Expression of human *NAA11* (*ARD1B*) gene is tissue-specific and is regulated by DNA methylation

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NAA10 gene encodes the catalytic subunit of N(alpha)-acetyltransferase NatA that catalyzes the acetylation of the N-termini of many eukaryotic proteins. A homologous gene called *NAA11* is also present in mammalian cells. hNaa10p and hNaa11p are reported to be co-expressed in human cell cultures. In mouse tissues, however, *Naa11* transcripts can only be detected in gonadal tissues whereas *Naa10* transcripts are present in various tissues. We re-examined the expression of *NAA11* in human cell lines and expanded the test to normal as well as cancerous human tissues. Surprisingly, we did not detect the expression of *NAA11* in human cell lines that previously were reported to express it. Similar to its mouse ortholog, *NAA10* displayed widespread expression in human tissues. *NAA11* transcripts, however, were only detected in testicular and placental tissues. The lack of *NAA11* expression was also demonstrated in eight different types of human cancerous tissues. By methylation-specific polymerase chain reaction and bisulfite sequencing, we found that the absence of *NAA11* expression correlated with hypermethylation of the CpG island located at the proximal promoter of *NAA11* gene. We also found that the cloned *NAA11* gene promoter fragment was active when introduced into non *NAA11*-expressing human cells and its promoter activity was lost upon in vitro DNA methylation. Taken together, our results indicate *NAA11* expression is tissue-specific and is epigenetically regulated by DNA methylation.

Introduction

In higher eukaryotes, protein acetylation occurs at either α-amino groups or ε-amino groups on polypeptide chains. While protein ε-acetylation at internal lysine residues is a reversible post-translational modification process,[1] protein α-acetylation is irreversible and takes place co-translationally when the first 25 to 50 amino acid residues of a nascent polypeptide emerge from the ribosome.[2] The transfer of an acetyl group from acetyl-CoA to N-termini of proteins is catalyzed by N(α)-acetyltransferases (NATs). At least three distinctive NAT complexes (namely NatA, NatB and NatC) exist in yeast and human cells.[3] Among them, NatA is the most studied. NatA is composed of a catalytic subunit called Naa10p (also known as ARREST DEFECTIVE 1A; ARD1A) and an auxiliary subunit called Naa15p (also known as NMDA-Receptor Regulated 1; NARG1; NATH; NAT1) that is responsible for docking the NatA to ribosomes.[4,5] Formation of ARD1A-NARG1 heterodimer is required for NAT activity.[6]

Recently, an alternative form of NatA, composed of hNaa10p and hNaa16p, was reported in HEK 293 cells.[7]

N(α)-acetylation is one of the most common protein modification processes occurring in eukaryotic proteins. Over 80% of mammalian proteins undergo this modification.[2,8] The widespread occurrence of protein N(α)-acetylation suggests that the enzymatic activity is essential for normal cellular functions. Nevertheless, the biological significance of this protein modification process remains largely unknown. Recent studies in yeast suggest that N(α)-acetylation creates specific degradation signals for proteins,[9] and prevents proteins from being targeted to the endoplasmic reticulum.[10] NatA was shown to regulate neuronal dendritic development in rodents[11] and it may also be involved in maintaining cellular proliferation.[12] Attenuation of NatA activity by silencing the expression of hNaa10p or hNaa15p with small interfering RNA results in a reduced viability and an increased level of apoptosis in HeLa cells.[13] Furthermore, hNaa10p was demonstrated to promote cancer cell proliferation.[14-17] An elevated

expression level of hNaa10p is observed in a variety of human cancers such as breast, colorectum and lung.[16,18-21] Overexpression of hNaa10p is also associated with a poor prognosis in cancers of colon[19] and lung.[21] Nevertheless, the proposition that hNaa10p is an oncoprotein remains controversial as the opposite effect has also been observed.[22,23] hNaa10p level is reduced in neoplastic thyroid tissue when compared to its non-neoplastic counterpart.[24] *NAA10* transcript level is significantly reduced in non-small cell lung cancer as contrasted to adjacent non-tumor lung tissue.[23] Increased levels of *NAA10* transcripts and hNaa10p proteins were respectively found to correlate with better clinical outcome in breast cancer patients[23] and survival of lung cancer patients.[25] Similar to the yeast Naa10p, mouse Naa10p alone does not display NAT activity.[12,26] However, hNaa10p alone was demonstrated to catalyze the acetylation of internal lysine residues in β-Catenin (CTNNB1),[15] Myosin Light Chain Kinase (MLCK),[22] and hNaa10p itself.[14] A shorter isoform of mNaa10p (mNaa10p_NP_001171436) was shown to stimulate the degradation of Hypoxia Inducible Factor 1α (Hif1α) by acetylating an internal lysine residue of the protein.[27] Interestingly, hNaa10p was shown in lung cancer cells to modulate the activity of DNA Methyltransferase 1 (DNMT1),[21] and to suppress metastasis[25] independently of its acetyltransferase activity.

A homolog of Naa10p, called Naa11p (also known as ARREST DEFECTIVE 1B; ARD1B; ARD2), was identified in the mouse[26] and human.[28] The genes encoding *Naa11* and *NAA11* are believed to be the functional autosomal copies of their respective X-linked progenitors (*Naa10* and *NAA10*) through retroposition.[26,28] Except for the divergent C-termini, the polypeptide sequences of Naa10p and Naa11p are highly conserved between the mouse and human. Both mNaa11p and hNaa11p can reconstitute a functional NAT in the presence of mNaa15p and hNaa15p, respectively. In the mouse, *Naa11* is expressed predominantly in the testis; its expression level is upregulated during meiosis when *Naa10* expression is downregulated.[26] In contrast, *Naa10* is expressed in somatic tissues that do not show *Naa11* expression. It is therefore believed that mNaa11p is expressed to compensate for the loss of mNaa10p during spermatogenesis.[26] On the other hand, *NAA11* was found to co-express with *NAA10* in several human cell lines.[17,25,28] The induction of differentiation of promyelocytic leukemia NB4 cells leads to a downregulation of hNaa10p and hNaa15p expression. However, the level of hNaa11p remains unchanged, which implies a role for hNaa11p in the cellular differentiation process.[28] The loss of heterozygosity in *NAA11* was shown to correlate with a poor prognosis in hepatocellular carcinoma patients.[29] Other than these, the biological functions of mNaa11p and hNaa11p are not known. The presence of two similar NatA complexes sharing the same ribosome docking subunit, but different catalytic subunits in the same human cells,[28] may imply a complementary role in regulating similar biological processes. Alternatively, the two NatA complexes may display different protein substrate specificity and thus, biological functions. Intrigued by this hypothesis, we examined whether co-expression of *NAA10* and *NAA11* is a common phenomenon in human tissues. Contrary to our expectation, we could not reproduce the co-expression of *NAA10* and *NAA11* in

human cell cultures. *NAA11* expression was detected only in the testis and placenta obtained from normal human subjects. Except for a few cases, *NAA11* expression was also absent in a variety of human cancer tissues. We examined the methylation status of the CpG island in *NAA11* gene promoter and tested the promoter activity in the presence or absence of DNA methylation. Our findings indicate the expression of *NAA11* gene is epigenetically regulated by DNA methylation of its proximal promoter, which explains the tissue-specific expression pattern of the gene.

Results

Expression analysis of *NAA10* and *NAA11* in human tissues and cell lines. To examine the tissue expression pattern of *NAA10* and *NAA11* in different adult human tissues, we performed RT-PCR experiments with PCR primers spanning the exon-intron junctions of both genes. *NAA11* transcripts were detected only in the testis and placenta (**Fig. 1A**). In contrast, *NAA10* was expressed in all tissues. These findings were confirmed in a separate Q-PCR analysis, from which the expression level of *NAA11* was more than 2-fold higher in human testis than placenta (**Fig. 1B**).

We further examined the expression of hNaa10p and hNaa11p in human tissues by western blotting. Despite the fact that the anti-hNaa11p antibody displayed cross-reactivity to hNaa10p, an immunoreactive band of hNaa11p, which appeared at a slightly smaller molecular weight than that of hNaa10p, was detected only in human testis (**Fig. 1C**). We did not observe similar immunoreactivity of hNaa11p in human placental tissues even when higher amounts of tissue lysates were used in the western blotting experiment (**Fig. 1C**). Consistent with the RT-PCR result, hNaa10p was expressed in different human tissues.

Based on the differential expression pattern of *NAA11* in human tissues we observed, we re-examined the expression pattern of *NAA10* and *NAA11* in HeLa and HEK 293 cells. *NAA10* transcripts were readily detected in these cells, but no *NAA11* expression could be detected (**Fig. 1D**). Similar findings were obtained when we examined the expression of hNaa11p in these and other human cell lines (**Fig. 1E**).

Expression analysis of *NAA10* and *NAA11* in cancer tissues by Q-PCR. To determine if *NAA10* and *NAA11* were co-expressed in the malignant state, we performed Q-PCR to examine the gene expression patterns in normal and cancerous tissues derived from breast, colon, kidney, liver, lung, ovary, prostate and thyroid of individual donors. Despite different levels of expression, *NAA10* transcripts could be detected in all cancerous tissues as well as their normal counterparts (**Fig. 2A**). In contrast, *NAA11* transcripts could only be detected in 5 of the 72 cancer tissues examined (one from breast cancer, two from liver cancer, one from ovarian cancer and one from thyroid cancer), and their relative abundance was much lower than that of *NAA10* (**Fig. 2B**). None of the tissue samples from normal donors showed *NAA11* expression.

Analysis of the methylation status of *NAA10* and *NAA11* promoters. The expression pattern of *NAA11* suggests its expression is regulated in a tissue-specific manner. In a search of regulatory sequence elements present in the upstream genomic regions
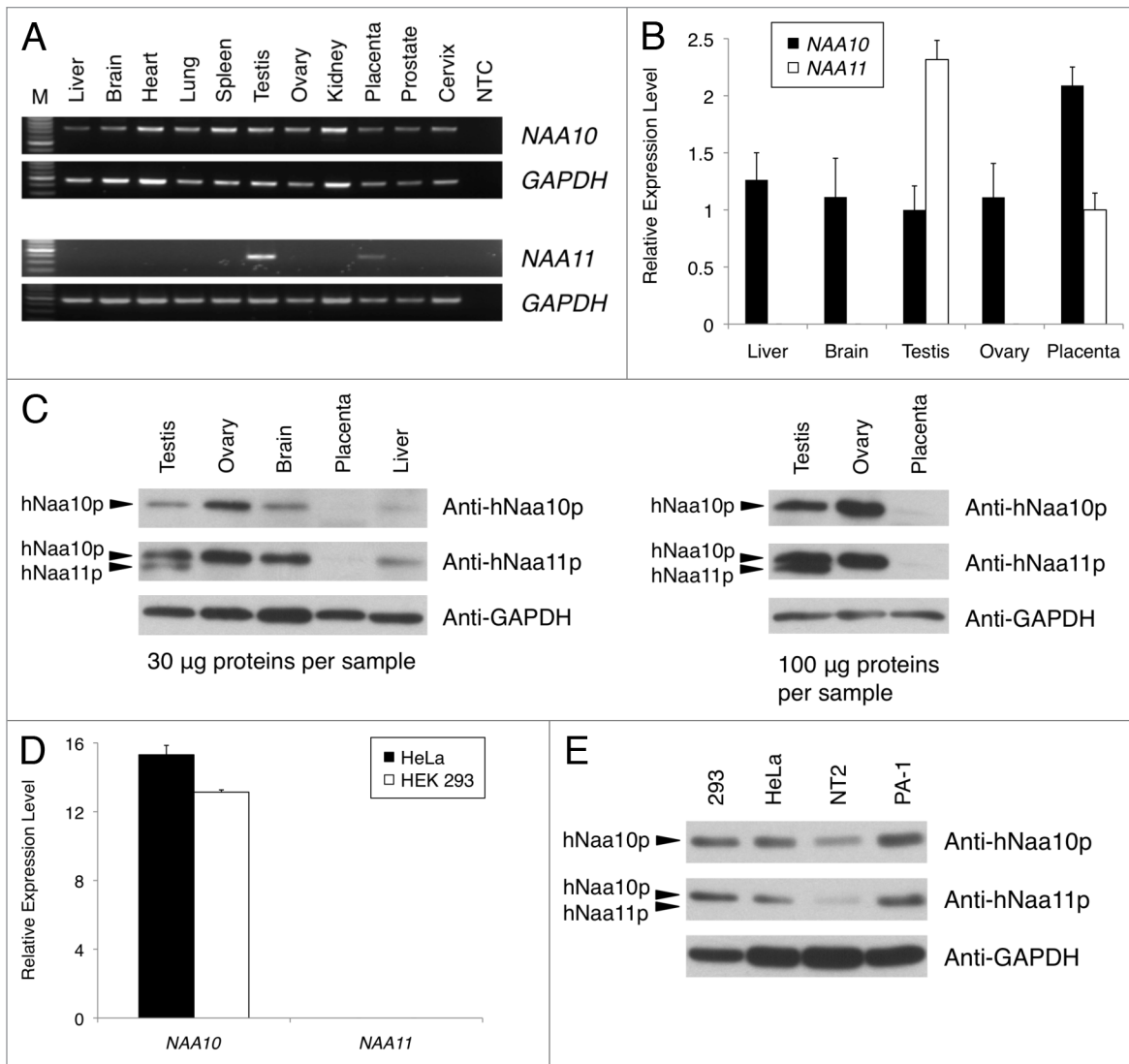
Figure 1. Expression analysis of *NAA10* (NM_003491) and *NAA11* (NM_032693) gene products in human tissues and cell lines. (A) Expression pattern of *NAA10* and *NAA11* transcripts in normal human tissues by RT-PCR. Expression of *GAPDH* was analyzed as an endogenous control. M: DNA molecular weight ladder. NTC: no-template control reaction. Sizes of amplicons: *NAA10* (708 bp), *NAA11* (382 bp) and *GAPDH* (452 bp). (B) Q-PCR analysis of *NAA10* and *NAA11* expression in selected human tissues. Relative expression levels of *NAA10* (black bars) and *NAA11* (white bars) after normalizing to that of *18S rRNA* were presented using the 2(-Delta Delta C(T)) method. (C) Expression analysis of hNaa10p and hNaa11p in human tissues by western blotting. The detection of GAPDH protein was performed as an endogenous control. The amount of tissue lysates used is listed. The immuno-reactive band corresponding to hNaa11p appears at a slightly smaller molecular weight than that of hNaa10p; and the anti-hNaa11p antibody was cross-reactive to hNaa10p. (D) Q-PCR analysis of *NAA10* and *NAA11* expression in HeLa and HEK 293 cells. Relative expression levels of *NAA10* and *NAA11* after normalizing to that of *18S rRNA* were presented using the delta C(T) method. *NAA10* expression was detected in HeLa cells (black bar) and HEK 293 cells (white bar), whereas no *NAA11* expression was detected. Data shown were averaged from triplicate measurements of duplicate preparations of total RNA samples from each cell line. (E) Expression analysis of hNaa10p and hNaa11p in human cell lines by western blotting. Thirty micrograms of total protein lysates were loaded per sample. No immuno-reactivity corresponding to hNaa11p was observed in any of the cell lysates. The detection of GAPDH protein was performed as an endogenous control. In figures (B and D), error bars represent the standard deviations of relative gene expression levels.

of *NAA10* and *NAA11* genes, a CpG island was identified at the proximal promoter region of both genes (**Fig. 3A**). In both cases, the CpG island spans the transcription start site (TSS) of the gene. The presence of CpG island at TSS suggests that the transcription of *NAA11* may be susceptible to regulation by DNA methylation. To examine this possibility, we performed MS-PCR to analyze the methylation status of the CpG islands in both *NAA10* and *NAA11* genes in human tissues (including testis, liver, kidney,

lung and placenta) and cell lines (HeLa and HEK 293). PCR primers were designed to detect the corresponding genomic loci in methylated (M) and non-methylated (U) states after bisulfite treatment. For *NAA10* gene, U primers generated PCR products of the expected size in all tissues and cell lines examined. On the other hand, reactions with M primers generated PCR products in kidney and placenta (**Fig. 3B**). The PCR products from the latter may be derived from non *NAA10*-expressing cell types present
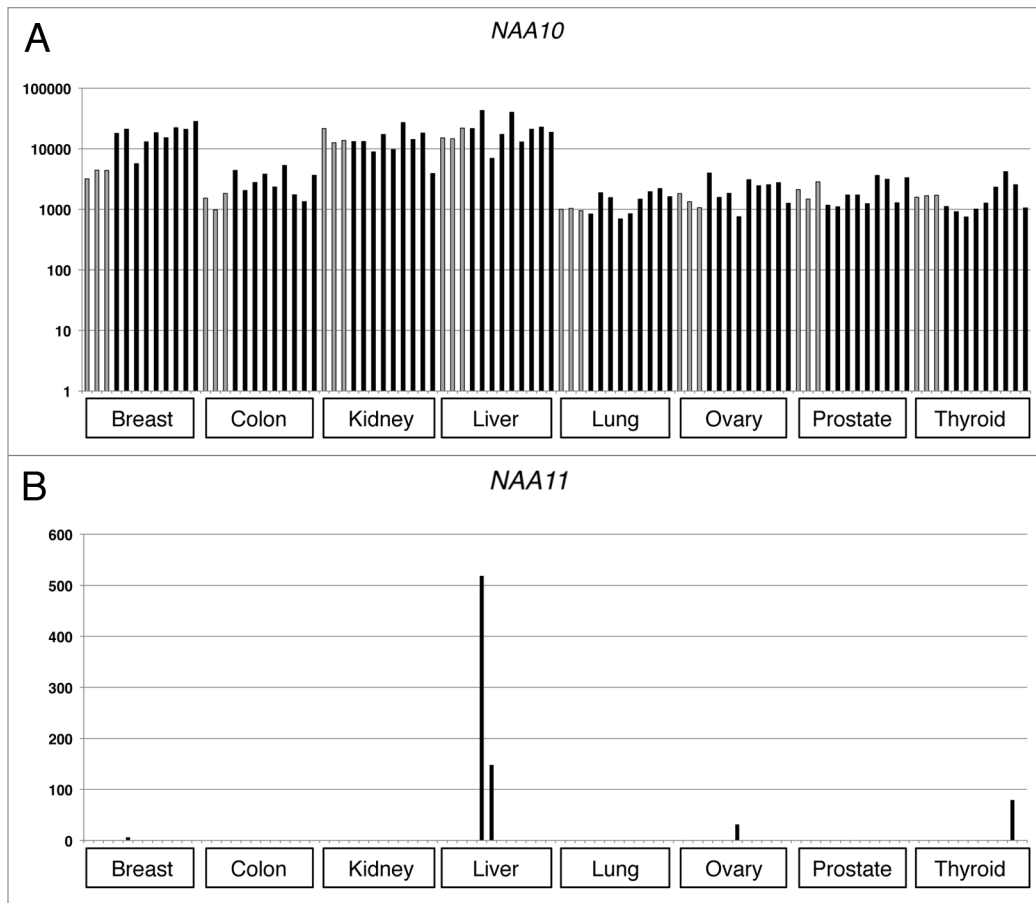
Figure 2. Expression analysis of *NAA10* and *NAA11* in cancerous tissues by Q-PCR. Y-axis: relative expression level of respective gene after normalizing to that of *ACTB*. Grey bars represent the gene expression levels obtained from normal tissues, whereas black bars refer to the expression levels detected in cancerous tissues. Br: breast; Co: colon; Ki: kidney; Lv: liver; Ln: lung; Ov: ovary; Pr: prostate; Ty: thyroid; N: normal tissues; T: cancer tissues. Numbers refer to the individual donors.

in these tissues. For *NAA11* gene, PCR products generated with U primers were limited to testis, placenta and surprisingly, HeLa cells. PCR products produced with M primers were detected readily in all tissues and cell lines examined.

It is noteworthy that the genomic DNA samples for HeLa and HEK 293 cells were extracted from the same preparations of cells used for *NAA10* and *NAA11* expression analysis (**Fig. 1D**). To obtain a better resolution of the methylation status of the CpG islands at single nucleotide level, we performed bisulfite sequencing on the same set of human tissues and cells. Owing to the large size (978 bp) of the CpG island in *NAA10* gene, we were not able to design primers that could amplify the corresponding genomic locus for sequencing analysis after bisulfite conversion. We focused on delineating the methylation status of the CpG island in *NAA11* gene. As shown in **Figure 3C**, the CpG island was hyper-methylated in liver, lung, kidney and HEK 293 cells which did not show *NAA11* expression. In both testis and placenta, we observed two distinctive CpG methylation profiles that were indicative of the presence of hyper-methylated and non-methylated CpG islands. This finding is consistent with the presence of non *NAA11*-expressing and *NAA11*-expressing cell types, respectively, in these tissues. On the contrary, the CpG

methylation profile in HeLa cells was less distinctive, with the CpG island displaying various degree of methylation among the different clones of sequences. The presence of non-methylated CpG dinucleotides allowed a productive MS-PCR with the U primers, which explained the detection of both methylated and non-methylated version of *NAA11* CpG island in HeLa cells. Nevertheless, our findings suggest the CpG island in *NAA10* gene is generally non-methylated in human tissues and cell lines. With the exception of HeLa cells, the CpG island in *NAA11* gene is extensively methylated in human tissues and cell lines that do not show *NAA11* expression; the genomic locus is non-methylated in tissues that show *NAA11* expression.

Suppression of *NAA11* promoter activity by CpG methylation. The MS-PCR and bisulfite sequencing data indicate the involvement of DNA methylation in the suppression of *NAA11* transcription. We therefore examined if DNA methylation would directly suppress *NAA11* promoter activity. A 427-bp contiguous genomic DNA fragment covering the proximal promoter and the first 7 codons of *NAA11* gene was cloned into reporter vector pGL3-Basic to generate the promoter construct pGL3-Basic/NAA11-pro (**Fig. 3A**). Upon transfection of HeLa cells, the promoter construct produced a significantly higher level of
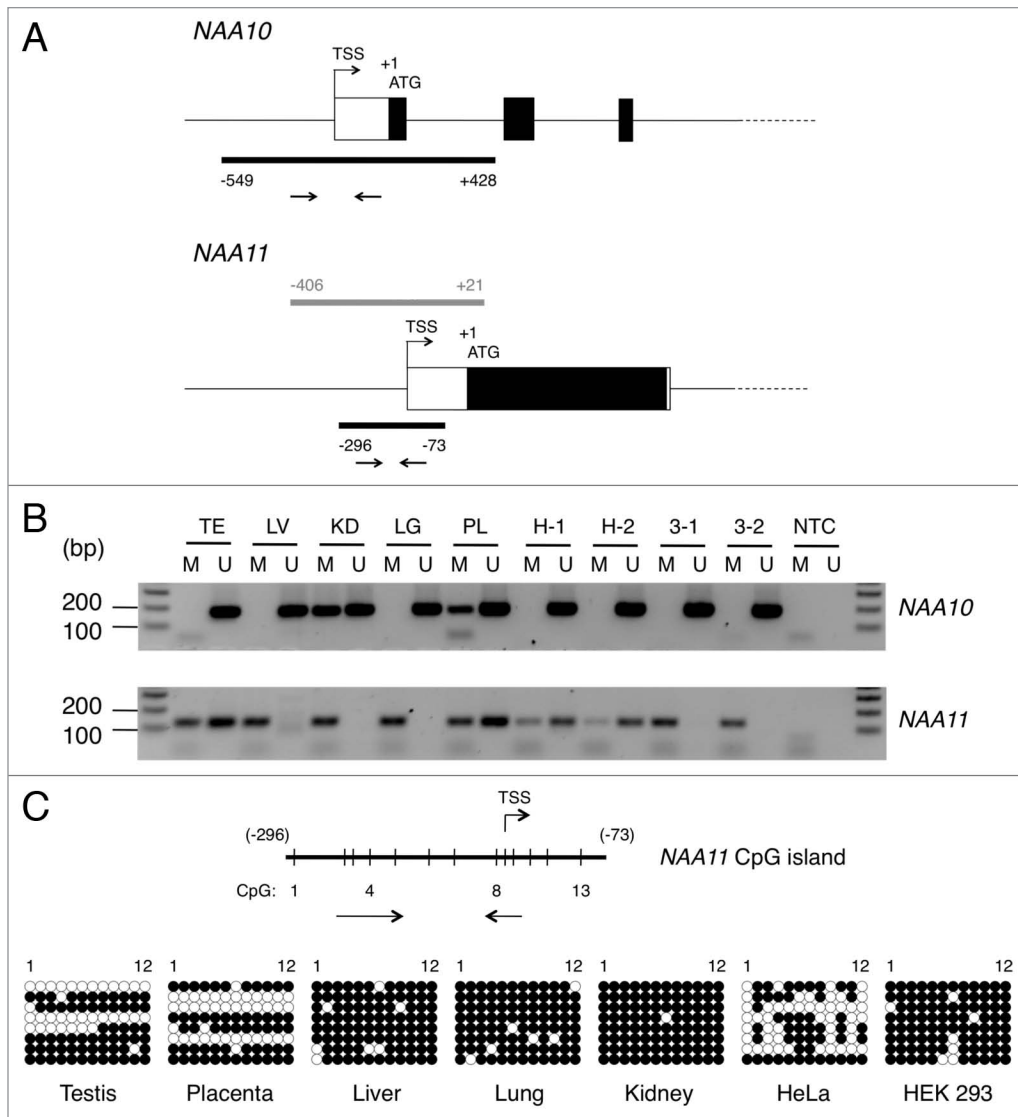
Figure 3. Identification of CpG islands in proximal promoter regions of *NAA10* and *NAA11* genes and analysis of their methylation status. (A) Genomic organization of *NAA10* and *NAA11* genes at their 5′ ends. Black bars represent the locations of CpG islands in both genes. The arrow pairs denote the positions of primers used for MS-PCR. Grey bar refers to the genomic fragment cloned for *NAA11* promoter assay. TSS, transcription start site. The genomic positions of CpG islands and *NAA11* promoter fragment are annotated with respect to the start codon (ATG) of the respective gene, with A denoted as +1. (B) Analysis of methylation status of *NAA10* and *NAA11* gene proximal promoters in human tissues and cell lines by MS-PCR. Sizes of amplicons: for *NAA10* gene: M primers (178 bp), U primers (183 bp); for *NAA11* gene: M primers (129 bp), U primers (126 bp). Duplicate sets of genomic DNA samples from HeLa (H-1 and H-2) and HEK 293 (3-1 and 3-2) cells, which were harvested from the same preparations of cells for *NAA10* and *NAA11* expression analysis in Figure 1D, were examined. TE, testis; LV, liver; KD, kidney; LG, lung; PL, placenta; NTC, no-template control reaction. Forty cycles of amplification were performed. (C) Bisulfite sequencing analysis of the CpG island in *NAA11* gene. Vertical lines on horizontal bar refer to the position of CpG dinucleotides (1–13) in the CpG island. Annotations are defined as in (A). Owing to the constraints in primer design, the 13th CpG dinucleotide was not analyzed in this experiment. For HeLa and HEK 293 cells, only one genomic DNA sample from each cell line was analyzed. Black circle, methylated CpG dinucleotide; white circle, non-methylated CpG dinucleotide.

luciferase activity than cells transfected with the empty vector (data not shown). The same effect was also observed in HEK 293 cells, suggesting the *NAA11* promoter fragment was active in both cell lines even though these cells do not express endogenous *NAA11*.

The same experiment was performed with cells transfected with in vitro methylated empty vector and promoter construct. In both cell lines, the luciferase activity produced by the methylated promoter construct was significantly lower than its non-methylated counterpart. To eliminate the interference that may be contributed by the vector sequence (i.e., to study the effect of DNA methylation on the *NAA11* promoter fragment only), we compared the luciferase activities between the methylated and non-methylated promoter construct after normalizing to those obtained from the methylated and non-methylated empty vector, respectively. From this, we observed a 25.4-fold reduction of *NAA11* promoter activity in HeLa cells after DNA methylation (**Fig. 4A**). In HEK 293 cells, a 7.4-fold reduction
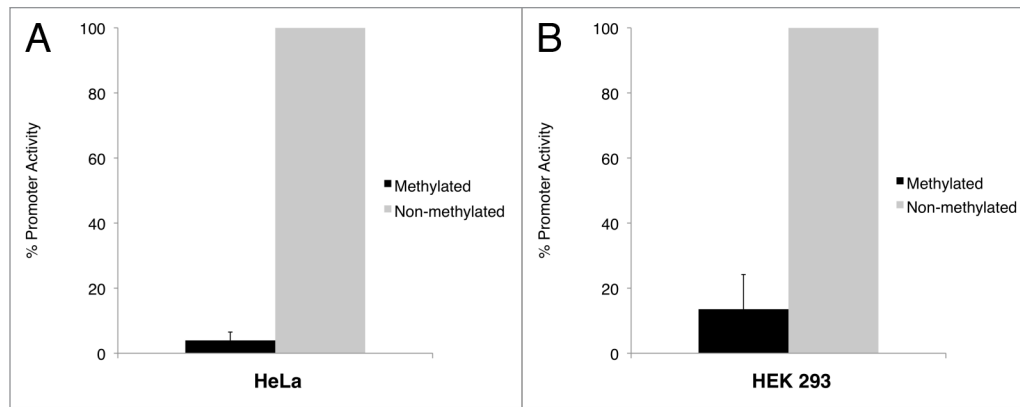
Figure 4. *NAA11* promoter activity is regulated by DNA methylation. Promoter assays were performed in HeLa and HEK 293 cells that were transiently transfected with pGL3-Basic and pGL3-Basic/NAA11-pro vectors with or without in vitro methylation treatment. Black bars and grey bars correspond to the promoter activities displayed by methylated and non-methylated *NAA11* promoter fragment, respectively. Data shown are averaged results from three independent experiments. Promoter activity displayed by the non-methylated *NAA11* promoter fragment was set to be 100%. Error bars represent the standard deviations of percentage promoter activities.

of *NAA11* promoter activity was obtained (**Fig. 4B**). Our data clearly demonstrated that the activity of *NAA11* promoter was susceptible to suppression by DNA methylation.

Discussion

Acetylation has long been a subject of interest in the study of protein co- and post-translational modification processes. The discovery of alternative substrate specificity of hNaa10p suggests the homologous hNaa11p may also acetylate amino groups other than α-amino groups on polypeptides. The human cell lines shown to co-express hNaa10p and hNaa11p[17,25,28] therefore represent a convenient platform to investigate the biological activity of the two catalytic subunits, as well as their associated enzymatic complexes. We sought to evaluate this possibility. In this study, we first compared the expression of *NAA10* and *NAA11* gene products in different normal human tissues and established human cell lines. Similar to the mouse *Naa11* gene, we found that *NAA11* was predominantly expressed in the human testis; a lower expression level of the gene was detected in human placenta. On the protein level, however, we observed the presence of hNaa11p only in human testis. We speculate the *NAA11* transcripts may be translationally repressed in placenta in a way similar to the *Naa11* transcripts in the mouse testis.[26] Alternatively, the expression of hNaa11p may be confined to a small population of cells within the placenta. As a result, the abundance of the protein may be lower than the detection limit of western blotting assay. The identification of the cellular expression pattern of *NAA11* gene products in placenta tissues will help explain the discrepancy.

Unexpectedly, we did not detect *NAA11* expression in the cell lines that were originally reported to express the gene. As these cells are either transformed or cancerous in nature, we also surveyed the expression of *NAA11* in specimens that were extracted from multiple types of human cancer. The cancer specimens examined (e.g., breast, liver, lung and thyroid) covered the tissue origins of some of the cell lines that were previously analyzed

for *NAA11* and hNaa11p expression.[17,25,28] *NAA11* expression was not detected in the cancerous tissues, with the exception in several cases of relatively weak expression of the gene. On the other hand, *NAA10* demonstrated a housekeeping expression pattern in all cases. All together, our data are consistent with our previous study on the mouse orthologs[26] that shows that the expression of *NAA10* is constitutive, but tissue-specific for *NAA11*. This observation implies that the regulatory mechanisms controlling the expression of the two genes are different. The fact that the *NAA11* promoter fragment is active in non *NAA11*-expressing cells suggests the tissue-restricted expression of *NAA11* is not entirely mediated by the action of cell- or tissue-specific factors. Instead, our data indicate the CpG island in the proximal promoter of *NAA11* gene is a target of transcriptional regulation.

DNA methylation at CpG dinucleotides is an epigenetic mechanism commonly associated with gene silencing. The process is involved in the control of developmental or cell-type specific gene expression patterns in normal cells.[30] Meanwhile, dysregulated gene expression owing to aberrant DNA methylation is frequently observed in cancer cells.[31] DNA methylation inhibits gene transcription by interfering directly with the binding of transcription factors to their cognate DNA sequences, or by recruiting methyl-CpG-binding domain proteins and their associated transcriptional co-repressors to induce a repressive chromatin state.[32,33] The effect of CpG island methylation on suppression of *NAA11* transcription was demonstrated in our promoter assay in which the activity of *NAA11* promoter was dramatically reduced after in vitro methylation. The involvement of DNA methylation in controlling *NAA11* expression was further corroborated by the MS-PCR and bisulfite sequencing analyses. We found that the CpG island in *NAA11* gene was hyper-methylated in non *NAA11*-expressing cells and tissues. *NAA11* gene products were also absent in HeLa cells despite the existence of a less extensively methylated CpG island. In contrast, the CpG island in *NAA10* gene was not methylated, which correlates with its housekeeping expression pattern across different cell types and tissues. Consistently, *NAA11* expression was present

in normal testis and placenta in which a non-methylated CpG island was detected. Similar to the observations from male germ cell-specific genes such as *Pdha2* [34] and *LDH-C* [35] and cancer testis antigens (e.g., *MAGE-A1* and *LAGE-1*),[36] our findings suggest that DNA methylation is the regulatory mechanism specifically utilized to suppress *NAA11* transcription in cells and tissues that do not require its activity. The low level of *NAA11* expression detected in the few cancer specimens may have resulted from a loss of DNA methylation that would lead to the re-activation of gene transcription. In this regard, the re-expression of *NAA11* could be mimicked by treating non *NAA11*-expressing cells with inhibitor of DNA methyltransferase (Pang et al. manuscript in preparation).

To conclude, we demonstrated that the expression of *NAA11* is tissue-specific and is governed by DNA methylation. Although *NAA10* and *NAA11* were found to be co-expressed in several human cell lines, our current observations point to the opposite: co-expression of *NAA10* and *NAA11* in the same cells is not a common phenomenon. In agreement with our previous findings,[26] the NAT constituted by mNaa10p/hNaa10p and mNaa15p/hNaa15p is the major form of NatA in mammalian cells. The NatA composed of mNaa11p/hNaa11p and mNaa15p/hNaa15p is present only in specialized cell types such as male germ cells. We do not know the exact reasons for the discrepancy in *NAA11* and hNaa10p expression pattern observed between our data and previous reports. The specificities of the different reagents and differences in experimental methodology may have contributed to the disparities. Other than that, the expression of *NAA11* may become "leaky" in response to environmental factors such as variations in cell culturing conditions; this awaits further investigation. No matter what the potential cause may be, a more extensive analysis of *NAA11* expression in various human cell lines is necessary before they can be used adequately as an in vitro model to study the biological roles of *NAA11* gene products.

Materials and Methods

Cell lines, genomic DNA and total RNA isolation. NT2/D1 (CRL-1973), PA-1 (CRL-1572), HeLa (CCL-2) and HEK 293 (CRL-1573) cell lines were obtained from ATCC and cultured according to ATCC recommendation. Genomic DNA samples from human tissues (testis: D1234260; liver: D1234149; kidney: D1234142; lung: D1234152; placenta: D1234200) were obtained from Biochain; those from human cell lines were extracted with Puregene Cell kit (Gentra D-5010A). Total RNA samples from human tissues were obtained from Ambion (AM6000); those from human cell lines were extracted using TRIzol reagent (Invitrogen 15596-018).

Reverse-transcription polymerase chain reaction (RT-PCR). One microgram of total RNA was reverse-transcribed in the presence of random hexamers (Invitrogen 48190-011) and SuperScript II reverse transcriptase (Invitrogen 18064-014). The first-strand cDNA sample was diluted 5 times in nuclease free water (Ambion AM9937). One microliter of the diluted cDNA was used in 20-μL PCR with 2.5 mM of MgCl$_2$, 0.5 mM of

Table 1. List of primers used in this study

For RT-PCR
NAA10-F: ATG AAC ATC CGC AAT GCG AGG
NAA10-R: CTA GGA GGC TGA GTC GGA GG
NAA11-F: CAG CAC ACT TTC TGA TTC TGA AG
NAA11-R: GTA ATG GCA GGT CTC AAA GTT C
GAPD-5: ACC ACA GTC CAT GCC ATC AC
GAPD-3: TCC ACC ACC CTG TTG CTG TA
For cloning of *NAA11* promoter*
NAA11-pro-F: <u>TAA GGC</u> ACG CGT TGT TAT ATG GGT TTC TCC AGT TTG TG
NAA11-pro-R: <u>TAA GGC</u> AAG CTT CTG AGC GTT GCG GAT GTT C
For DNA sequencing
M13-R: GGA AAC AGC TAT GAC CAT
M13-20: GTA AAA CGA CGG CCA GTG
For MS-PCR
M-NAA10-1F: GTT TCG CGT ATT GGT TAA GC
M-NAA10-1R: GTA AAA ACG CAA TCA ACT ACC G
U-NAA10-1F: GTT TGT TTT GTG TAT TGG TTA AGT G
U-NAA10-1R: CAT AAA AAC ACA ATC AAC TAC CAC C
M-NAA11-F: TGC GTA CGT ATA CGT ATA ATG AAG C
M-NAA11-R: ATA AAA AAC GAA TAA CGA AAA AAC G
U-NAA11-F: GTG TAT GTA TAT GTA TAA TGA AGT GA
U-NAA11-R: AAA AAA CAA ATA ACA AAA AAA CAA A
For bisulfite sequencing
BSS-NAA11-F: ATT TAA ATT AAA AAG TTA AAA TTG AAA TAA
BSS-NAA11-R: AAA AAA CAC TAT TTA CCT CAA AAA TC
BSS-NAA11-NR: AAT CCA AAA AAC TAA CAC CAC C

All primers are listed in 5' to 3' orientation. *Underlined sequence represents a random hexameric sequence included to protect the downstream *Mlu*I (ACGCGT) and *Hin*dIII (AAGCTT) restriction sites.

dNTPs, 0.5 μM of primers and 1 unit of Platinum *Taq* DNA polymerase (Invitrogen 10966-083). PCR products were resolved on 2% TAE agarose gel (EmbiTec GE-3642). The sequences of primers used for RT-PCR of *NAA10*, *NAA11* and *GAPDH* were listed in **Table 1**.

Gene expression in cancer tissues by quantitative real-time polymerase chain reaction (Q-PCR). The expression level of *NAA10*, *NAA11* and *18S rRNA* in normal tissues and cell lines were analyzed with cDNA samples generated as described above. The expression of *NAA10*, *NAA11* and *ACTB* in cancer tissues was analyzed on the TissueScan Cancer Survey Panel 96-I (OriGene Technologies CSRT501). Q-PCR was performed with Taqman gene expression assays for *NAA10* (Hs00185854_m1), *NAA11* (Hs00261953_m1) and *18S rRNA* (TaqMan Ribosomal RNA Control Reagents 4308329) in the presence of Taqman Universal PCR master mix (4364338), all from Applied Biosystems. For measurement of *ACTB* transcript level, SYBR Green PCR master mix (Applied Biosystems 4364344) was used in the presence of *ACTB*-specific primer mix provided with the TissueScan Cancer Survey Panel 96-I. Expression levels of *NAA10* and *NAA11* were normalized to that of *18S rRNA* or

ACTB and calculated by 2(-Delta Delta C(T)) or delta C(T) method as specified.

Western blotting. Total protein lysates from NT2/D1, PA-1, HeLa and HEK 293 cells were extracted in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with Halt protease inhibitor cocktail (Pierce 87786). Total protein lysates from human tissues were obtained from Biochain (testis: P1234260; ovary: P1234183; brain: P1234035; liver: P1234149; placenta: P1234200). Proteins were transferred to PVDF membranes (BioRad 162-0177) in Protean transfer cell (BioRad 170-3930) according to standard procedure. Primary antibodies were diluted in blocking solution (1x PBS, 0.05% Tween-20 and 5% non-fat dry milk) as specified below: goat-anti-hNaa10p (Santa Cruz Biotechnology sc-33256) at 1:2,000; rabbit-anti-hNaa11p (ProteinTech Group 15670-1-AP) at 1:1,000; chicken-anti-GAPDH (Genway 15-288-22763) at 1:20,000. Different horseradish peroxidase (HRP)-conjugated secondary antibodies (all diluted 10,000-fold in blocking solution) were used according to the origins of primary antibodies, including goat-anti-rabbit IgG-HRP (BioRad 170-6515), goat-anti-chicken IgY-HRP (abcam ab6877) and donkey-anti-goat IgG-HRP (Santa Cruz Biotechnology sc-2020). Immunoreactivities were visualized by film exposure after developing in SuperSignal West Pico chemiluminescent substrate (Pierce 34080). Membranes were re-probed after stripping in Restore western blot stripping buffer (Pierce 21059).

Cloning of *NAA11* promoter fragment. The -406 to +21 region of *NAA11* gene (GenBank accession number: NM_032693, with the adenosine of start codon assigned as +1) was amplified by PCR in the presence of 1x HF buffer, 0.5 μM of each of NAA11-pro-F and NAA11-pro-R primers (**Table 1**), 0.5 mM of dNTPs, 20 ng of human testicular genomic DNA and 0.4 unit of Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes F-540L). The PCR product, with *Mlu*I and *Hind*III restriction sites incorporated at the respective 5' end, was cloned into ZeroBlunt pCR4-TOPO vector (Invitrogen K2875-20) for DNA sequencing with M13 primers (**Table 1**). The promoter fragment was harvested by restriction digestion with the specified enzymes from New England Biolabs (*Mlu*I: R0198S; *Hind*III: R0104S) and ligated into firefly luciferase reporter vector pGL3-Basic (Promega E1751) to generate the pGL3-Basic/NAA11-pro vector. CpG islands on *NAA10* and *NAA11* genes were predicted using EMBOSS Cpgplot (www.ebi.ac.uk/emboss/cpgplot).

In vitro methylation. The pGL3-Basic and pGL3-Basic/NAA11-pro vectors were separately methylated in vitro by *M.Sss*I CpG methyltransferase (New England Biolabs M0226S). Each microgram of plasmid DNA was incubated at 37°C for 3 hours with 4 units of *M.Sss*I CpG methyltransferase and 0.32 mM of S-adenosylmethionine. Completion of CpG methylation was confirmed by digestion of methylated plasmid with methylation-sensitive restriction enzyme *Hha*I (New England Biolabs R0139S). Methylated plasmid was purified using the Wizard SV

Gel and PCR Clean-Up System (Promega A9282) and re-quantified by absorbance measurement using the NanoVue spectrophotometer (GE Healthcare 28924402).

Reporter assay. Eighty nanograms of pGL3-Basic vector or pGL3-Basic/NAA11-pro vector, and 1 ng of Renilla luciferase normalization vector pGL4.73[hRluc/SV40] (Promega E6911) were co-transfected into HeLa and HEK 293 cells seeded at a density of 20,000 cells/well in 24-well culture plates. In parallel, the same transfection experiment was performed with pGL3-Basic and pGL3-Basic/NAA11-pro vectors, both in vitro methylated. Two days after transfection, the transfected cells were harvested for reporter assay using the Dual Luciferase Reagent (Promega E1910). The ratios of luciferase activities from the reporter vector (pGL3-Basic series) and the normalization vector (pGL4.73[hRluc/SV40]) from all experiments (pGL3-Basic with or without methylation and pGL3-Basic/NAA11-pro with or without methylation) were obtained after background subtraction. To identify the effect of DNA methylation on reporter activity regulated by the *NAA11* promoter fragment, the luciferase activity ratio from non-methylated pGL3-Basic/NAA11-pro was divided by the ratio from non-methylated pGL3-Basic and compared to that obtained from methylated pGL3-Basic/NAA11-pro and pGL3-Basic. The promoter activity of methylated *NAA11* promoter fragment was expressed as a fraction of that of non-methylated *NAA11* promoter fragment.

Bisulfite treatment of genomic DNA, methylation specific-polymerase chain reaction (MS-PCR) and bisulfite sequencing. Genomic DNA samples were bisulfite-converted using the EZ DNA Methylation-Gold kit (Zymo Research D5005) and re-quantified with the NanoVue spectrophotometer. For MS-PCR, 10 ng of bisulfite-converted DNA was used per reaction in the presence of primers that would distinguish between methylated (M) and non-methylated (U) promoters of *NAA10* and *NAA11* genes after bisulfite conversion. For bisulfite sequencing, the modified CpG island in *NAA11* gene from individual samples was amplified by regular PCR in the presence of 20 ng of bisulfite-converted DNA and primers BSS-NAA11-F and BSS-NAA11-R; followed by a hemi-nested PCR with 2 μL of the ten-fold diluted PCR product and primers BSS-NAA11-F and BSS-NAA11-NR. The resulting 272-bp PCR products were separately cloned into pCR4-TOPO vector and multiple clones were selected for sequencing analysis. All MS-PCR and bisulfite sequencing primers (**Table 1**) were designed using the MethPrimer tool.[37]

Disclosure of Potential Conflicts of Interest

Acknowledgments

References

1. Yang XJ. Lysine acetylation and the bromodomain: a new partnership for signaling. Bioessays 2004; 26:1076-87.

2. Driessen HP, de Jong WW, Tesser GI, Bloemendal H. The mechanism of N-terminal acetylation of proteins. CRC Crit Rev Biochem 1985; 18:281-325.

3. Starheim KK, Gromyko D, Velde R, Varhaug JE, Arnesen T. Composition and biological significance of the human Nalpha-terminal acetyltransferases. BMC Proc 2009; 3:3.

4. Gautschi M, Just S, Mun A, Ross S, Rucknagel P, Dubaquie Y, et al. The yeast N(alpha)-acetyltransferase NatA is quantitatively anchored to the ribosome and interacts with nascent polypeptides. Mol Cell Biol 2003; 23:7403-14.

5. Polevoda B, Brown S, Cardillo TS, Rigby S, Sherman F. Yeast N(alpha)-terminal acetyltransferases are associated with ribosomes. J Cell Biochem 2008; 103:492-508.

6. Park EC, Szostak JW. ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity. EMBO J 1992; 11:2087-93.

7. Arnesen T, Gromyko D, Kagabo D, Betts MJ, Starheim KK, Varhaug JE, et al. A novel human NatA Nalpha-terminal acetyltransferase complex: hNaa16p-hNaa10p (hNat2-hArd1). BMC Biochem 2009; 10:15.

8. Arnesen T, Van Damme P, Polevoda B, Helsens K, Evjenth R, Colaert N, et al. Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci USA 2009; 106:8157-62.

9. Hwang CS, Shemorry A, Varshavsky A. N-terminal acetylation of cellular proteins creates specific degradation signals. Science 327:973-7.

10. Forte GMA, Pool, Martin R, Stirling, Colin J. N-Terminal Acetylation Inhibits Protein Targeting to the Endoplasmic Reticulum. PLoS Biol 2011; 9:1001073.

11. Ohkawa N, Sugisaki S, Tokunaga E, Fujitani K, Hayasaka T, Setou M, et al. N-acetyltransferase ARD1-NAT1 regulates neuronal dendritic development. Genes Cells 2008; 13:1171-83.

12. Sugiura N, Adams SM, Corriveau RA. An evolutionarily conserved N-terminal acetyltransferase complex associated with neuronal development. J Biol Chem 2003; 278:40113-20.

13. Arnesen T, Gromyko D, Pendino F, Ryningen A, Varhaug JE, Lillehaug JR. Induction of apoptosis in human cells by RNAi-mediated knockdown of hARD1 and NATH, components of the protein N-alpha-acetyltransferase complex. Oncogene 2006; 25:4350-60.

14. Seo JH, Cha JH, Park JH, Jeong CH, Park ZY, Lee HS, et al. Arrest defective 1 autoacetylation is a critical step in its ability to stimulate cancer cell proliferation. Cancer Res 2010; 70:4422-32.

15. Lim JH, Park JW, Chun YS. Human arrest defective 1 acetylates and activates beta-catenin, promoting lung cancer cell proliferation. Cancer Res 2006; 66:10677-82.

16. Yu M, Ma M, Huang C, Yang H, Lai J, Yan S, et al. Correlation of expression of human arrest-defective-1 (hARD1) protein with breast cancer. Cancer Invest 2009; 27:978-83.

17. Fisher TS, Etages SD, Hayes L, Crimin K, Li B. Analysis of ARD1 function in hypoxia response using retroviral RNA interference. J Biol Chem 2005; 280:17749-57.

18. Yu M, Gong J, Ma M, Yang H, Lai J, Wu H, et al. Immunohistochemical analysis of human arrest-defective-1 expressed in cancers in vivo. Oncol Rep 2009; 21:909-15.

19. Jiang B, Ren T, Dong B, Qu L, Jin G, Li J, et al. Peptide mimic isolated by autoantibody reveals human arrest defective 1 overexpression is associated with poor prognosis for colon cancer patients. Am J Pathol 2010; 177:1095-103.

20. Ren T, Jiang B, Jin G, Li J, Dong B, Zhang J, et al. Generation of novel monoclonal antibodies and their application for detecting ARD1 expression in colorectal cancer. Cancer Lett 2008; 264:83-92.

21. Lee CF, Ou DS, Lee SB, Chang LH, Lin RK, Li YS, et al. hNaa10p contributes to tumorigenesis by facilitating DNMT1-mediated tumor suppressor gene silencing. J Clin Invest 2010; 120:2920-30.

22. Shin DH, Chun YS, Lee KH, Shin HW, Park JW. Arrest defective-1 controls tumor cell behavior by acetylating myosin light chain kinase. PLoS One 2009; 4:7451.

23. Kuo HP, Lee DF, Chen CT, Liu M, Chou CK, Lee HJ, et al. ARD1 stabilization of TSC2 suppresses tumorigenesis through the mTOR signaling pathway. Sci Signal 2010; 3:9.

24. Arnesen T, Gromyko D, Horvli O, Fluge O, Lillehaug J, Varhaug JE. Expression of N-acetyl transferase human and human Arrest defective 1 proteins in thyroid neoplasms. Thyroid 2005; 15:1131-6.

25. Hua KT, Tan CT, Johansson G, Lee JM, Yang PW, Lu HY, et al. N-alpha-acetyltransferase 10 protein suppresses cancer cell metastasis by binding PIX proteins and inhibiting Cdc42/Rac1 activity. Cancer Cell 2011; 19:218-31.

26. Pang AL, Peacock S, Johnson W, Bear DH, Rennert OM, Chan WY. Cloning, characterization and expression analysis of the novel acetyltransferase retrogene Ard1b in the mouse. Biol Reprod 2009; 81:302-9.

27. Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, et al. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 2002; 111:709-20.

28. Arnesen T, Betts MJ, Pendino F, Liberles DA, Anderson D, Caro J, et al. Characterization of hARD2, a processed hARD1 gene duplicate, encoding a human protein N-alpha-acetyltransferase. BMC Biochem 2006; 7:13.

29. Huang GL, Li BK, Zhang MY, Zhang HZ, Wei RR, Yuan YF, et al. LOH analysis of genes around D4S2964 identifies ARD1B as a prognostic predictor of hepatocellular carcinoma. World J Gastroenterol 2010; 16:2046-54.

30. Illingworth RS, Bird AP. CpG islands-'a rough guide'. FEBS Lett 2009; 583:1713-20.

31. Berdasco M, Esteller M. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. Dev Cell 2010; 19:698-711.

32. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 2006; 31:89-97.

33. Portela A, Esteller M. Epigenetic modifications and human disease. Nat Biotechnol 2010; 28:1057-68.

34. Iannello RC, Young J, Sumarsono S, Tymms MJ, Dahl HH, Gould J, et al. Regulation of Pdha-2 expression is mediated by proximal promoter sequences and CpG methylation. Mol Cell Biol 1997; 17:612-9.

35. Tang H, Goldberg E. *Homo sapiens* lactate dehydrogenase c (Ldhc) gene expression in cancer cells is regulated by transcription factor Sp1, CREB and CpG island methylation. J Androl 2009; 30:157-67.

36. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 1999; 19:7327-35.

37. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002; 18:1427-31.