 (78B2, #2546), Rb (4H1, #9309), phospho-Rb (Ser807/811) (#9308), $p21^{WAF/CIP1}$

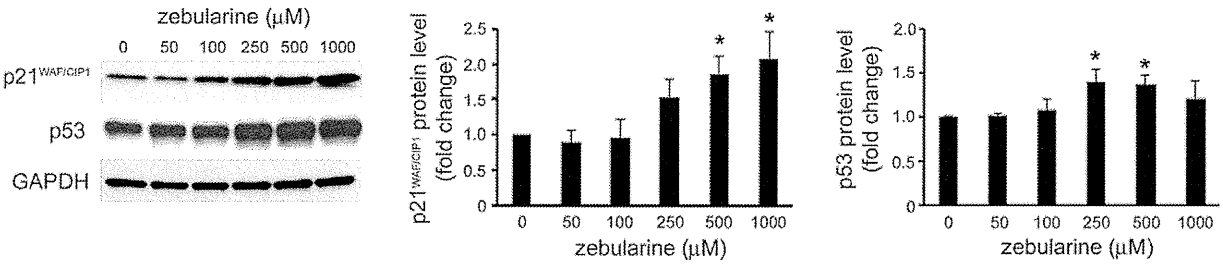

Figure 4. Effects of zebularine on the protein expression of $p21^{WAF/CIP1}$ and p53. The expression of $p21^{WAF/CIP1}$ and p53 after zebularine treatment for 24 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the protein level of $p21^{WAF/CIP1}$ and p53. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g004

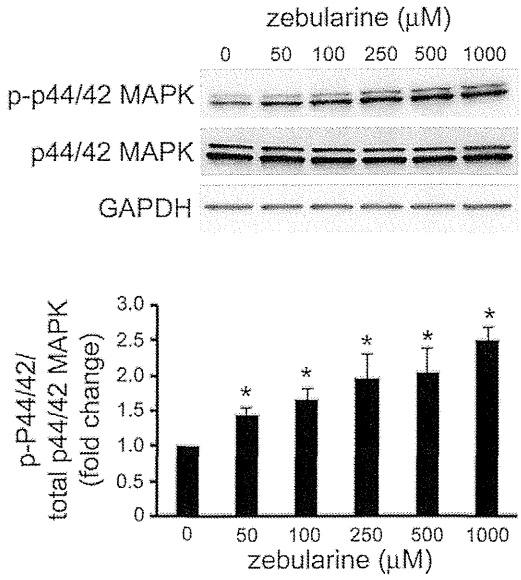

Figure 5. Effects of zebularine on phosphorylation of p44/42 MAPK. The phosphorylation and expression of p44/42 MAPK after zebularine treatment for 24 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the phosphorylated and total p44/42 MAPK protein level. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g005

(12D1, #2947), p44/42 mitogen-activated protein kinase (MAPK) (137F5, #4695), phospho-p44/42 MAPK (The202/Thy204) (#4370), Bax (D2E11, #5023), Bcl-2 (50E3, #2870), PKR (N216, #2766), DNMT1 (D63A6, #5032) (Cell Signaling Technology Japan, Tokyo, Japan), phospho-PKR (E120, ab32036, abcam, Tokyo, Japan), p53 (M 7001, Dako Japan, Tokyo, Japan), DNMT3a (sc-20703), DNMT3b (sc-81252) (Santa Cruz Biotechnology, Santa Cruz, CA), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#MAB374, Millipore, Temecula, CA) antibodies, and then with peroxidase-conjugated secondary antibodies (NA931 or NA940, GE Healthcare Japan, Tokyo, Japan). The bound antibodies were detected using the ECL system (GE Healthcare Japan).

Statistics

All experiments were performed at least three times. Values are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired Student's *t*-test or two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference as a post-hoc test. $p<0.05$ was considered to indicate statistical significance.

Results

The effects of zebularine on HepG2 cell viability

In order to investigate the effect of zebularine on HepG2 cell viability, we performed WST assay after zebularine exposure. WST assay indicated that zebularine affected cell viability. Exposure of cells to zebularine for 72 h resulted in a decrease in cell viability (Fig. 1A). To further determine whether zebularine could inhibit the proliferation of HepG2 cells, we conducted BrdU incorporation assay after zebularine treatment for 24 h. Although WST assay indicated that zebularine could not affect cell viability after 24 h (Fig. 1B), BrdU incorporation assay clearly showed that the uptake of BrdU by HepG2 cells was already reduced after 24 h exposure to zebularine (Fig. 1C). At a concentration of 250 μM, the uptake of BrdU was reduced to 22.1±0.6% compared with 0 μM and a similar reduction of BrdU uptake (20.1±1.5%) was observed at a concentration of 1000 μM. In addition, we examined whether zebularine could induce HepG2 cell death. Terminal deoxynucleotidyl transferase dUTP nick end labeling

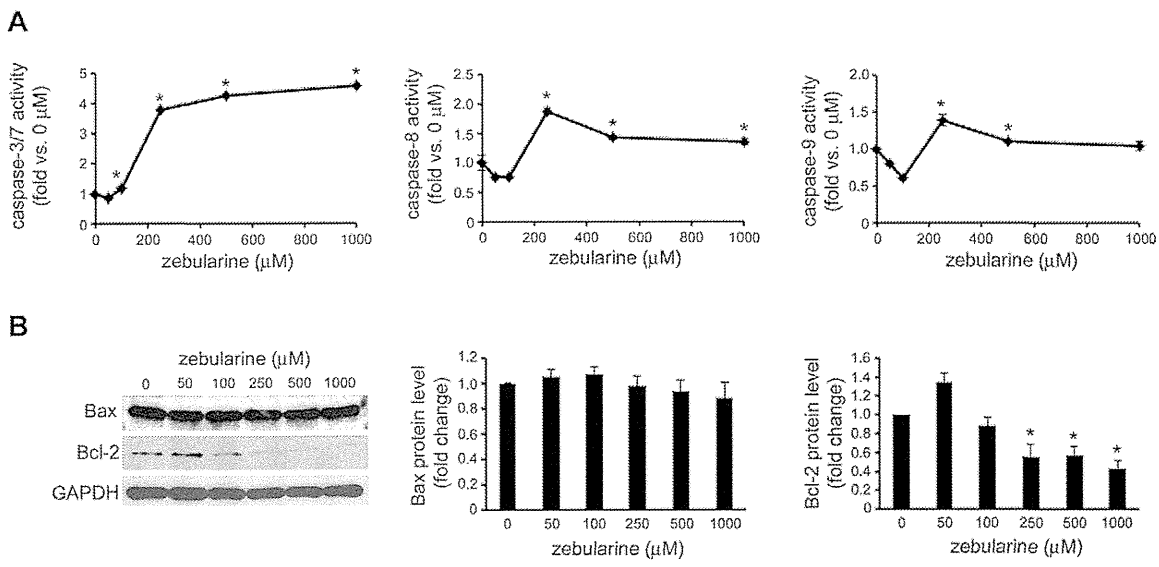

Figure 6. The effect of zebularine on apoptosis-related proteins. HepG2 cells were treated with zebularine at indicated concentrations for 72 h. (A) Caspase-3/7, -8, and -9 activities were determined using Caspase-Glo Assays. The data are expressed as fold-increase relative to the respective untreated samples (RLU/60 min/μg protein). (B) The protein level of Bax and Bcl-2 after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the protein level of Bax and Bcl-2. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g006

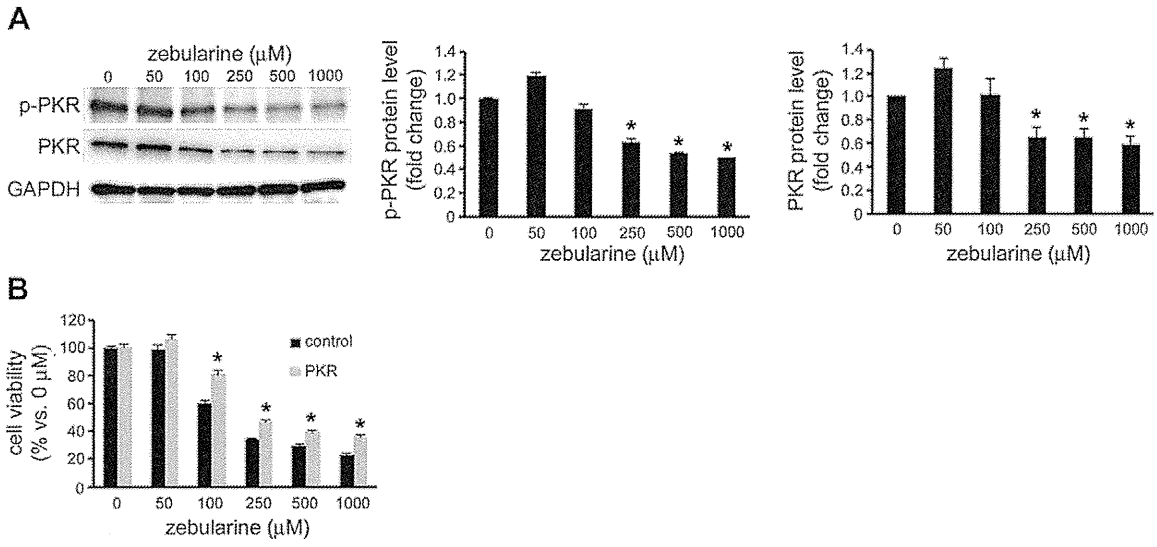

Figure 7. Effects of zebularine on phosphorylation of PKR. (A) The phosphorylation and expression of PKR after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the phosphorylated and total PKR protein level. GAPDH was used as a loading control. *$p<0.05$, compared to 0 μM. (B) Effect of the overexpression of PKR in zebularine-induced cell death. The forward transfection of the empty vector (Halo Tag control vector) as the control or the plasmid-containing PKR cDNA sequence (pFN21A-hPKR) was performed, and the cells were then treated with different concentrations of zebularine for 72 h. *$p<0.05$, compared to control. Data are the means ± SEM of results from at least three independent experiments.
doi:10.1371/journal.pone.0054036.g007

(TUNEL) assay demonstrated that zebularine induced apoptotic cell death on HepG2 cells. Exposure of cells to zebularine for 72 h resulted in an increase in the number of apoptotic cells (Fig. 1D). These results indicated that DNA replication was blocked and apoptotic cell death was induced by treatment with zebularine, which resulted in reduced HepG2 cell viability.

Zebularine affects HepG2 cells growth arrest and apoptosis via DNA methylation-independent pathway

Because of zebularine's activity as a DNMT inhibitor in other model systems [29,30], its effect on the expression of DNMTs in HepG2 cells was examined. As expected, zebularine treatment was associated with a statistically significant dose-dependent depletion of DNMT1, DNMT3a, and DNMT3b (Fig. 2A).

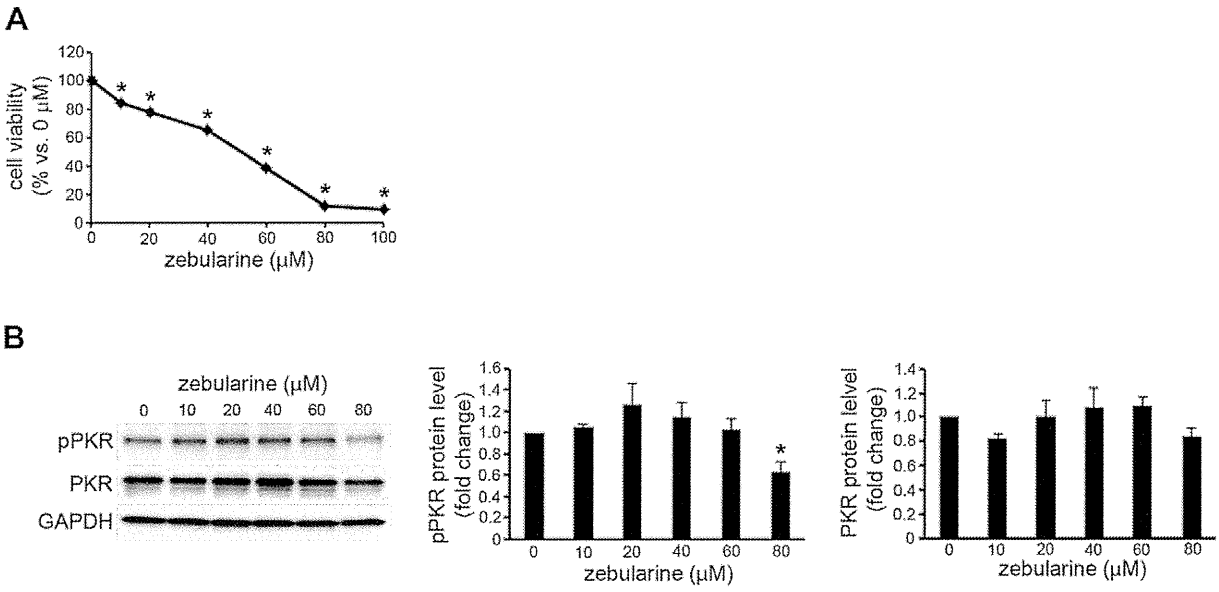

Figure 8. Effects of zebularine on phosphorylation of PKR in HeLa cells. (A) HeLa cells were treated with zebularine at indicated concentrations for 72 h. Cell growth was measured by WST assay. (B) The phosphorylation and expression of PKR after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the phosphorylated and total PKR protein level. GAPDH was used as a loading control. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g008

Since zebularine decreased DNMT protein levels, to determine whether the growth inhibition and/or apoptosis induction in HepG2 cells by zebularine are a result of a change in DNA methylation, we obtained the genome-wide methylation profiles of zebularine-treated and -untreated (control) HepG2 cells using an Illumina Infinium HumanMethylation450 BeadChip (GEO accession number GSE42490). Among 482,421 assays for CpG sites, 482,260 assays fulfilled our quality control criteria (detection p value <0.01 and no missing beta value for both groups) and were subjected to the following analysis. For each assay, delta-beta value (= average of the beta values of three zebularine-treated samples − average of those of three controls) was calculated. As shown in Fig. 2B, the methylation profiles were highly similar between zebularine-treated and -untreated HepG2 cells. The number of CpG sites whose delta-beta values are >0.1 and <-0.1 was 35 and 162, respectively. At the majority (99.96%) of CpG sites, methylation levels were nearly the same under the two conditions. To further assess whether these minor methylation changes are observed at specific genes or genomic regions, we conducted region-level methylation analysis using the IMA package [28]. Among 26,659 CpG islands (CGIs), only five showed a significant change (adjusted p value <0.05 and $|$delta-beta value$|$ >0.1) of the methylation level upon zebularine treatment (Table S1). All five CGIs were found to be highly methylated in control HepG2 cells (beta value >0.8), and to be partially hypomethylated (delta-beta range -0.11–-0.21) in zebularine-treatment cells. One CGI is located in an intron of the AGAP1 gene that encodes ArfGAP with GTPase domain, ankyrin repeat, and PH domain 1 protein. Another CGI is located 10 kb downstream of the USP18 gene that encodes ubiquitin specific peptidase 18. The other three CGIs are not associated with any RefSeq gene structure (within 50 kb distance). It is unlikely that the slight decrease in DNA methylation at these five CGIs causes growth arrest and apoptosis in HepG2 cells. These results suggest that the administration of zebularine has little effect on DNA methylation in HepG2 cells, and that the inhibited cell growth and induced apoptosis observed in HepG2 cells upon zebularine treatment are caused by unknown mechanisms that are independent of DNA methylation.

Zebularine inhibited CDK and phosphorylation of protein retinoblastoma

To estimate the mechanism by which zebularine inhibits HepG2 cell proliferation, we investigated the change in CDK2 expression that was associated with cell-cycle regulation after zebularine treatment. Our results showed that the levels of CDK2 were downregulated in HepG2 cells at 24 h by zebularine treatment (Fig. 3). Protein retinoblastoma (Rb) plays a critical role in governing cell-cycle progression, especially for the transition from the G1 to the S phase [31], where the total and phosphorylation level of Rb was detected. Our results revealed that phosphorylated Rb (p-Rb) decreased in a concentration-dependent manner 24 h after zebularine treatment, which was accompanied by a reduction in total Rb (Fig. 3).

Zebularine increased $p21^{WAF/CIP1}$ and p53 level in HepG2 cells

Previous studies have demonstrated that tumor suppressor protein $p21^{WAF/CIP1}$ and p53 play an important role in G0/G1 arrest in HepG2 cells [32]. Therefore, in order to determine whether these two proteins play a role in inhibiting cell proliferation, the HepG2 cells were exposed to zebularine and analyzed for change on the protein level of $p21^{WAF/CIP1}$ and p53. The results showed that after 24 h of zebularine treatment, the $p21^{WAF/CIP1}$ and p53 protein level was higher in HepG2 cells than in the control (Fig. 4).

The effect of zebularine on p44/42 MAPK expression

To further clarify the mechanism of the proliferation inhibitory effect of zebularine on HepG2 cells, we examined the expression of p44/42 MAPK in HepG2 cells after zebularine treatment. As shown in Fig, 5, zebularine increased the level of phosphorylated p44/42 MAPK, whereas total p44/42 MAPK was unaffected by the zebularine treatment, as judged by comparisons with GAPDH as a loading control. This data indicates that zebularine can increase the phosphorylation of p44/42 MAPK.

Zebularine induced apoptosis via caspase pathway

To investigate whether zebularine-induced apoptosis was associated with the caspase family proteins, the activity of caspase-3/7, -8, and -9 was examined after zebularine treatment at 72 h. As shown in Fig. 6A, the activity of caspase-3/7 was significantly increased at an apoptosis-inducible concentration of zebularine. In addition to caspase-3, the activity of caspase-8 and -9 was also increased with zebularine treatment. The expression of the proapoptotic factor Bax and the antiapoptotic factor Bcl-2 was examined by western blotting. The result demonstrated that Bax expression was not affected. On the other hand, Bcl-2 expression decreased with an increasing amount of zebularine (Fig. 6B).

Zebularine decreases the activity of PKR in HepG2 cells

A previous study showed that PKR regulates the protein expression level and phosphorylation of Bcl-2 and plays an anti-apoptotic role in HepG2 cells [33]. Since zebularine can reduce the Bcl-2 protein level, we examined PKR and the phosphorylated PKR level with zebularine treatment. Our results showed that zebularine can reduce the phosphorylated PKR level; this was accompanied by a reduction in total PKR (Fig. 7A). To determine whether PKR has an anti-apoptotic effect in HepG2 cells treated with zebularine, we overexpressed the PKR gene in HepG2 cells and exposed the cells to zebularine. We found that zebularine-induced cell death was reduced by overexpression of PKR (Fig. 7B).

The effect of zebularine on the activity of PKR in other cancer cells

Zebularine also inhibits the growth of bladder cancer, breast cancer, and cervical cancer cells [29,30,34]. Since PKR is ubiquitously expressed, we examined whether zebularine decreases the activity of PKR in other cancer cells. It was recently reported that zebularine inhibits the growth of HeLa cervical cancer cells via cell-cycle arrest and caspase-dependent apoptosis [30]. We also observed that zebularine inhibited the growth of HeLa cells, which coincided with the results of the previous study (Fig. 8A). However, our results showed that cell growth inhibiting concentration of zebularine did not reduce the phosphorylated PKR and total PKR levels in HeLa cells (Fig. 8B).

Discussion

In the present study, we investigated the effect of zebularine on human hepatic carcinoma cells and the possible mechanism. To the best of our knowledge, this is the first study to demonstrate that zebularine inhibits hepatic carcinoma cell HepG2 proliferation by inducing cell growth arrest and apoptosis via intrinsic and extrinsic apoptotic pathways.

In this study, we observed that zebularine decreased the level of DNMT1, DNMT3a, and DNMT3b in HepG2 cells. These results

were similar to the reports that DNMT inhibitor induces the depletion of DNMT1, 3a, or 3b protein in human bladder, breast, and cervical cancer cells [24,30,35]. Because tight covalent complexes of zebularine and DNMT could lead to compositional change in DNMT protein, it is plausible that DNMTs can be degraded via a ubiquitination system, consequently being observed in the reduction of its expression [30]. On the other hand, our results suggest that zebularine has little effect on DNA methylation in HepG2 cells. Thus, it seems that the cell-cycle arrest and apoptosis observed in HepG2 cells upon zebularine treatment are caused by mechanisms that are independent of DNA methylation.

Eukaryotic cell proliferation is a highly regulated system that is controlled by CDK-cyclin complexes. The cell-cycle transition from the G1 to the S phase was the major regulatory checkpoint in this process. This transition is characterized by the phosphorylation of Rb, and the CDK-cyclin complex catalyzes the reaction [36,37]. In this study, we found that zebularine inhibited the CDK2 and p-Rb accompanied by a decrease in total Rb, which resulted in cell-cycle arrest and the exertion of its antiproliferative effect. Cell-cycle inhibitor $p21^{WAF/CIP1}$ plays an important role in the G1/S progression process. It may inhibit the activity of the CDK-cyclin complex to regulate cell-cycle progression. These effects can be mediated through p53-dependent or -independent machinery according to the types of stimuli [38–43]. There are two p53-binding elements located at the $p21^{WAF/CIP1}$ gene promoter that can be transactivated by the accumulated nuclear p53 after DNA damage [44]. It is reported that p53-dependent G1 growth arrest is mediated by $p21^{WAF/CIP1}$, and $p21^{WAF/CIP1}$ is the CDK inhibitory protein transcriptionally regulated by p53 [45]. Our results showed that the $p21^{WAF/CIP1}$ level was increased after zebularine treatment. In addition, zebularine also upregulated p53 protein. Thus, in the present study, both p53 and $p21^{WAF/CIP1}$ may perform their function by inhibiting the kinase activities of CDK-cyclin complexes to stimulate cell-cycle arrest, which was attributed to the zebularine effect.

MAPKs are essential components of the intracellular signal transduction pathways that regulate cell proliferation and apoptosis. One subgroup of MAPKs, p44/42 MAPK (ERK1/2), is an important target in the diagnosis and treatment of cancer and has been reported to be required for the upregulation of $p21^{WAF/CIP1}$ that results in cell-cycle arrest [46–48]. Furthermore, the high-intensity p44/42 MAPK signal leads to the repression of CDK2 kinase activity for p-Rb, which mainly regulates the proliferation of HepG2 cells [49]. In the present study, MAPK signaling pathway regulation after zebularine treatments was investigated. We found that zebularine treatment upregulated the phosphorylation of p44/42 MAPK. Therefore, it is suggested that the p44/42 MAPK pathway plays a role in zebularine-induced cell-cycle arrest by regulating the activity of $p21^{WAF/CIP1}$ and Rb.

During the process of apoptosis, caspases are essential for the initiation and execution of cell death in a self-amplifying cascade in response to various stimuli [50]. Two major apoptotic pathways have been identified: the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by death receptors, which recruit initiator caspase-2, -8, or -10 through adaptor molecules, whereas the intrinsic signals result in the activation of caspase-9. These initiator caspases can sequentially cleave and activate the effector caspase (caspase-3, -6, and -7), which play an important role in mediating cellular destruction [51]. Our results showed that zebularine appeared to induce the apoptosis of HepG2 cells via the intrinsic pathway, as shown by the activation of caspase-9, and the extrinsic pathway, as shown by the activation of caspase-8, which led to caspase-3 activation. Proteins from the Bcl-2 family can be divided into two groups: suppressors of apoptosis (e.g., Bcl-2, Bcl-XL, and Mcl-1) and activators of apoptosis (e.g., Bax, Bok, Hrk, and Bad). These proteins are key regulators of the intrinsic pathway of apoptosis, setting the threshold for engagement into the death machinery [52,53]. Among these, the anti-apoptotic Bcl-2 protein acts to suppress apoptosis by preventing the release of apoptogenic proteins, such as cytochrome c, that reside in the intermembrane space of mitochondria. Functionally, Bax acts in opposition to Bcl-2 and facilitates the release of these mitochondrial apoptogenic factors by translocation and oligomerization [54–56]. Thus, the ratio of Bax/Bcl-2 determines, in part, the susceptibility of cells to death signals and might be a critical factor in a cell's threshold for apoptosis [57]. In this study, the expression of Bax and Bcl-2 proteins in zebularine-treated HepG2 cells was examined by western blot assay. We found that although Bax protein levels were not affected, Bcl-2 protein level was downregulated with zebularine treatment, which led to a marked increase in the Bax/Bcl-2 ratio and then apoptosis.

Initially identified as an antiviral protein, PKR is best known for triggering cell defense responses and initiating innate immune responses by arresting general protein synthesis and inducing apoptosis during virus infection [58]. Activated PKR, known as a eukaryotic initiation factor 2-alpha (eIF-2α) kinase, induces the phosphorylation of eIF-2α [59], which inhibits the initiation of translation through the tRNA-40S ribosomal subunit. On the other hand, PKR is involved in controlling the transcription of Bcl-2 in HepG2 cells, mediated by the transcription factor NF-κB [33]. In this study, we observed that zebularine can reduce the phosphorylation of PKR, which indicates the activated PKR. In addition, overexpression of PKR reduced zebularine-induced cell death. Thus, our results suggest that zebularine decreases the activity of PKR and results in apoptotic cell death via reduced NF-κB activity and the downregulation of Bcl-2. The fact that zebularine inhibits the growth of bladder, breast, and cervical cancer cells [29,30,34] and that PKR is ubiquitously expressed led us to hypothesize that zebularine induced the cell growth arrest via the downregulation of PKR in other cancer cells. When we examined the effect of zebularine on PKR expression in HeLa cells, we observed, however, that zebularine did not decrease the phosphorylation of PKR and the total PKR level. These results suggest that there are differences in the mechanism by which zebularine inhibits cell growth among the different types of carcinomas. The action and mechanisms of zebularine must therefore be further investigated in other cancer cells.

In conclusion, our observation indicated that zebularine inhibited cell growth and induced apoptotic cell death, which contributed to its antiproliferation effects against hepatocellular carcinoma HepG2 cells. The most likely mechanism underlying the zebularine-induced growth arrest involves an initial induction of p44/42 phosphorylation and an increase in $p21^{WAF/CIP1}$ expression, which leads to a reduction in G1-related CDKs such as CDK2 protein and p-Rb, and then ultimately arrests the HepG2 cell cycle. Furthermore, zebularine decreased the activity of PKR, and resulted in apoptotic cell death via the downregulation of Bcl-2.

Supporting Information

Table S1 List of CGIs showing a significant change in DNA methylation level upon zebularine-treatment in HepG2 cells.
(XLS)

Author Contributions

Conceived and designed the experiments: K. Nakamura KH AT. Performed the experiments: K. Nakamura KA K. Nakabayashi NK. Analyzed the data: K. Nakamura K. Nakabayashi KH JY AT. Contributed reagents/materials/analysis tools: JY. Wrote the paper: K. Nakamura K. Nakabayashi AT.

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Characterization of Novel Paternal ncRNAs at the *Plagl1* Locus, Including *Hymai*, Predicted to Interact with Regulators of Active Chromatin

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Abstract

Genomic imprinting is a complex epigenetic mechanism of transcriptional control that utilizes DNA methylation and histone modifications to bring about parent-of-origin specific monoallelic expression in mammals. Genes subject to imprinting are often organised in clusters associated with large non-coding RNAs (ncRNAs), some of which have cis-regulatory functions. Here we have undertaken a detailed allelic expression analysis of an imprinted domain on mouse proximal chromosome 10 comprising the paternally expressed *Plagl1* gene. We identified three novel *Plagl1* transcripts, only one of which contains protein-coding exons. In addition, we characterised two unspliced ncRNAs, *Hymai*, the mouse orthologue of *HYMAI*, and *Plagl1it* (*Plagl1* intronic transcript), a transcript located in intron 5 of *Plagl1*. Imprinted expression of these novel ncRNAs requires DNMT3L-mediated maternal DNA methylation, which is also indispensable for establishing the correct chromatin profile at the *Plagl1* DMR. Significantly, the two ncRNAs are retained in the nucleus, consistent with a potential regulatory function at the imprinted domain. Analysis with catRAPID, a protein-ncRNA association prediction algorithm, suggests that *Hymai* and *Plagl1it* RNAs both have potentially high affinity for Trithorax chromatin regulators. The two ncRNAs could therefore help to protect the paternal allele from DNA methylation by attracting Trithorax proteins that mediate H3 lysine-4 methylation.

Submitted GenBank nucleotides sequences: Plagl1it: JN595789 Hymai: JN595790

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Introduction

Genomic imprinting is an epigenetic form of transcriptional regulation that results in the monoallelic expression of genes from the paternal or maternal allele [1]. Currently there are around 120 confirmed imprinted genes in the mouse, with approximately 60 showing conserved imprinted expression in humans (http://igc.otago.ac.nz/home.html). Imprinted genes have been shown to play important roles in development, and code for proteins with diverse biological activities.

The allele-specific expression of imprinted genes is mediated by CpG rich sequence elements that show allelic DNA methylation [2]. These differentially methylated regions (DMRs) result from methylation deposition during oogenesis or spermatogenesis, specifically by the DNMT3A/DNMT3L *de novo* methyltransferase complex [3–5]. Following fertilization, the allelic methylation is maintained throughout development. In somatic tissues, most DMRs are also marked by allelic histone modifications, highlighting interplay between these two epigenetic systems [6]. Recently, non-coding RNAs (ncRNAs) have

been shown to be important in recruiting histone methyltransferases to imprinted gene promoters, thus revealing the diversity of epigenetic mechanisms involved in the imprinting process [7,8].

The *Plagl1* (also known as *Zac1*) imprinted gene maps to mouse chromosome 10. The human orthologue is located on human chromosome 6 [9,10]. This paternally expressed gene encodes a zinc finger transcription factor with seven C_2H_2-type zinc-fingers that regulates apoptosis and cell cycle [11]. Loss of *PLAGL1* expression is frequently observed in many human tumours, consistent with its proposed role as a tumour-suppressor gene [12]. Over-expression of the human *PLAGL1* gene is thought to be responsible for Transient Neonatal Diabetes Mellitus (TNDM), a genetic disease characterised by severe intrauterine growth restriction and insulin dependence in neonates [13]. This over-expression can result from paternal uniparental isodisomy, paternally inherited duplications of 6q24–q25 or epigenetic mutations in which the maternal allele adopts a paternal epigenotype, resulting in biallelic expression [14]. A paternally expressed ncRNA, *HYMAI*, located in the first intron of human *PLAGL1*, is also over-expressed in TNDM patients, but the function of this transcript remains unknown [13].

To explore the mechanisms regulating *PLAGL1* imprinted expression, we performed a comparative characterisation of the orthologous domain on mouse chromosome 10. We identified numerous paternally expressed ncRNAs, which we propose may be involved in maintaining the paternal allele in a transcriptionally permissive state.

Results

Novel Imprinted *Plagl1* Isoforms

To first determine the size of the *Plagl1* gene in mouse, we interrogated the working draft sequence browser (NCBI26/mm8, Feb 2006). In accordance with previous reports, we find that the *Plagl1* gene covers ~71 kb and contains 12 exons [10]. These include numerous alternatively spliced exons in the 5′UTR originating from two promoter regions embedded within two different CpG islands (Figure 1A). The majority of transcripts arise from the promoter (P1) within the DMR, whereas less abundant transcripts originate from an unmethylated CpG island ~30 kb upstream (P2) (reference EST FJ425893). The open reading frame (ORF) for these transcripts is restricted to the last two exons, resulting in a full-length protein of 705 amino acids. All full-length transcripts share a common 3′UTR, with a polyadenylation signal 24 bp from the stop codon.

As a result of expressed sequence tag (EST) alignments, we identified three additional *Plagl1* transcripts (Figure 1A). A novel *Plagl1* transcript (reference EST BM894919) originates from a unique promoter region (P3) 5′ to the exon 7 acceptor site (gtccaag//GTCTCTT or ctcacag/GTTTGAG) of P1-*Plagl1* transcript, with a 5′UTR that extends at least 300 bp into the upstream intron mapping to an interval containing a cluster of CAGE (5′Cap Analysis Gene Expression) tags. This transcript includes the last three exons and therefore incorporates the full-length *Plagl1* ORF. The remaining two transcripts (reference ESTs CJ065374 and AI607573) originate from within the *Plagl1*-DMR region but terminate after exons 4 and 5 respectively. These different RNAs contain unique 3′UTRs, extending beyond the exon boundaries into the P1-*Plagl1* introns and do not include the *Plagl1* ORF. Northern blot analysis using a *Plagl1* exon 2–3 probe revealed, in addition to the 2 major splice variants, multiple transcripts between 700 bp and 1.7 kb (Figure S1). Using various strategically designed RT-PCR primers, we were able to confirm paternal expression of all novel *Plagl1* transcripts in RNA derived from E18.5 (B x C) F1 mouse tissues (Figure 1B).

Conserved Expression of *Hymai* in Mouse

The human *PLAGL1* region contains the paternally expressed *HYMAI* transcript. This non-coding RNA has a transcription start site located within the *PLAGL1*-DMR. However, DNA sequence from this region shows only weak conservation between humans and mouse (data not shown) and no mouse *Hymai* is described on the UCSC sequence browser or in Genbank databases. We set out to determine whether this non-coding RNA is conserved in mouse. We utilised allelic RT-PCR amplifications restricted to intron 1 of P1-derived *Plagl1* transcript. We observed paternal expression of an RNA in various mouse tissues from E18.5 embryos (Figure 1B). Using 5′ and 3′ RACE, we were able to map the extent of this transcript, which we named '*Hymai*'. We identified four different transcriptional start sites (TSS) for *Hymai*, spread over a 19 bp interval embedded within the *Plagl1*-DMR (Figure S2). Using the same RACE-ready cDNA from E18.5 embryos, we were able to show that P1-*Plagl1* transcript originates from an overlapping 47 bp region, with neither P1-*Plagl1* nor *Hymai* being associated with a TATA-box. Using 3′RACE, we show that *Hymai* terminates ~5 kb from the TSS interval, with multiple 3′ RACE products (last base chr10: 12815696 and chr10: 12815706 of mouse genome NCBI37/mm9), the longest transcript terminating 46 bp after a canonical polyadenylation signal (AATAAA). We were unable to confirm a single band on northern blot analysis, since the expression of this transcript is below the detectable limits of the technique. Analysis of the open reading frame revealed that *Hymai* has no obvious ORF (Figure S 2).

Paternal Expression of a Novel *Plagl1* Internal Transcript, *Plagl1it*

Through examination of the UCSC sequence browser we identified 12 ESTs of various sizes transcribed from the same (+) strand as *Plagl1*, located within intron 5 of P1-*Plagl1*. The largest EST, AK087432, is 2964 bp, representing an intronless transcript with no ORF, that we named *Plagl1 intronic transcript* (*Plagl1it*) (Figure 1B; Figure S2). Using RACE, we found that this transcript initiates within intron 5 of P1-*Plagl1* and is at least 3.6 kb, with its 5′ end overlapping the 3′UTR of the paternally expressed EST AI607573 by ~400 bp. Northern blot analysis confirmed the presence of a faint band of between 3.5–kb (Figure S1). Using RACE and RT-PCR we were unable to link *Plagl1it* to *Plagl1*, confirming this is an independent overlapping transcript and not an alternative *Plagl1* exon or UTR (Figure S2). Using allele-specific RT-PCR, we were able to show that this transcript is expressed solely from the paternal chromosome in different mouse tissues (Figure 1B).

Expression of *Hymai* and *Plagl1it* is Uniformly Low Throughout Development

Next, we set out to analyse the tissue-specificity of expression for the novel transcripts. Using quantitative RT-PCR we determined the abundance of the transcripts in placenta, brain and decapitated embryos at E11.5, E12.5, E14.5, E18.5 and in addition to brain, liver, kidney and muscle from both newborn and adult mice (Figure S1). We observed that *Plagl1* expression was consistently higher than both *Hymai* and *Plagl1it* in all tissues and developmental stages analysed. All genes show a marked decrease in expression after birth, in both newborn and adult tissues.

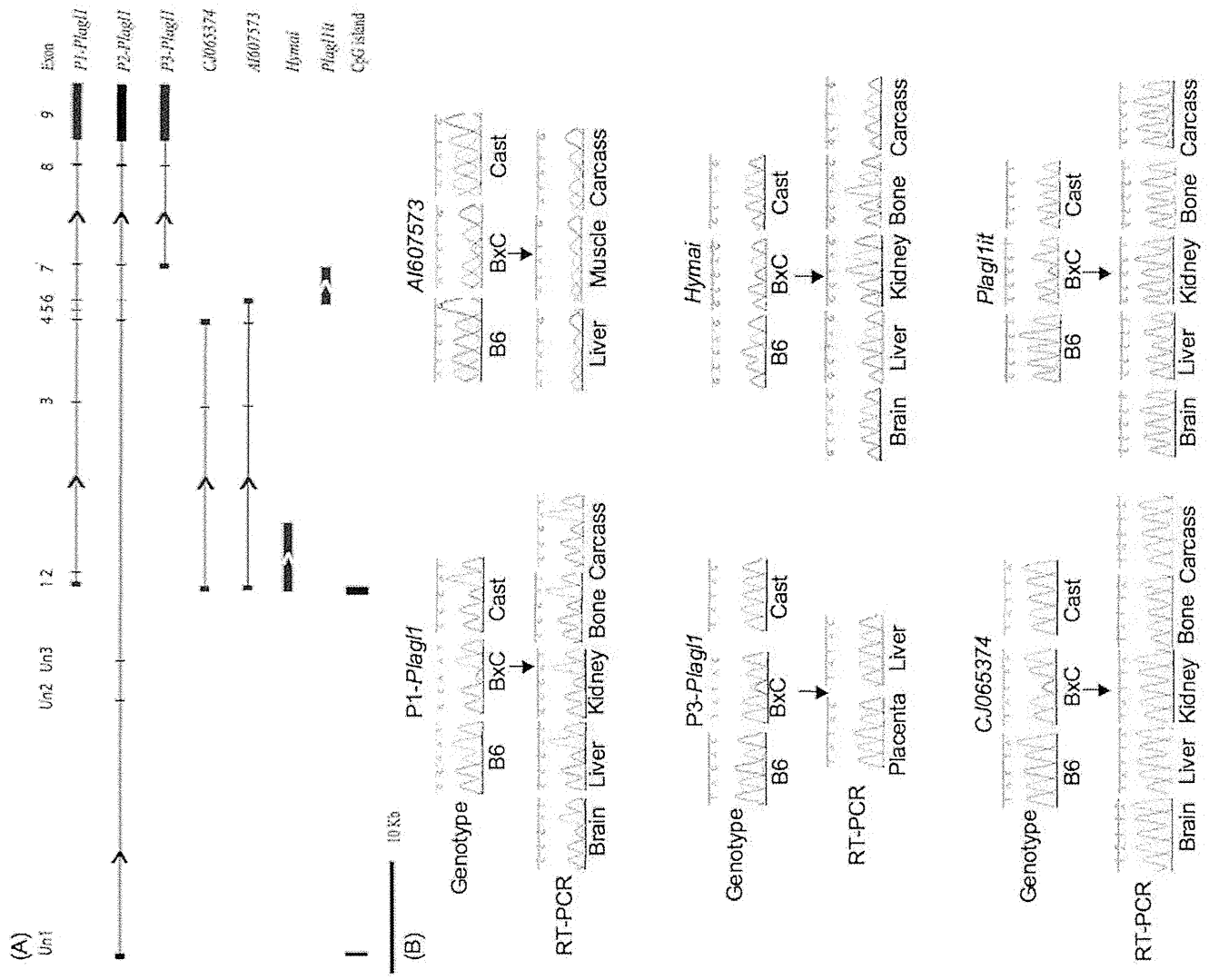

Figure 1. Schematic overview of the mouse chromosome 10 imprinted domain. (A) Map of the *Plagl1* locus, showing the location of the various imprinted transcripts and CpG islands (paternally expressed transcripts are in blue; biallelically expressed transcripts are in grey). Arrows represent direction of transcription. (B) The allelic expression of the various transcripts in embryonic tissues in reciprocal mouse crosses (for clarity only (B×C) F1 tissues are shown).
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The ncRNAs are Nuclear Retained, Unstable Transcripts

As a first step to explore whether *Hymai* and *Plagl1it* could have functional roles, we determined the cellular localisation of these ncRNAs. We performed qRT-PCR on nuclear, cytoplasmic and total RNA isolated from mouse embryonic fibroblasts (MEF) cells. The efficiency of the nuclear separation was confirmed using the *U937 snoRNA* and paternally expressed *Airn* ncRNAs that have been shown previously to not be exported to the cytoplasm. We observed residual *Airn* in the cytoplasmic fraction, suggesting slight nuclear RNA contamination only detectable when analysing highly expressed nuclear retained transcripts. The *Igf2r* mRNA was used as a control for a transcript that is exported to the cytoplasm [15]. Quantitative RT-PCR analysis revealed that the *Plagl1* transcript is efficiently exported to the cytoplasm for translation, whereas the *Hymai* ncRNA is retained in the nucleus. The *Plagl1it* transcript is present in both the nucleus and cytoplasm, but is more abundant in the nuclear fraction (Figure 2A).

To determine the stability of *Hymai* and *Plagl1it* in MEFs, actinomycin (ActD) was used to inhibit transcription. We used *C-Myc* and the unspliced *Airn* transcripts as controls for RNAs with short half-life and *Gapdh* and *Igf2r* as control for RNAs with long half-lives [8,15]. Figure 2B shows that after 12 hours treatment with ActD the *C-Myc* and *Airn* mRNAs are largely depleted, whereas *Gapdh* and *Igf2r* are not affected. The *Plagl1* transcript remains abundant under these ActD conditions, suggesting that it is a highly stable transcript. However, both *Hymai* and *Plagl1it* are diminished after 12 hours to levels that are similar to *C-Myc* and *Airn*, indicating that these ncRNAs are unstable transcripts.

DNMT3L is Indispensable for *Hymai*, *Plagl1it* and *Plagl1* Imprinting

DNA methylation inherited from the maternal germline requires the DNMT3L/DNMT3A complex [3,4]. Using bisulphite DNA sequencing, we were able to confirm that the CpG island overlapping the P1-*Plagl1* and *Hymai* transcription start sites

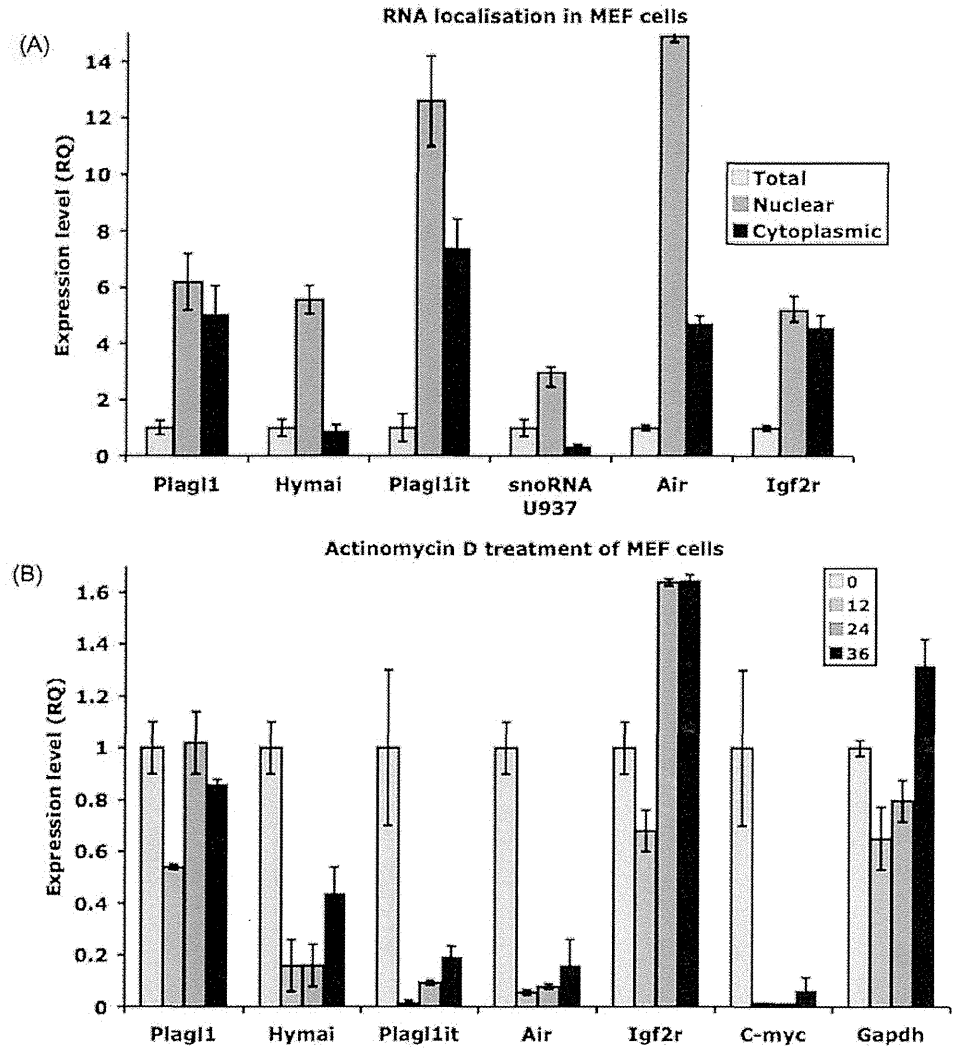

Figure 2. Cellular localization and RNA stability of the ncRNAs. (A) Distribution of the various transcripts in the nuclear (dark grey) and cytoplasmic (black) fractions, compared to total RNA (light grey). *U937 snoRNA* and *Airn* are nuclear-retained controls, whereas *Igf2* is cytoplasm-exported control. (B) Abundance of the various transcripts after exposure to Actinomycin D to determine RNA stability. The relative expression values of the control untreated samples are set to 1 (light grey bars) for each transcript. *C-Myc* and *Airn* are control transcripts for with short half-life; *Gapdh* and *Igf2r* are long half-life controls.
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is differentially methylated, whereas P2-*Plagl1* arises from an unmethylated CpG island. The promoters of *Plagl1it* and P3-*Plagl1* initiate from regions of low CpG content that display partial, but not allelic DNA methylation (Figure 3A). To assess if the maternal allelic silencing of *Hymai*, *Plagl1it* and the various *Plagl1* transcripts requires maternal germline DNA-methylation, we used qRT-PCR on mouse embryos that had inherited a deletion of the *Dnmt3l* gene from a homozygous mutant mother [3]. Lack of this essential imprinting factor led to the loss of maternal methylation at the *Plagl1*-DMR, and increased expression of all transcripts in targeted E8.5 embryos due to reactivation of the maternal allele (Figure 3B).

The *Plagl1*-DMR Chromatin Profile Requires Allelic DNA Methylation

Recent studies have suggested that there is a mechanistic link between DNA and histone methylation at imprinted DMRs [6]. To determine if there was a link between allelic DNA-methylation and any histone modifications present at the *Plagl1*-DMR, we first looked for the presence of modifications by allelic chromatin immunoprecipitation on whole embryos followed by discrimination of the parental alleles in the precipitated chromatin fractions. Our analysis focused on different modifications of histone H3 and H4; pan-acetylation of H3, acetylation of H3 lysine-9 (H3K9ac) and H3 lysine 4 dimethylation (H3K4me2) as markers of active chromatin; and the repressive marks of H3 lysine 9 trimethylation (H3K9me3) and H3 lysine 27 trimethylation (H3K27me3), along with the histone H4 lysine 20 trimethylation (H4K20me3).

We ascertained allelic enrichment using a polymorphic region between inbred mouse strains that maps within 200 bp of the CpG island associated with the *Plagl1*-DMR. Within this region H3K4me2 and H3K9ac were strongly enriched specifically on the unmethylated paternal allele (Figure 3C). The same regions showed precipitation of the repressive markers H3K9me3, H3K27me3 and H4K20me3 on the DNA-methylated maternal allele. We extended our analysis to include the promoter regions of P2-*Plagl1*, which maps within an unmethylated CpG island, and *Plagl1it*, whose promoter is not associated with a CpG island. In

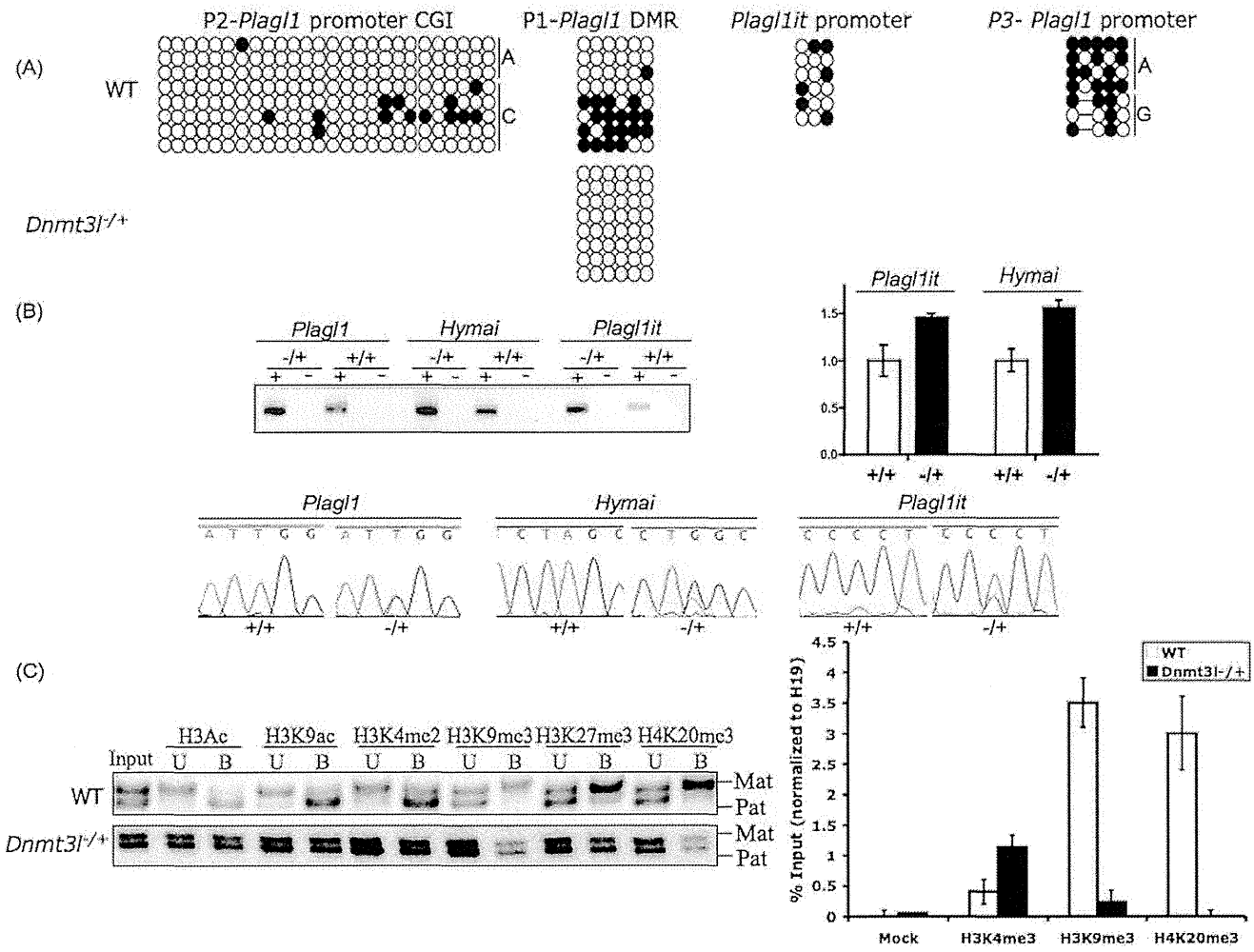

Figure 3. Analysis of *Plagl1* region in *Dnmt3l* −/+. (A) The methylation status of the *Plagl1* promoter regions in wild type +/+ and *Dnmt3l* −/+ embryos examined by bisulphite PCR. Each circle represents a single CpG dinucleotide on a DNA strand, a methylated cytosine (●) or an unmethylated cytosine (○). (B) RT-PCRs on cDNA generated with (+) and without (−) reverse transcriptase show an increase in the expression of the imprinted transcripts in *Dnmt3l*−/+ embryos as a result of reactivation of the maternal allele. (C) The histone modification signature of the *Plagl1*-DMR in wild type B×C embryos, and after targeted deletion of the *Dnmt3l* gene. DNA extracted from antibody bound (B) and unbound (U) chromatin fractions were subject to either qPCR or PCR and SSCP analysis with primers that can discriminate parental alleles.
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both cases, we failed to detect allelic precipitation, suggesting that the presence of allelic histone modifications is restricted to the DMR region (data not shown).

To assess whether the allelic histone modifications we observe at the *Plagl1*-DMR require the maternally derived DNA methylation, we performed allelic ChIP on *Dnmt3l* −/+ embryos. In agreement with observations at other imprinted DMRs [6], we detect a dramatic effect on histone modification distribution, with the lack of allelic enrichment due to "paternalization" of the maternal allele, as a result of increased H3K4me3 and a concomitant reduction of H3K9me3 and H4K20me3 (Figure 3C).

Hymai and *Plagl1it* Potentially Interact with Active Chromatin Regulatory Factors

To determine whether *Hymai* and/or *Plagl1it* could be involved in maintaining the active state of the paternal allele of the *Plagl1*-DMR, we performed a prediction of their interaction propensities against four Trithorax proteins (ASH1/KMT2H, MLL1/KTM2A, WDR5, CFP1) using the recently published catRAPID method [16]. CatRAPID allows evaluation of the interaction potential of polypeptides and RNAs using their physiochemical properties, with initial studies revealing high interactions propensities for the ncRNAs *Xist* and *HOTAIR* with Polycomb repressive complex proteins (interaction propensities 76–99% and 69–99%, respectively). In addition, CatRAPID was able to accurately predicted RNA binding of the human RNase P proteins (interaction propensities 68–99%) and discriminate RNA binding (interaction propensity >65%) and non-binding (interaction propensity <5%) proteins of the human ribonuclease mitochondrial RNA processing (MRP) complex [16].

In our analysis we used ncRNAs *Evx1as* and *HOTTIP* as controls because they are known from experimental work to directly recruit MLL1 and WDR5 proteins to *HOX* gene loci [17,18]. We observed moderate to high interaction propensities between *Evx1as* and various functional domains of the MLL1 protein, and between *HOTTIP* and WDR5 (Figure 4A). Interestingly both are predicted to interact strongly with the CFP1 PHD and Ash1 SET-postSET regions. Subsequent analysis using our imprinted ncRNAs revealed that *Hymai* and *Plagl1it* are highly prone to interaction; in particular they have strong binding

propensity with Trithorax proteins. We observe that *Hymai* and *Plagl1it* have negligible propensity for interaction with the Polycomb repressive complex protein EZH2, which trimethylates H3K27 to repress transcription (Figure 4B). Finally, we compared the interaction propensities for *Hymai* and the human orthologue *HYMAI*. We observe that despite having different sequences, and *HYMAI* being subject to splicing, the two transcripts have similar potential interactions (Figure 4C), with 3′ regions having the highest interaction propensities (data not shown). Overall the murine *Hymai* could interact with MLL1 slightly less than human *HYMAI*, but both display high interaction propensities for ASH1 SET-postSET domains and for CFP1 (Figure 4C). Taken together, our results suggest that both *Hymai* and *Plagl1it* may interact with chromatin machinery that confers a permissive chromatin state.

Discussion

Here we show a detailed investigation of the genomic organisation of the mouse *Plagl1* domain. As in humans, *Plagl1* transcripts can originate from multiple promoters, one of which is a DMR previously shown to be methylated in the female germline and therefore likely to be the ICR for this region [10,19]. A second alternative promoter located ~30 kb upstream is within an unmethylated CpG island. This promoter is orthologous with the human P2-*PLAGL1* which gives rise to biallelically expressed transcripts in lymphocytes and pancreas [20]. In mouse, transcription from this promoter is low in somatic tissues, however the primary function of this promoter may be to allow transcription across the P1-*Plagl1* promoter CpG island in growing oocytes. This has been proposed to be important for the establishment of the allelic DNA-methylation at this DMR [21]. In addition to the alternative transcripts of *Plagl1*, we show the presence of two additional ncRNAs, *Hymai* and *Plagl1it*. In keeping with other reported ncRNAs, these are expressed at a lower level than nearby mRNAs, consistent with the hypothesis that ncRNAs may fulfil a regulatory function [22]. We were able to successfully map the TSS and polyadenylation sites for both *Hymai* and *Plagl1it* using RACE-ready cDNAs, indicating that these transcripts comprise rare ncRNAs that are polyadenylated and have 5′-Caps. The reason for the nuclear enrichment of these ncRNA is unknown, as the majority of polyadenylated RNAs are exported to the cytoplasm [23,24]. However, the lack of RNA splicing may be a significant factor in the nuclear retention, as has been described for the various full-length and spliced isoforms of *Airn* [16] and other mRNAs [24].

The precise roles of *Hymai/HYMAI* and *Plagl1it* are unclear, but it is likely that they have a different function to the other known imprinted long ncRNAs such as *Airn* and *Kcnq1ot1* due to their different affinities for chromatin remodelling enzymes. *Airn* and *Kcnq1ot1* have been shown to attract histone methyltransferases G9a/KTM1C and EZH2/KMT6, and are involved in *cis*-silencing of nearby genes [8,24,25]. However, recent studies demonstrated that large ncRNAs can also guide the permissive H3K4 histone methyltransferase machinery to target genes in mouse ES cells and MEFs [17,18] and can act as local enhancers [26]. Thus, unlike other imprinted "repressive" ncRNAs, our data suggests that *Hymai* and *Plagl1it* could act to keep the paternal allele unmethylated and in a transcriptionally permissive state. In fitting with this hypothesis, we observe that *Hymai* and *Plagl1it* are unstable transcripts, which presumably ensures they stay near the site of transcription, preventing their action in *trans* on the maternal allele within the same nucleus. Our *in silico* analysis using catRAPID suggests that *Plagl1it* and the mouse and human *Hymai/HYMAI* may interact with various components of the Trithorax group proteins, with potentially the highest specificity for SET-proSET and zinc finger CXXC domains, in agreement with previous *in vitro* experiments showing that these domains can bind RNA [27,28]. *In vitro* demonstration of these interactions is technically challenging since *Hymai* and *Plagl1it* are not expressed at the levels required for RNA-ChIP in MEF cells. However, we observe that WDR5 does precipitate preferentially on the paternal unmethylated allele of the *Plagl1*-DMR (Figure S3) substantiating our hypothesis.

Conclusions

Germline loss of methylation at the maternal allele of the *PLAGL1*-DMR is known to result in TNDM [13,29]. In addition, *PLAGL1* has been suggested to play a role in numerous cancers, including ovarian, breast and pituitary adenomas, with somatic deletions or gains in methylation resulting in loss of expression of this tumour suppressor gene [30]. We hypothesise that the newly identified ncRNA could potentially guide the H3K4 methylation machinery to the paternal allele of the *PLAGL1*-DMR, and thus protect this region from pathological hypermethylation.

Materials and Methods

Mouse Crosses and Cell Lines

For the analysis of expression, wild type mouse embryos and placentas were produced by crossing C57BL/6 (B) with *Mus musculus castaneus* (C) mice. RNA and DNA from *DNMT3L*$^{-/+}$ mice (B×C) was isolated and extracted as previously described [3]. Animal husbandry and breeding were licensed by Direction Departementale des Services Veterinaires (authorization number 34–104). Homozygous C57BL/6 mice of various gestational ages were used for expression analysis. Mouse embryonic fibroblast cell lines were established from both wild-type (B x C) F1 (Bourc'his laboratory) and C57BL/6 (B) with *Mus musculus molossinus* (JF1) F1 (Feil laboratory) mice. The Institutional Review Board of Bellvitge Institute for Biomedical Research granted scientific and ethical approval for this study (PR232/09).

RNA Preparations

Total RNA from (B×C) F1 wild type embryos, *Dnmt3l*−/+ embryos and MEF cells was isolated using Trizol reagent (Invitrogen) and subjected to double DNase 1 treatment to ensure preparations were free of contaminating DNA. 1 ug of RNA was used for first strand cDNA synthesis using Promega reagents according to the manufacturer's instructions. Nuclear and cytoplasmic RNA was isolated from MEF cells using the Norgen kit (Biotek corporation, Ontario, Canada) following manufacturers instructions. cDNA was generated using 0.5 ug of cytoplasmic, nuclear and total RNA.

Actinomycin Treatment

5×10^5 MEF cells seeded per 10 cm dish were cultured for 36 hrs. At time point 0, the medium was removed; cells were washed with PBS and then incubated with medium supplemented with 10 mg/ml Actinomycin D (dissolved in ethanol). At each time point (0, 12, 24 and 36 hrs) cells from a treated dish were harvested for RNA using Trizol (Invitrogen).

5′ and 3′ RACE

Mouse E18.5 embryo Marathon-Ready cDNA (Clontech) was used for RACE using the Advantage 2 polymerase kit (Clontech). The PCR step was performed with the gene-specific primers located in ESTs for *Plagl1* and *Plagl1it* in combination with nested adaptor oligonucleotides following manufacturers recommenda-