

## Derepression of *CLDN3* and *CLDN4* during ovarian tumorigenesis is associated with loss of repressive histone modifications

Mi Jeong Kwon, Sung-Su Kim, Yoon-La Choi<sup>1</sup>, Hun Soon Jung, Curt Balch<sup>2</sup>, Su-Hyeong Kim<sup>3</sup>, Yong-Sang Song<sup>3,4</sup>, Victor E. Marquez<sup>5</sup>, Kenneth P. Nephew<sup>2</sup> and Young Kee Shin\*

Laboratory of Molecular Pathology, Department of Pharmacy, College of Pharmacy, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Korea, <sup>1</sup>Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea, <sup>2</sup>Medical Sciences, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Bloomington, IN, 47405-4401, USA, <sup>3</sup>Cancer Research Institute and <sup>4</sup>Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul 110-744, Korea and <sup>5</sup>Laboratory of Medicinal Chemistry, Center for Cancer Research, National Cancer Institute, Frederick, MD, 21702-1201, USA

\*To whom correspondence should be addressed. Tel: +82 2 880 9126;  
Fax: +82 2 882 9751;  
Email: ykeeshin@snu.ac.kr

**Unlike epigenetic silencing of tumor suppressor genes, the role of epigenetic derepression of cancer-promoting genes or oncogenes in carcinogenesis remains less well understood. The tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer and their overexpression was previously reported to promote the migration and invasion of ovarian epithelial cells. Here, we show that the expression of claudin-3 and claudin-4 is repressed in ovarian epithelial cells in association with promoter ‘bivalent’ histone modifications, containing both the activating trimethylated histone H3 lysine 4 (H3K4me3) mark and the repressive mark of trimethylated histone H3 lysine 27 (H3K27me3). During ovarian tumorigenesis, derepression of *CLDN3* and *CLDN4* expression correlates with loss of H3K27me3 in addition to trimethylated histone H4 lysine 20 (H4K20me3), another repressive histone modification. Although *CLDN4* repression was accompanied by both DNA hypermethylation and repressive histone modifications, DNA methylation was not required for *CLDN3* repression in immortalized ovarian epithelial cells. Moreover, activation of both *CLDN3* and *CLDN4* in ovarian cancer cells was associated with simultaneous changes in multiple histone modifications, whereas H3K27me3 loss alone was insufficient for their derepression. *CLDN4* repression was robustly reversed by combined treatment targeting both DNA demethylation and histone acetylation. Our study strongly suggests that in addition to the well-known chromatin-associated silencing of tumor suppressor genes, epigenetic derepression by the conversely related loss of repressive chromatin modifications also contributes to ovarian tumorigenesis via activation of cancer-promoting genes or candidate oncogenes.**

### Introduction

In addition to genetic DNA alterations, including mutations, deletions, amplifications, rearrangements and translocations, epigenetic abnormal-

**Abbreviations:** ChIP, Chromatin immunoprecipitation; DZNep, 3-deazaneplanocin A; 5-aza-dC, 5-aza-2'-deoxycytidine; FFPE, formalin-fixed paraffin-embedded; IOSE, immortalized ovarian surface epithelial; MSP, methylation-specific PCR; NOSE, normal ovarian surface epithelial; PCR, polymerase chain reaction; siRNA, small interfering RNA; TSA, trichostatin A.

ities are now known to be intimately involved in multistep carcinogenesis (1,2). The role of epigenetic silencing of key tumor suppressors by DNA hypermethylation or histone modifications in their promoter regions is now well established in tumorigenesis (2–4). Although less well understood, the loss of epigenetic repression also plays a role in tumorigenesis, by facilitating the activation of oncogenes or cancer-promoting genes, and global DNA hypomethylation at repetitive sequences and imprinted genes also contributes to tumorigenesis by promoting chromosomal instability or loss of imprinting (2,5,6).

Claudins are a 24-member family of proteins that are the major components of tight junctions, epithelial cell–cell contacts that play crucial roles in cell polarity maintenance and control of paracellular ion flux (7). Although loss of claudins has been associated with tumorigenesis (probably by allowing cell detachment and migration), claudin-3 and claudin-4 have been demonstrated to be overexpressed in several cancers including those of the breast, prostate and uterus (7,8). More frequently, however, claudin-3 and claudin-4 have been shown to be overexpressed in ovarian cancer (7–9), a malignancy that is atypical in that it actually retains or gains (rather than loses) epithelial characteristics during tumor progression (10,11). It is believed that claudin-3 and claudin-4 overexpression in ovarian cancer enhances tumor cell motility, invasiveness and survival, possibly by enhancing proteolytic activation of basement membrane-degrading matrix metalloproteinases (12). Due to their consistent overexpression in ovarian cancer and the association of claudin-3 overexpression with poor prognosis, claudin-3 and claudin-4 are under investigation as diagnostic or prognostic biomarkers (7,8). Furthermore, as claudins are transmembrane proteins with two extracellular loops, they represent promising targets for therapeutic antibodies (7), and interestingly, claudin-3 and claudin-4 are receptors for the *Clostridium perfringens* enterotoxin (7,9,13), representing a possible targeted therapeutic using *C.perfringens* enterotoxin in ovarian cancer (7,9,13), whereas a *CLDN3* small interfering RNA (siRNA) demonstrated potent suppression of tumor growth and metastasis of mouse and human ovarian tumor xenografts (14).

Despite the importance of the upregulation of claudin-3 and claudin-4 in ovarian cancer, the mechanism by which their overexpression occurs remains under investigation, although recent studies have suggested the crucial roles of epigenetic modifications, including DNA hypomethylation and histone H3 acetylation in the upregulation of two genes in ovarian cancer cells (9,15,16). Interestingly, *CLDN3* and *CLDN4* are adjacent genes on chromosome 7q11.23, located only ~60 kb apart and being transcribed in opposite directions, suggesting their possible coordinated regulation by sharing regulatory regions.

In addition to DNA methylation, the ‘histone code’ hypothesis posits that covalent modification of histone tail residues acts in concert to govern DNA packaging and thus access of transcription machinery to coding sequences (17,18). Histone modifications on specific residues correlate with either active or repressive transcription (3,19). Specifically, trimethylation of histone H3 lysine 9 (H3K9me3), H3 lysine 27 (H3K27me3) and H4 lysine 20 (H4K20me3), facilitates transcriptional repression, whereas histone acetylation of histone H3 (H3Ac) and H4 (H4Ac), and trimethylation of H3 lysine 4 (H3K4me3) are associated with active transcription (3,19). Methylation of specific histone lysine residues is mediated by their cognate histone methyltransferases, and the recent discovery of histone lysine demethylases has indicated that the ‘histone code’ is highly signal responsive and dynamic (19,20). In embryonic stem cells, a ‘bivalent’ colocalization of the activating H3K4me3 and the repressive H3K27me3 of

development-associated genes, followed by lineage-specific loss of the H3K4me3 or H3K27me3, has been reported to allow differentiated tissue silencing or expression (21,22). To fine-tune gene regulation, ‘crosstalk’ between various epigenetic modifications has also been reported (23–26). In particular, it is well known that histone H3K9 methylation and H3K27me3 are linked to DNA methylation (24,27–29).

In this study, we describe epigenetic mechanisms that regulate *CLDN3* and *CLDN4* expression during ovarian tumorigenesis and suggest that epigenetic derepression by loss of repressive histone modifications is one possible mechanism underlying the overexpression of *CLDN3* and *CLDN4*. These results demonstrate that DNA methylation-independent epigenetic activation of previously repressed cancer-promoting genes, in addition to the better understood process of epigenetic silencing of tumor suppressor genes, may represent an additional mechanism of tumor progression.

## Materials and methods

### Cell lines and drug treatment

Human ovarian cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) (Caov3, OV-90, SW626 and TOV-112D) or from the Korean Cell Line Bank (Seoul, Korea) (OVCAR3, SK-OV3, SNU8 and SNU119). Human immortalized ovarian surface epithelial (IOSE) cell lines were obtained from Dr Nelly Auersperg (University of British Columbia, Vancouver, Canada) (IOSE80UBC and IOSE385) and from Dr Michael Birrer (National Cancer Institute, Bethesda, MD) (IOSE80NIH and IOSE120) and grown in 1:1 mixture of Medium 199 and MCDB 105 supplemented with 5% fetal bovine serum. Primary cultures of human normal ovarian surface epithelial (NOSE) cells were obtained from Dr Yong-Sang Song of the Department of Obstetrics and Gynecology, Seoul National University College of Medicine, following Institutional Review Board approval, using resected healthy ovaries, followed by ovarian surface epithelium exfoliation and culturing as described previously (30).

Cells were treated with 5  $\mu$ M 3-deazaneplanocin A (DZNep; obtained from Dr Victor E. Marquez of National Cancer Institute, MD) or 5  $\mu$ M 5-aza-2'-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO) for 72 h and 200 nM trichostatin A (TSA; Sigma) for 24 h as described previously (31). Twenty micromolar LY294002, a PI3K-Akt inhibitor (Sigma), was added to cells for 24 h (32).

### Tissue samples and immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissues of human NOSE, ovarian adenoma, borderline tumor and ovarian carcinoma which are de-identified and anonymized, were obtained from the Samsung Medical Center (Seoul, Korea), following approval by that institution's review board. Immunohistochemistry was performed as described previously (8) using Zymed antibodies against EZH2 (36-6300), claudin-3, (18-7340) or claudin-4 (18-7341). We used the same ovarian carcinoma FFPE tissues that were used for tissue microarray analysis of the expression levels of claudin-3 and claudin-4 by Choi *et al.* (8) for EZH2 immunohistochemistry.

### Quantitative real-time reverse transcription–polymerase chain reaction

Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA) or the Allprep (Qiagen GmbH, Hilden, Germany) kits and reverse transcribed to complementary DNA using the Superscript<sup>TM</sup> II First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Following complementary DNA synthesis, quantitative polymerase chain reaction (PCR) was carried out as described previously (33) in a dual system LightCycler (Roche Applied Science, Mannheim, Germany) using the primers, Universal Probe Library (Roche Applied Science) probe sequences are listed in supplementary Table S1 (available at *Carcinogenesis* Online), with the *HPRT1* Taqman probe (TIB MOLBIOL, Berlin, Germany) used as a ‘reference gene’ to normalize gene expression.

### Western blot analysis

Whole cell lysates were prepared by cell incubation with RIPA lysis buffer and 20–30  $\mu$ g of total proteins were used for immunoblotting with antibodies to claudin-3 (Zymed Laboratories, South San Francisco, CA; 18-7340), claudin-4 (Zymed, 18-7341), actin (Santa Cruz Biotechnology, Santa Cruz, CA; sc-1616), H3K27me3 (Millipore, Temecula, CA; 07-449), EZH2 (Cell signaling, Danvers, MA; 4905), SUZ12 (Abcam, Cambridge, UK; ab12073) or EED (Millipore, 09-927).

### Luciferase reporter assays

*CLDN3* promoter luciferase reporter plasmids were constructed by PCR using a bacterial artificial chromosome clone as a template (AC093168, RP11-

148M21/DH10B; Invitrogen) and the primers are listed in supplementary Table S2 (available at *Carcinogenesis* Online). The PCR product was then cloned into a pGL3-Basic (Promega, Madison, WI) and the correct product confirmed by DNA sequencing. Cells were harvested 16 h after cotransfection with luciferase reporter and pCMV-lacZ plasmids using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions and firefly luciferase activity was measured using a luciferase assay system (Promega). All experiments were repeated three times.

### Methylation-specific PCR and bisulfite sequencing PCR

*CLDN3* and *CLDN4* CpG islands were located using CpGIE (34) with the following criteria: GC%  $\geq$ 55; observed to expected ratio  $\geq$ 0.65; length  $\geq$ 500. Genomic DNA was extracted from (i) cells, using G-spin (iNtRON Biotechnology, Seongnam-si, Korea) or Allprep (Qiagen) genomic DNA extraction kits or (ii) ovarian FFPE tissues, using the ChargeSwitch gDNA Micro Tissue Kit (Invitrogen) according to the manufacturer's protocols. One microgram of genomic DNA was bisulfite modified using the EzWay<sup>TM</sup> DNA methylation Detection Express Kit (KOMABIOTECH, Seoul, Korea) and PCR amplified using the primers and Taqman probes for methylation-specific PCR (MSP), quantitative MSP and bisulfite sequencing PCR listed in supplementary Tables S2 and S3 (available at *Carcinogenesis* Online). For quantitative MSP, methylation levels were calculated as percentage of methylated reference (%) by dividing *CLDN3* or *CLDN4:ACTB* ratio of a sample by the *CLDN3* or *CLDN4:ACTB* ratio of SssI-treated (and thus fully methylated) human leukocyte genomic DNA (from the Samsung Medical Center) and multiplying this value by 100.

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions with 5  $\mu$ g of normal rabbit IgG (Cell signaling, 2729) as a negative control and 5  $\mu$ g Millipore antibodies against H3K4me3 (05-745), H3K27me3 (07-449), H3K9me2 (07-441), H3K9me3 (07-442), H3Ac (06-599), H4Ac (06-598), H4K20me3 (Abcam, 9053). ChIP primers and Taqman probes are shown in supplementary Tables S2 and S3 (available at *Carcinogenesis* Online). Fold enrichment by quantitative ChIP was calculated as the value of the immunoprecipitated sample divided by the value of the negative control (IgG).

### Transient siRNA transfection

Control (siGENOME, non-targeting siRNA #1) and gene-specific (*EZH2*, *SUZ12*, On Target plus SMART pool) siRNAs were purchased from Dharmacon (Lafayette, CO). After one or more siRNA transfections using Oligofectamine (Invitrogen), cells were harvested at various time points and used for quantitative real-time reverse transcription–PCR, western blotting and ChIP assays.

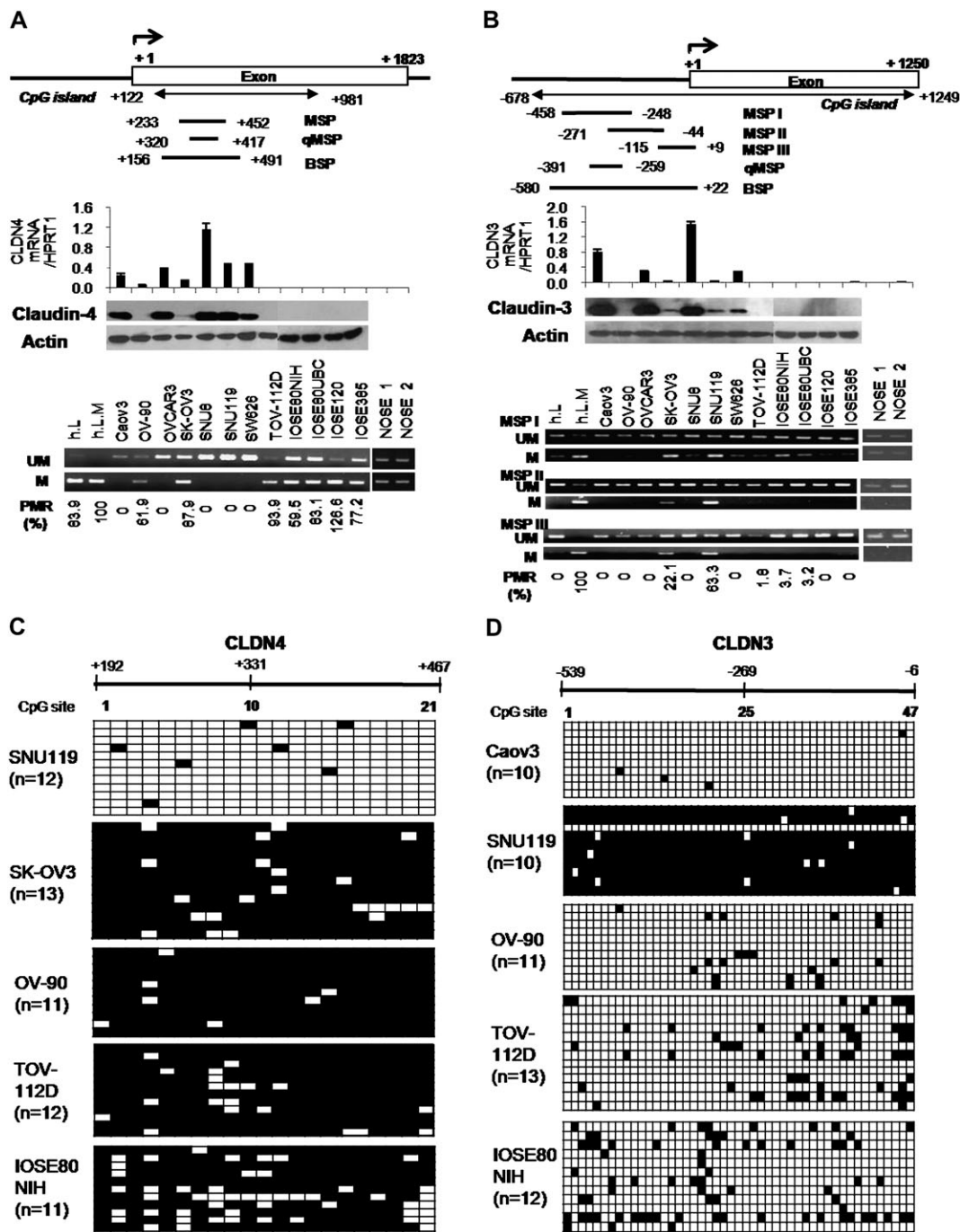
### Establishment of stable cell lines

TOV-112D cells stably expressing H3K27me3 dominant-negative mutation (TOV-K27R) or wild-type histone H3 (TOV-WtH3) were established through G418 (Invitrogen) selection after transfection with the plasmids described previously (29). Transformants were first selected by resistance to G418 (800 or 1000  $\mu$ g/ml), and single-cell clones were then isolated by limiting dilution, with plasmid stable expression confirmed by enhanced green fluorescent protein fluorescence.

## Results

### Repression of *CLDN3* in ovarian surface epithelial cells is independent of promoter DNA methylation

Using eight ovarian cancer and four IOSE cell lines, we first confirmed a positive correlation between *CLDN3* and *CLDN4* messenger RNA and protein levels, demonstrating both to be regulated at the level of transcription (Figure 1A and B). Based on previous studies suggesting the upregulation of *CLDN3* and *CLDN4* in ovarian cancer cells via hypomethylation of promoter CpG islands (9,15,16), we next performed MSP and quantitative MSP to further explore those previous correlations. MSP analyses of normal and malignant ovarian cell lines demonstrated that *CLDN4* messenger RNA levels inversely correlate with *CLDN4* promoter DNA methylation (Figure 1A), with DNA hypermethylation in *CLDN4*-repressed IOSE cells being consistent with the NOSE cells used by Litkouhi *et al.* (9). To further confirm *CLDN4* methylation level, we performed bisulfite sequencing PCR to quantitatively examine the methylation levels of individual CpG dinucleotides. Bisulfite sequencing results were similar with the MSP data, showing *CLDN4* hypermethylation in *CLDN4*-repressed



**Fig. 1.** DNA methylation in the *CLDN3* and *CLDN4* promoters in ovarian cell lines and NOSE cells. (A) *CLDN4* and (B) *CLDN3* DNA methylation status by MSP analysis in ovarian cell lines and NOSE cells. The location of CpG islands and regions for MSP and bisulfite sequencing PCR (BSP) are indicated in the genomic sequence of *CLDN3* and *CLDN4* (top). The transcript and protein levels for *CLDN3* and *CLDN4* were measured by quantitative real-time reverse transcription-PCR (qRT-PCR) and western blot, respectively (middle). The qRT-PCR values are represented as mean  $\pm$  SD. DNA methylation status was analyzed by MSP and quantitative MSP (qMSP) (bottom). Unmethylated (UM) and methylated (M) bands are shown and the quantitative methylation levels by qMSP are represented as percentage of methylated reference (PMR, %). Human leukocytes (h.L), methylated *in vitro* by *SssI* treatment (h.L.M), were used as a positive control. (C) *CLDN4* and (D) *CLDN3* DNA methylation status by bisulfite sequencing in ovarian cell lines. CpG sites analyzed by BSP in the *CLDN4* (21 CpG sites) and *CLDN3* promoter region (47 CpG sites) are indicated. At least 10 clones were sequenced and each row represents the methylation pattern of an individual cloned PCR product (filled square, methylated CpG sites; open square, unmethylated CpG sites).

cells (SK-OV3, OV-90, TOV-112D and IOSE80NIH) (Figure 1C). These findings confirm that *CLDN4* derepression in ovarian cancer cell lines strongly associates with DNA hypomethylation, as compared with its promoter hypermethylation-induced silencing in primary NOSE or IOSE cells (Figure 1A).

However, unexpectedly, we observed little or no DNA methylation in the *CLDN3* promoter of six cell lines lacking *CLDN3* expression

(OV-90, TOV-112D and four IOSE cells), in contrast to *CLDN3* promoter hypermethylation in SK-OV3 and SNU119 cells, two cell lines that express *CLDN3* at relatively low levels (Figure 1B). As shown in Figure 1D, bisulfite sequencing PCR analysis also corroborated the MSP data, further confirming a lack of DNA methylation in *CLDN3*-repressed cells, whereas *CLDN3* expression (absent) and DNA methylation levels (little or none) in primary cultured NOSE



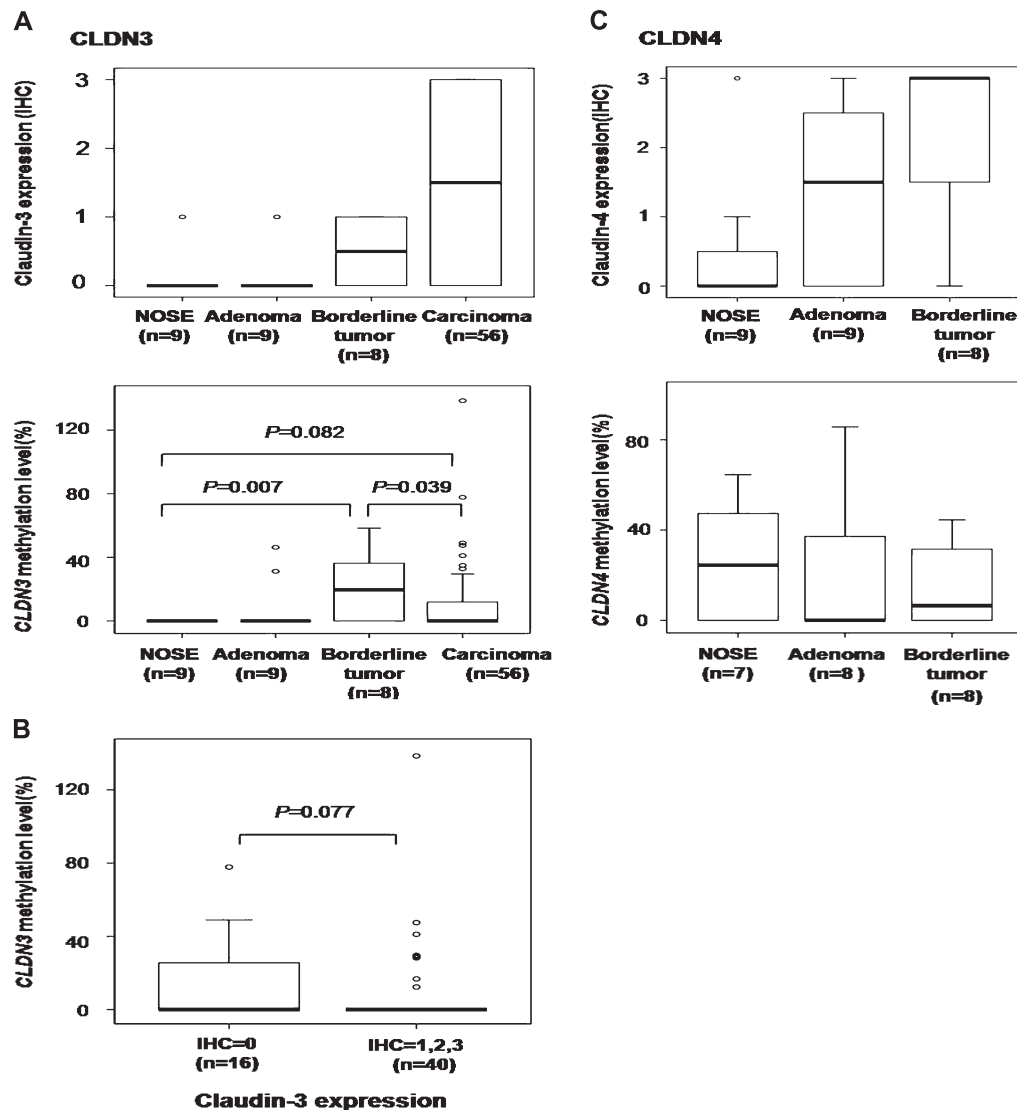
cells were similar to IOSE cells. Taken together, our results demonstrate a lack of DNA hypermethylation in the *CLDN3* promoter region in *CLDN3*-repressed cells, suggesting another mechanism(s) for gene repression.

*CLDN3* DNA methylation occurs in borderline tumors, whereas *CLDN4* is methylated in NOSE tissues

Based on different DNA methylation pattern between *CLDN3*-repressed and *CLDN4*-repressed ovarian cells, we further compared *CLDN3* and *CLDN4* promoter DNA methylation levels in FFPE samples from NOSE, ovarian adenoma, borderline tumors and ovarian carcinomas. Similar to IOSE cells, *CLDN3* was not methylated in NOSE tissues (0%, 0/9), despite undetectable claudin-3 expression in those normal tissues (Figure 2A; supplementary Table S4 is available at *Carcinogenesis* Online). Interestingly, in precancerous border-

line tumors, which had little or no claudin-3 expression (supplementary Table S4 is available at *Carcinogenesis* Online), *CLDN3* DNA promoter methylation (62.5%, 5/8) was significantly increased, as compared with NOSE tissues ( $P = 0.007$ ) (Figure 2A). However, in ovarian carcinomas (26.8%, 15/56), *CLDN3* methylation was significantly lower compared with that in borderline tumors ( $P = 0.039$ ) (Figure 2A; supplementary Table S5 is available at *Carcinogenesis* Online). These results suggest that *CLDN3* promoter methylation increases during ovarian premalignant tumorigenesis, but is lost in fully neoplastic tumors, in agreement with a previous study (16).

In contrast to the lack of association between DNA methylation and claudin-3 expression in premalignant tissues, the frequency and level of *CLDN3* methylation in the claudin-3-non-expressing group were higher than in the claudin-3-expressing group (frequency:  $P = 0.07$ ; level:  $P = 0.077$ ) in ovarian carcinomas (Figure 2B; supplementary Table S5 is available at *Carcinogenesis* Online), demonstrating that



**Fig. 2.** DNA methylation status in the *CLDN3* and *CLDN4* promoters in ovarian FFPE tissues. (A) *CLDN3* DNA methylation status in ovarian FFPE tissues. Claudin-3 expression in ovarian tissues was examined by immunohistochemistry and expression levels were scored on a scale of 0–3 (upper panel). Immunohistochemistry (IHC) = 0 indicates negative expression and a value >0 was regarded as positive expression. The methylation level (y-axis) was calculated as the mean percentage of methylated reference (PMR) value of two to six repeated quantitative MSP (qMSP) assays (lower panel). A PMR >0 was regarded as methylated. A horizontal line within the box indicates the median value. Outliers and extremes are indicated as open circles in the box plots. Differences in methylation levels between two groups of ovarian FFPE tissues were analyzed by the non-parametric Mann–Whitney test. A  $P$ -value < 0.05 (two-sided) was regarded as statistically significant. Statistical analyses were carried out using SPSS Version 12. (B) Relationship of DNA methylation with claudin-3 expression in ovarian carcinoma tissues. The methylation level was also compared between claudin-3-expressing (IHC = 1, 2, 3) and non-claudin-3-expressing tissues (IHC = 0) in ovarian carcinoma FFPE tissues. (C) *CLDN4* DNA methylation status in ovarian FFPE tissues. Claudin-4 expression was determined by immunohistochemistry (upper panel) and DNA methylation level by qMSP (lower panel).

*CLDN3* promoter DNA methylation at least partially associates with protein expression in ovarian carcinoma tissues.

We also examined *CLDN4* promoter DNA methylation and protein expression in normal and malignant ovarian FFPE tissues. Consistent with the cell line data, *CLDN4* methylation was frequently found in NOSE tissues at frequencies and methylation levels similar to those found in ovarian adenoma and borderline tumor tissues (Figure 2C; supplementary Table S6 is available at *Carcinogenesis* Online). These FFPE analyses indicate that in contrast to *CLDN3*, *CLDN4* methylation is maintained during early-stage tumorigenesis. Interestingly, there was no inverse correlation between claudin-4 expression and DNA methylation in ovarian adenoma and borderline tumor tissues (supplementary Table S4 is available at *Carcinogenesis* Online), despite the strong inverse correlation observed in the cell lines studies (Figure 1A), suggesting additional mechanisms of *CLDN4* dysregulation during premalignant tumor progression. In ovarian carcinomas, *CLDN4* was surprisingly methylated, with no significant difference in the levels and frequency of DNA methylation between the claudin-4-expressing and claudin-4-non-expressing groups (supplementary Table S6 is available at *Carcinogenesis* Online). However, we note the small number ( $n = 7$ ) of claudin-4-repressed carcinomas, in addition to possible leukocyte contamination of the FFPE tissues, as *CLDN4* was also found hypermethylated in human leukocytes (Figure 1A, 'h.L' bar).

To determine whether *CLDN3* repression in IOSE cells and ovarian cancer cell lines was due to absent transcription factors, we examined luciferase reporter activity following deletion analysis of *CLDN3* promoter in four cell lines possessing varying levels of endogenous *CLDN3* expression. As shown in supplementary Figure S1 (available at *Carcinogenesis* Online), luciferase activity of the various *CLDN3* promoter fragments was similar among the four cell lines examined (regardless of their *CLDN3* expression status), in agreement with a similar study of the *CLDN3* promoter (16). These promoter regulation studies confirm that the basal transcription machinery remains intact in both *CLDN3*-repressed and *CLDN3*-expressing cells, supporting the role of other possible mechanism(s) of *CLDN3* regulation during ovarian tumorigenesis. Similarly with *CLDN3*, the same pattern of luciferase activity of the *CLDN4* promoter was also reported in ovarian cancer cells regardless of their *CLDN4* expression in previous study (15).

*Repression of CLDN3 and CLDN4 in IOSE cells is associated with bivalent histone modifications, whereas their derepression in ovarian cancer cells associated with loss of repressive H3K27me3 and H4K20me3*

To determine whether histone modifications in the *CLDN3* promoter mediate transcriptional repression, independently of DNA methylation, we performed ChIP studies of three distinct *CLDN3* promoter regions and quantitative ChIP of one region. Interestingly, the *CLDN3* promoter showed a 'bivalent' colocalization of both repressive H3K27me3 and activating H3K4me3 histone mark in *CLDN3*-repressed IOSE cells in addition to *CLDN3*-repressed ovarian cancer cell lines (OV-90 and TOV-112D), whereas H3K27me3 was lost in *CLDN3*-expressing cells (Caov3 and SNU119) (Figure 3A). The most disparately altered chromatin modification between *CLDN3*-repressed and *CLDN3*-expressing cells was the repressive H3K27me3 histone mark, although loss of two other repressive marks, H4K20me3 and H3K9me3, likewise associated with *CLDN3* derepression, also independently of altered DNA methylation. Detectable H3K9me2 was not found in the *CLDN3*-repressed cells and active chromatin marks, H3K4me3 and H3Ac, were observed in the *CLDN3* promoters of all cells, regardless of *CLDN3* expression (Figure 3A). In contrast to H3Ac, however, only low levels of H4Ac were detected in the *CLDN3* promoter of the three cell lines (Caov3, SNU119 and IOSE80NIH) having varying levels of *CLDN3* expression.

Similar to the *CLDN3* promoter, we found a bivalent pattern of *CLDN4* promoter histone methylations in *CLDN4*-repressed IOSE

cells, which were DNA hypermethylated (Figure 3B). In addition to DNA hypomethylation, loss of the repressive H3K27me3 and H4K20me3 histone mark was observed in *CLDN4*-expressing cells (SNU119 and Caov3), as compared with *CLDN4*-repressed cells. In contrast to *CLDN3*, the *CLDN4* promoter possessed detectable H3K9me2, in addition to DNA hypermethylation, in cells lacking its gene expression (TOV-112D and IOSE80NIH) (Figure 3B), suggesting a link between these two repressive modifications in the *CLDN4* regulation, in accord with previous studies supporting such an association (24,35). The levels of active histone mark H4Ac and H3K4me3 were correlated with *CLDN4* expression (Figure 3B), whereas H3Ac levels were high regardless of *CLDN4* expression similar to *CLDN3*. These results indicate that in normal and malignant ovarian epithelial cells, repressive histone methylations (H3K27me3, H3K9me2 and H4K20me3) in the *CLDN4* promoter, together with DNA hypermethylation, strongly repress *CLDN4*, whereas activating histone marks (H3K4me3 and H4Ac), in conjunction with DNA hypomethylation, allow *CLDN4* transcriptionally permissive.

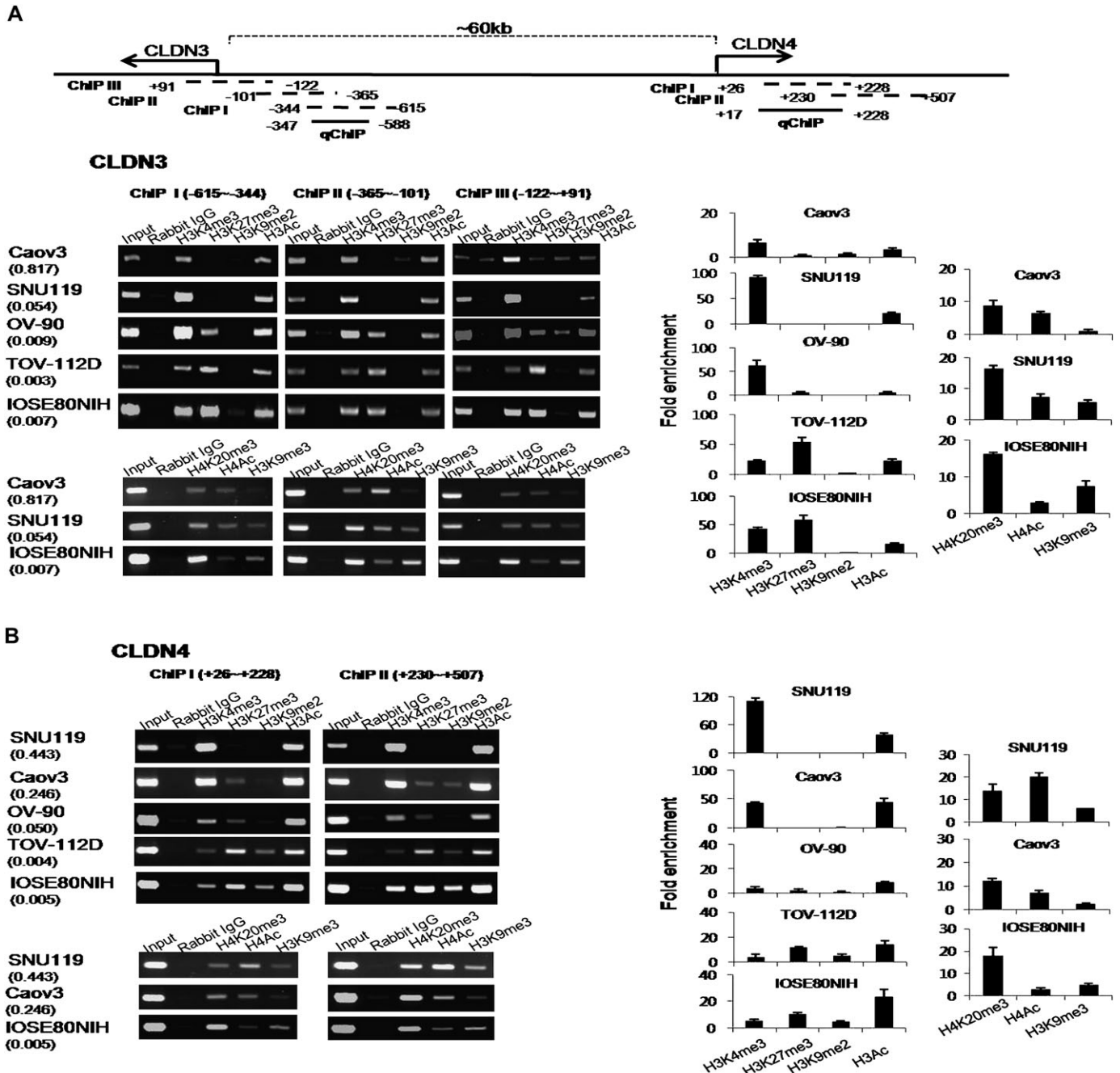
*CLDN3 and CLDN4 are not direct targets of EZH2 and loss of H3K27me3 alone does not induce their derepression in ovarian cells*

Trimethylation of H3K27 is catalyzed by the histone methyltransferase EZH2, a member of the Polycomb repressive complex-2 that is also overexpressed in embryonic stem cells and in highly aggressive epithelial malignancies (36). To examine the role of EZH2 in the regulation of *CLDN3* and *CLDN4* expression, we utilized siRNA transfection to knock down EZH2 in TOV-112D cells, which normally repress *CLDN3* and *CLDN4* in association with promoter H3K27me3. Reduced levels of *EZH2* messenger RNA and protein corresponded with significantly decreased H3K27me3 binding in both the *CLDN3* and *CLDN4* promoter region ( $P < 0.05$ ) (Figure 4A). However, the loss of these repressive chromatin marks did not correspondingly induce *CLDN3* or *CLDN4* expression, as compared with the control siRNA treatment (Figure 4A). Similar results were observed in *CLDN3*- and *CLDN4*-repressed OV-90 cells (supplementary Figure S2 is available at *Carcinogenesis* Online). Further attempts to augment EZH2 knock-down by repeated *EZH2* siRNA transfections or cotransfection of *EZH2* siRNA with an siRNA against *SUZ12* in TOV-112D cells also did not restore the expression of both genes (data not shown). We also treated TOV-112D cells with the PI3K-Akt inhibitor LY294002 to inhibit EZH2 serine-21 phosphorylation and thus upregulate its H3K27 methyltransferase activity (32). This treatment actually increased *CLDN3* and *CLDN4* expression (Figure 4B), rather than decreasing the expression. Additional evidence against direct EZH2 regulation of *CLDN3* and *CLDN4* expression was provided by a lack of correlation between EZH2 and claudin-3/claudin-4 expression by immunohistochemistry in ovarian carcinoma tissues (supplementary Figure S3 is available at *Carcinogenesis* Online).

We also established stable cell lines with a dominant-negative histone H3 lysine 27 mutation in TOV-112D cells (TOV-K27R) to rule out the possibility of incomplete H3K27me3 by transient siRNA transfection and the existence of other methyltransferases involved in the methylation of H3K27. In agreement with our EZH2 knock-down findings, the TOV-K27R cells did not derepress *CLDN3* or *CLDN4*, as compared with untransfected TOV-112D or TOV-WtH3 cells (Figure 4C). Overall, these results suggest that the expression of *CLDN3* and *CLDN4* is not solely regulated by EZH2, or that H3K27me3 loss alone is insufficient to derepress their expression consistent with findings in previous reports (37,38), and lend support to the idea that additional mechanisms might be involved in their regulation.

*Increased histone acetylation and simultaneous loss of repressive H3K27me3 and H4K20me3 result in increased expression of CLDN3 and CLDN4 in ovarian cancer cells*

Recently, DZNep was found to deplete components of the Polycomb repressive complex-2 complex and inhibit histone methylations, including H3K27me3 and H4K20me3, whereas it had little effect on the

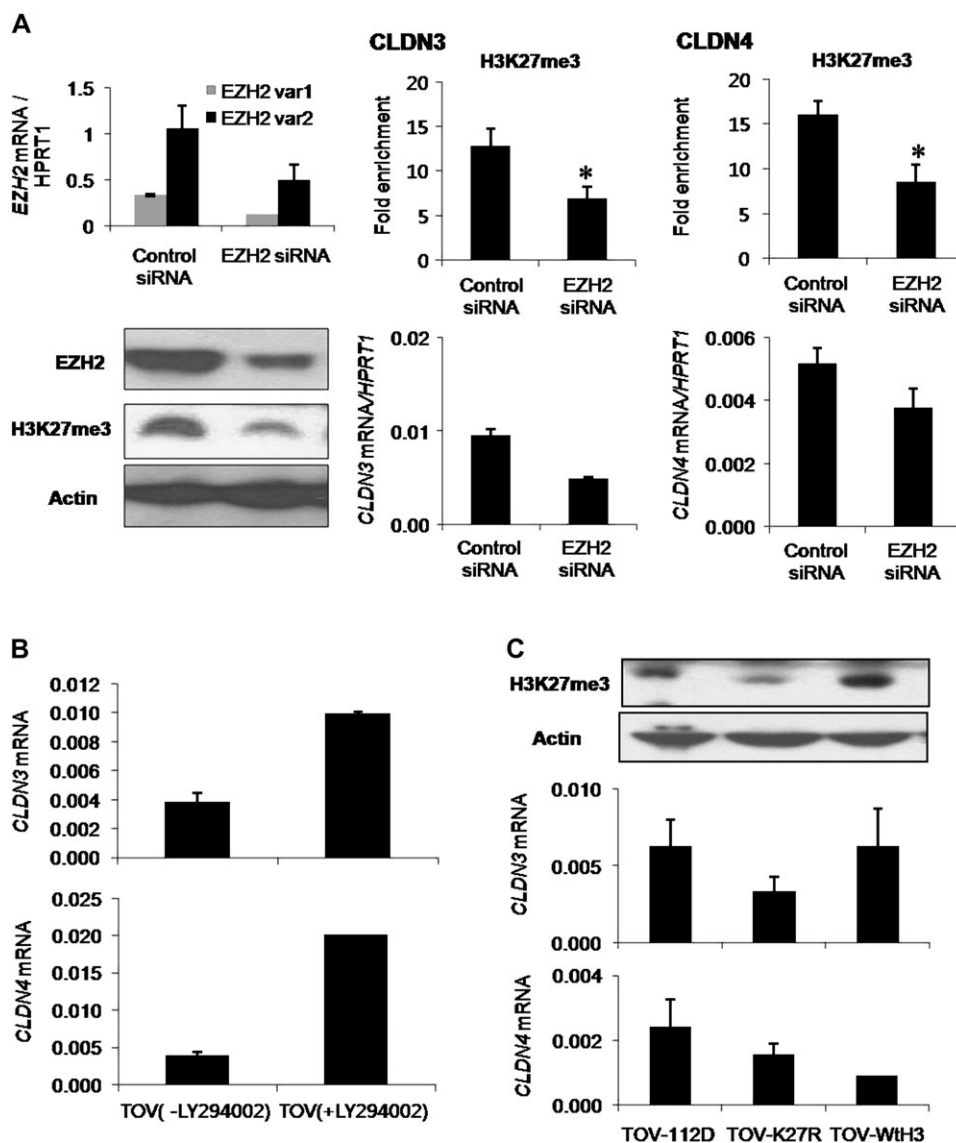


**Fig. 3.** Histone modifications in the *CLDN3* and *CLDN4* promoters in ovarian cancer cell lines. (A) Histone modifications in the *CLDN3* and (B) *CLDN4* promoters. Location of the regions analyzed by the ChIP assay is indicated (top). Three regions of *CLDN3* (ChIP I to ChIP III) and two regions of *CLDN4* (ChIP I to ChIP II) were analyzed for histone modifications by ChIP assays (left). Quantitative enrichment of histone modifications in the *CLDN3* and *CLDN4* promoters was also assessed by quantitative ChIP (qChIP) assays using Taqman probe (right). y-axis represents the fold enrichment relative to IgG control. Gene expression levels of *CLDN3* and *CLDN4* in each cell line are indicated under the name of cell line.

levels of H3K9me3 or H3Ac (39). To test whether DZNep, alone or in combination with another epigenetic modulator, could regulate *CLDN3* expression in cells lacking its expression (TOV-112D, OV-90 and IOSE80NIH), we assessed *CLDN3* transcript levels after mono- or co-treatment with the histone deacetylase inhibitor, TSA. By themselves, DZNep or TSA treatment alone only slightly induced *CLDN3* expression in each cell line (Figure 5A). However, we detected a strong (3- to 5-fold) increase in *CLDN3* expression in all three cell lines following treatment with DZNep combined with TSA (Figure 5A), suggesting possible crosstalk of histone methylations (H3K27me3 and/or H4K20me3) with histone acetylation.

Interestingly, 5-aza-dC treatment induced *CLDN3* expression in TOV-112D and IOSE80NIH cells with very low DNA methylation in contrast to no induction in OV-90 cells having little DNA methylation (supplementary Figure S4 is available at *Carcinogenesis* Online). However, it was difficult to detect the concomitant DNA demethylation at *CLDN3* promoter in TOV-112D and IOSE80NIH cells after 5-aza-dC treatment (data not shown), suggesting that increased *CLDN3* expression by 5-aza-dC might occur independently of DNA demethylation.

To examine possible mechanisms of DZNep/TSA-mediated *CLDN3* upregulation, we assessed changes in histone

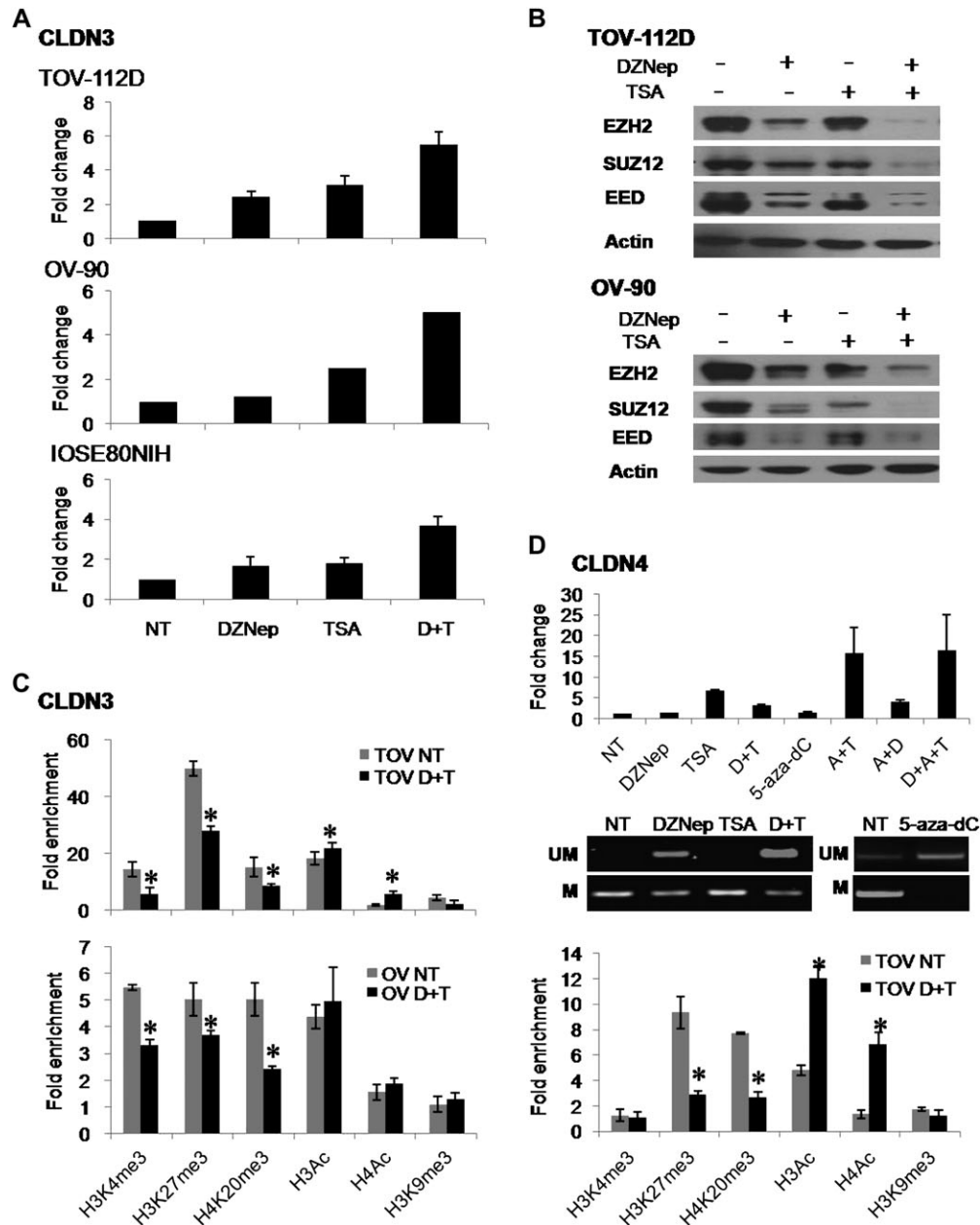


**Fig. 4.** Effect of the loss of H3K27me3 on *CLDN3* and *CLDN4* expression in ovarian cancer cells. (A) Effect of EZH2 knockdown by *EZH2* siRNA on *CLDN3* and *CLDN4* expression. Knockdown of *EZH2* transcripts (*EZH2* var1 and *EZH2* var2) and the decrease in protein levels of H3K27me3 were assessed in TOV-112D cells 72 h after siRNA treatment (100 nM). The effect of *EZH2* knockdown on H3K27me3 enrichment in the *CLDN3* and *CLDN4* promoters was also evaluated by quantitative ChIP in TOV-112D cells. \* $P < 0.05$ , Mann-Whitney test. *CLDN3* and *CLDN4* messenger RNA (mRNA) levels after *EZH2* siRNA treatment were compared with the levels after control siRNA treatment. (B) Effect of LY294002 on *CLDN3* and *CLDN4* expression. *CLDN3* and *CLDN4* expression levels were determined by quantitative real-time reverse transcription-PCR (qRT-PCR) in TOV-112D cells after a 24 h treatment with 20  $\mu$ M LY294002. (C) *CLDN3* and *CLDN4* expression in TOV-K27R cells. The level of H3K27me3 in TOV-112D, TOV-K27R and TOV-WtH3 cells was assessed by western blotting and the mRNA levels of *CLDN3* and *CLDN4* were assessed by qRT-PCR.

modifications in the *CLDN3* promoter following DZNep/TSA-mediated gene induction in TOV-112D and OV-90 cells. Polycomb repressive complex-2 proteins (EZH2, SUZ12 and EED) levels were further depleted when DZNep/TSA cotreated, as compared with DZNep alone (Figure 5B). As shown in Figure 5C, the combined treatment significantly reduced *CLDN3* promoter-localized H3K27me3 and H4K20me3 ( $P < 0.05$ ), with a slight decrease in H3K9me3, while concomitantly increasing the activating mark H3Ac and H4Ac ( $P < 0.05$ ) in TOV-112D cells. These results demonstrate that in addition to loss of repressive histone methylations, histone acetylations on both H3 and H4 may also regulate *CLDN3* expression. In OV-90 cells, both H3K27me3 and H4K20me3 were significantly decreased ( $P < 0.05$ ) without a marked increase in histone acetylation (Figure 5C), suggesting that a reduction in multiple repressive histone methylation modifications alone can allow gene induction when high levels of active marks are already present.

As *CLDN4* was repressed with DNA hypermethylation in ovarian cells (Figure 1A and C), in addition to H3K27me3 and histone acetylation, we also assessed the effects of a DNA methylation inhibitor, 5-aza-dC (alone or in combinations with DZNep or TSA), on *CLDN4* expression in promoter-hypermethylated TOV-112D cells. We found that combined 5-aza-dC/TSA treatment resulted in robust *CLDN4* induction (~16-fold), with only minimal induction by 5-aza-dC monotherapy (Figure 5D, top). DNA demethylation was observed in 5-aza-dC-treated cells (Figure 5D, middle), indicating that increased *CLDN4* expression by 5-aza-dC/TSA treatment is related to DNA demethylation. Fairly similar to *CLDN3*, *CLDN4* was induced nominally (~3-fold) by DZNep/TSA, although that combination induced simultaneously significant decreases in repressive marks (H3K27me3 and H4K20me3) and increases in active marks (H3Ac and H4Ac) in the *CLDN4* promoter ( $P < 0.05$ ; Figure 5D, bottom). Unlike *CLDN3*, marked increases in the H3Ac and H4Ac were observed, with H4Ac increased greater (5.1-fold) than H3Ac (2.5-fold).





**Fig. 5.** Effects of DZNep, 5-aza-dC and TSA treatment on *CLDN3* and *CLDN4* expression and epigenetic modifications in ovarian cell lines. **(A)** Changes in *CLDN3* expression in ovarian cells treated with 5  $\mu$ M DZNep (D), 200 nM TSA (T) or combination of DZNep/TSA. *CLDN3* induction by quantitative real-time reverse transcription-PCR is represented as the fold change (y-axis), calculated relative to no treatment (NT). **(B)** Effect of pharmacological treatments on Polycomb repressive complex-2 (EZH2, EED and SUZ12) protein levels. **(C)** ChIP analysis of the *CLDN3* promoter in TOV-112D and OV-90 cells untreated or treated with DZNep/TSA. Fold enrichment was measured by quantitative ChIP. \* $P < 0.05$ . **(D)** Effect of DZNep, 5-aza-dC and/or TSA on the expression, methylation status and histone modifications of *CLDN4* in TOV-112D cells. Changes in *CLDN4* expression (top), DNA methylation (middle) and histone modifications (bottom) after DZNep, TSA, 5-aza-dC (A, 5 $\mu$ M) or their combined treatments are represented, respectively. \* $P < 0.05$ .

These results indicate that concurrent histone acetylation and DNA hypomethylation are required for maximal *CLDN4* derepression. Interestingly, DZNep treatment of TOV-112D cells induced the loss of DNA methylation with a concurrent increase in demethylation of the promoter region of *CLDN4* (Figure 5D, middle), suggesting an interaction between DNA methylation and Polycomb group proteins (PcG)-mediated histone methylation in the regulation of *CLDN4* expression, in agreement with a previous report (27).

These results indicate that *CLDN3* and *CLDN4* repression can be robustly reversed by simultaneous changes in multiple epigenetic modifications using combined treatment targeting several chromatin modifications.

## Discussion

Several candidate oncogenes, including *BORIS*, *SNGG*, *IGF2* and tumor progression-associated *CLDN4*, have been shown to be induced by the loss of promoter DNA methylation in ovarian and other cancers (40). However, little is known about activation of tumorigenesis-associated genes due to the loss of other repressive epigenetic marks, including silencing histone methylations.

In the present study, we first demonstrate that *CLDN3* and *CLDN4* overexpression in ovarian cancer cells is associated with decrease of the repressive histone methylation marks H3K27me3 and H4K20me3 in their promoter regions. Interestingly, we found *CLDN3* to be



repressed in NOSE cells and tissues, despite a lack of promoter DNA methylation. Promoter bivalency, simultaneously possessing activating (H3K4me3) and repressive (H3K27me3) histone mark, is found in numerous development-related genes in embryonic and hematopoietic stem cells (21,41). This histone pattern is associated with a low level of gene expression, keeping genes poised for activation (21,41). Similarly, we found that *CLDN3* and *CLDN4* repression is associated with promoter bivalency, with their upregulation associated with loss of H3K27me3 and H3K4me3 retention. It has been recently demonstrated that a H3K27me3 demethylase UTX forms a complex with the H3K4 trimethyltransferase MLL2 (42), suggesting counter-modulation of these two opposing histone marks in the loss of bivalency during gene upregulation (20,42,43), and further studies could investigate a role for this complex in tumorigenesis-associated *CLDN3* or *CLDN4* reexpression.

As expected, in highly gene-repressed cells, we observed the presence of additional repressive histone marks, including H3K9me3, H4K20me3 and hypoacetylated H4 in *CLDN3*-repressed cells and H3K9me2, H4K20me3 and hypoacetylated H4 in *CLDN4*-repressed cells, consistent with previous reports (44,45). Although *CLDN3* was repressed in IOSE cells by histone modifications alone, *CLDN4* additionally possessed DNA hypermethylation in *CLDN4*-repressed IOSE cells and NOSE tissues, consistent with previous cell line studies (9,15), indicating a more pronounced gene-suppressive chromatin environment for that gene.

Previous studies have linked bivalent chromatin pattern or H3K27me3 to mark genes for subsequent DNA methylation (28,46), and in *CLDN4* promoter, we indeed observed the presence of both repressive marks. Although *CLDN3* possessed H3K27me3, but not DNA methylation, we did observe *CLDN3* DNA methylation in premalignant borderline tumor tissues consistent with the histone mark acting as an identifier for subsequent DNA methylation. Similarly, DNA methylation has been associated with both H3K9me2 and H3K9me3 (24); however, although DNA-methylated *CLDN4*-repressed cells possessed both histone marks, DNA methylation-independent *CLDN3* repression associated with H3K9me3 alone, suggesting that H3K9me2, rather than H3K9me3, is more likely to be linked to DNA methylation.

In contrast to some genes whose expression was reactivated by *EZH2* siRNA alone (47,48) or in H3K27 dominant-negative mutant cells (29), *CLDN3* and *CLDN4* expression was not induced by *EZH2* knockdown or loss of H3K27me3, suggesting that *CLDN3* and *CLDN4* are not direct targets of *EZH2* and PcG-mediated H3K27me3 depletion alone insufficient to derepress their expression, in agreement with previous studies (37,38). However, DZNep treatment in combination with TSA resulted in considerable *CLDN3* induction with changes in multiple histone modifications in the *CLDN3* promoter. These results indicate that simultaneous changes in multiple epigenetic modifications are required for *CLDN3* expression induction, in agreement with previous reports (31,38).

Moreover, in *CLDN4*-repressed cells with DNA hypermethylation, 5-aza-dC treatment strongly induced *CLDN4* expression in combination with TSA (~16-fold) or with DZNep (~4-fold), whereas inhibition of DNA methyltransferases or DZNep alone resulted in only minimal reexpression, indicating the need for loss or gain of other chromatin marks in addition to DNA hypomethylation for *CLDN4* strong reexpression.

Although it needs to be biochemically determined whether strong synergy by DZNep/TSA for *CLDN3* or 5-aza-dC/TSA for *CLDN4* is generated by direct interaction between epigenetic modifications, simultaneous changes in epigenetic marks by combined treatment in our study reflect possible interplay between histone modifications in the regulation of *CLDN3* expression or link between histone modification and DNA methylation in the regulation of *CLDN4* expression. Further studies will be required to address mechanistically if *CLDN3* and *CLDN4* are regulated by multiple chromatin modifications via their crosstalk.

Although previous studies (15,16) reported the similar epigenetic mechanism regulating *CLDN3* and *CLDN4* expression in ovarian

cancer, our study shows that different epigenetic mechanism is involved in the alteration of claudin-3 and claudin-4 expression during ovarian tumor progression. This might be due to different chromatin-binding proteins including transcriptional factors in their upstream regulatory elements because transcription regulatory factors are involved in recruiting multiple chromatin modifying proteins, which in turn regulate epigenetic modifications in the promoter region (49). These findings raise the possibility of more complex upstream mechanism regulating *CLDN3* and *CLDN4* and further allow different epigenetic strategy targeting claudin-3 or claudin-4 for cancer therapy.

Finally, our findings suggest that epigenetic therapies capable of targeting multiple histone modifications in the promoter regions of *CLDN3* and *CLDN4* may have the potential to treat ovarian cancer. Since the overexpression of *CLDN3* and *CLDN4* is related to ovarian malignancy, repression of these genes in ovarian cancer by an epigenetic approach could be used for treatment of ovarian cancer. Conversely, as claudin-3 and claudin-4 have been demonstrated to be receptors for the *C.perfringens* enterotoxin, it is possible that their further epigenetic upregulation could enhance the sensitivity to that compound, similar to currently studied methods of derepressing cell surface antigens for immunotherapy (50,51).

In conclusion, we have elucidated the epigenetic mechanisms underlying the overexpression of *CLDN3* and *CLDN4* in ovarian cancer. Although there exists a growing number of examples of DNA hypomethylation-related induction of candidate oncogenes, we further demonstrate the crucial importance of histone modifications in tumorigenesis-associated gene activation and suggest the epigenetic derepression by loss of repressive histone modifications as one possible mechanism underlying the overexpression of cancer-related genes during ovarian tumorigenesis. Our study offers novel insight into cancer epigenetics and the current understanding of ovarian tumorigenesis, and further suggests novel epigenetic approaches that could be used to target these proteins for ovarian cancer therapy.

### Supplementary material

Supplementary Tables S1–S6 and Figures S1–S4 can be found at <http://carcin.oxfordjournals.org/>

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