ASBEL, an ANA/BTG3 antisense transcript required for tumorigenicity of ovarian carcinoma

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Mammalian genomes encode numerous antisense non-coding RNAs, which are assumed to be involved in the regulation of the sense gene expression. However, the mechanisms of their action and involvement in the development of diseases have not been well elucidated. The ANA/BTG3 protein is an antiproliferative protein whose expression is downregulated in prostate and lung cancers. Here we show that an antisense transcript of the ANA/BTG3 gene, termed ASBEL, negatively regulates the levels of ANA/BTG3 protein, but not of ANA/BTG3 mRNA and is required for proliferation and tumorigenicity of ovarian clear cell carcinoma. We further show that knockdown of ANA/BTG3 rescues growth inhibition caused by ASBEL knockdown. Moreover, we demonstrate that ASBEL forms duplexes with ANA/BTG3 mRNA in the nucleus and suppresses its cytoplasmic transportation. Our findings illustrate a novel function for an antisense transcript that critically promotes tumorigenesis by suppressing translation of the sense gene by inhibiting its cytoplasmic transportation.

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Recent studies have revealed that most mammalian genes express antisense transcripts1,2. The majority of antisense transcripts are non-coding RNAs (ncRNAs) complementary to a region of the sense mRNA. Sense-antisense transcript pairs so far reported include genes involved in various biological processes, development and diseases, suggesting critical roles of antisense transcripts in mammalian gene expression. In contrast to microRNAs, antisense transcripts have been suggested to exert their function through a variety of mechanisms. For example, duplex formation between sense and antisense RNAs in the nucleus can modulate mRNA alternative splicing, editing and transport3–5. Sense-antisense duplex formation in the cytoplasm can change sense mRNA stability and translation efficiency6–10. It has also been suggested that some antisense transcripts bind to the corresponding DNA strand and recruit DNA methyltransferases or histone-modifying enzymes, thereby modulating sense gene expression11–15. However, the exact mechanisms underlying these functions remain to be further elucidated.

ANA/BTG3 is a member of the TOB/BTG family of antiproliferative genes that regulates cell cycle progression in a variety of cell types16. It has also been reported that loss of ANA/BTG3 in normal cells induces cellular senescence via the ERK-JMJD3-p16(INK4a) signaling axis17. ANA/BTG3 expression is also known to be induced by DNA damage in a p53-dependent manner and directly represses E2F1-mediated transactivation18. In addition, ANA/BTG3 interacts with the CCR4 transcription factor-associated protein Cfl17, suggesting its involvement in cytoplasmic mRNA deadenylation and turnover. Furthermore, ANA/BTG3 expression is downregulated in prostate cancer through promoter hypermethylation19. ANA/BTG3 expression is also reduced in the majority of lung adenocarcinoma19. Thus, increasing evidence suggests that ANA/BTG3 functions as a tumor suppressor.

It has been reported that the tumor suppressor functions of p53 and WT1 are regulated by their antisense transcripts20,21. We therefore searched for antisense transcripts encoded in other tumor suppressor genes. We found that ANA/BTG3 encodes an antisense transcript although most of the important tumor suppressor genes, including RB, APC, BRCA1, BRCA2, NF1 and NF2, do not. Here we show that an antisense transcript of ANA/BTG3 termed ASBEL is required for the regulation of ANA/BTG3 protein expression and tumorigenicity of ovarian cancer.
Results

*ASBEL*, an antisense transcript of *ANA/BTG3*. Examination of the GenBank database revealed that a highly conserved gene is encoded by the DNA strand opposite the *ANA/BTG3* gene (Fig. 1a). This gene encodes a conserved ~2-kb ncRNA (termed *ASBEL* [antisense ncRNA in the *ANA/BTG3* (three) locus]), the 5’ region of which is complementary to a portion of the 5’ untranslated region (UTR) and the first exon of *ANA/BTG3* mRNA. Strand-specific RT-PCR analysis confirmed that *ASBEL* was indeed transcribed from the DNA strand opposite to the *ANA/BTG3* gene (Fig. 1a). Northern blotting analyses showed that *ASBEL* was detected (Fig. 1b). Subcellular fractionation and RT-PCR analysis revealed that *ASBEL* was present in the nucleus (Fig. 1c and Supplementary Fig. S1), consistent with the fact that *ASBEL* is a ncRNA.

*ASBEL* is required for the tumorigenicity of ovarian cancer. We examined *ASBEL* expression in human ovarian cancerous tissues and adjacent non-cancerous tissues (5 serous adenocarcinoma (SA), 2 endometrioid adenocarcinoma (EA), 2 clear cell adenocarcinoma (CCC), 1 mucinous adenocarcinoma (MA), 1 dysgerminoma (Dys)). The expression of *ASBEL* was higher in 8 out of 11 ovarian cancerous tissues than in the non-cancerous tissues (Fig. 2a). Thus, to clarify the importance of *ASBEL* in ovarian tumorigenesis, we knocked down *ASBEL* expression in the JHOC5 cells by infecting with a lentivirus expressing an shRNA targeting *ASBEL* (shASBEL) (Fig. 2b). MTT assays revealed that knockdown of *ASBEL* caused a significant reduction in the growth of JHOC5, JHOC9 and OVISE cells (Fig. 2c). CellTiter-Glo assays also revealed that knockdown of *ASBEL* using siRNA caused a significant reduction in the growth of the CCC cell lines JHOC5, JHOC9, and OVISE and the serous adenocarcinoma cell lines OV1063 and 2008 cells (Fig. 2d). Furthermore, AnnexinV assays showed that knockdown of *ASBEL* induced apoptosis of JHOC5 cells (Fig. 2e). When JHOC5 cells stably expressing shASBEL were transplanted into nude mice, cell growth was significantly retarded compared to JHOC5 cells infected with control lentivirus (Fig. 2f and Supplementary Fig. S2). Immunohistochemical analyses of tumor xenografts demonstrated that tumor cells were arranged in solid, tubular and partially papillary patterns, which represents an important feature of human ovarian cancer (Supplementary Fig. S2). These results suggest that *ASBEL* may be required for the tumorigenicity of ovarian cancer.

*ASBEL* downregulates *ANA/BTG3* protein expression. Since antisense transcripts have been reported to regulate the expression of their overlapping sense transcripts1-2, we investigated whether knockdown of *ASBEL* could increase the expression of *ANA/BTG3* in JHOC5, JHOC9 and OVISE cells. RT-PCR and immunoblotting analyses revealed that knockdown of *ASBEL* using siRNA (siASBEL) resulted in an increase in the levels of *ANA/BTG3* protein, but not mRNA (Fig. 3a, b). Knockdown of *ASBEL* using shASBEL also did not affect the levels of *ANA/BTG3* mRNA (Supplementary Fig. S3). By contrast, overexpression of *ASBEL* decreased the levels of *ANA/BTG3* protein, but not mRNA, compared to that of *antisense ASBEL* (Fig. 3c). These results raise the possibility that the reduction in cell growth caused by *ASBEL* knockdown is due to an increase in the expression of *ANA/BTG3* protein. Thus, we examined whether knockdown of *ANA/BTG3* could restore the growth of cells transfected with siASBEL. We found that knockdown of *ANA/BTG3* using shRNA (shANA/BTG3) could partially rescue JHOC5 cells from siASBEL-mediated growth inhibition (Fig. 3d, e). Knockdown of *ANA/BTG3* did not affect *ASBEL* expression levels (Fig. 3e and Supplementary Fig. S4). Thus, ASBEL-mediated downregulation of *ANA/BTG3* may be critical for the proliferation of ovarian cancer.

*ASBEL-ANA/BTG3* duplexes are retained in the nucleus. To clarify the mechanisms underlying *ASBEL*-mediated downregulation of *ANA/BTG3* expression, we attempted to detect *ASBEL-ANA/BTG3* RNA duplexes. We overexpressed *ASBEL* fused to an oligonucleotide coding for an RNA hairpin that binds bacteriophage pp7 coat protein (pp7-*ASBEL*) in JHOC5 cells and performed RNA immunoprecipitation (RIP) analysis. Lysates prepared from JHOC5 cells transfected with the pp7-*ASBEL*, *ANA/BTG3* mRNA and FLAG-tagged pp7 coat protein expression constructs were subjected to immunoprecipitation with anti-FLAG antibody. RT-PCR analysis revealed that *ANA/BTG3* mRNA was associated with the pp7-*ASBEL* immunoprecipitates (Fig. 4a, b). By contrast, *ANA/BTG3* mRNA was not co-precipitated when pp7-*antisense ASBEL* or pp7-mutant ASBELs (Fig. 4a, *ASBEL*-Del-1 and -4) that lack the region complementary to *ANA/BTG3* was transfected instead of pp7-*ASBEL* (Fig. 4b, c). In addition, pp7-mutant ASBELs (Fig. 4a, *ASBEL*-Del-2 and -3) that contain the region complementary to *ANA/BTG3* efficiently coprecipitated *ANA/BTG3* mRNA (Fig. 4c). These results suggest that *ASBEL* is able to hybridize and generate *ASBEL-ANA/BTG3* RNA duplexes.

Because only *ANA/BTG3* protein, but not its mRNA levels are regulated by *ASBEL*, we speculated that *ASBEL-ANA/BTG3* RNA duplexes are retained in the nucleus. Subcellular fractionation and RT-PCR analyses showed that knockdown of *ASBEL* resulted in an increase in the amount of *ANA/BTG3* mRNA and *ANA/BTG3* protein present in the cytoplasm (Fig. 4d). To directly test the possibility that *ASBEL* may influence translation, lysates from JHOC5 cells transfected with siASBEL were fractionated through sucrose.
gradients. Knockdown of ASBEL did not change the polysome distribution profiles (Supplementary Fig. S5, lower panel), indicating that ASBEL does not affect global translation. qRT-PCR analysis of RNA isolated from each fraction revealed that knockdown of ASBEL did not induce changes in the size of polysomes translating ANA/BTG3 and HPRT1 mRNAs. These results suggest that ASBEL does not affect the translation of ANA/BTG3 mRNA. Thus, ASBEL may inhibit ANA/BTG3 protein expression by forming ASBEL–ANA/BTG3 RNA duplexes, which are retained in the nucleus (Fig. 5).

Discussion

In the present study, we have shown that ASBEL, a natural antisense transcript of the ANA/BTG3 gene, negatively regulates the expression of ANA/BTG3. This is the first report showing the mechanism underlying the regulation of ANA/BTG3 expression. Furthermore, we showed that ASBEL is required for proliferation and tumorigenicity of ovarian cancer. Thus, we speculate that downregulation of ANA/BTG3 by ASBEL is critical for proliferation and tumorigenicity of ovarian cancer. This notion is supported by the results of our rescue experiments showing that knockdown of ANA/BTG3 using shRNA rescues growth inhibition caused by ASBEL knockdown. These results are consistent with previous reports that ANA/BTG3 plays a critical role in cell proliferation and is downregulated in prostate and lung cancers14–16,18,19. It is possible that ASBEL is also involved in the tumorigenicity of cancers other than ovarian cancer, including prostate and lung cancers. Indeed, our preliminary results show that ASBEL is required for proliferation and tumorigenicity of colon cancer cells.

It has been reported that antisense transcripts regulate gene expression at various steps in the post-transcriptional processing of mRNAs. We showed that ASBEL hybridizes and generates ASBEL–ANA/BTG3 RNA duplexes in vivo. It is well known that mRNA expression of several genes, including p53, β-secretase-1 (BACE1) and inducible nitric oxide synthase (iNOS), is regulated by duplex
splicing, our results showed that nuclear-retained ncRNA mic transportation (Fig. 5). Although some ncRNAs such as the levels of ANA/BTG3 protein. Furthermore, our subcellular frac-
not affect remains to be elucidated. Investigation of the normal function ASBEL-ANA/BTG3 of of mRNA. The mechanism by which ASBEL-ANA/BTG3 expression in JHOC5 cells infected with a lentivirus expressing shRNA targeting (a) qRT-PCR analysis of ASBEL and ANA/BTG3 expression in ovarian cancer cell lines transfected with siRNA targeting ASBEL. Prior to fold-change calculation, the values were normalized to the signal generated from GAPDH mRNA. (b) Cell lysates from ovarian cancer cell lines transfected with siRNA targeting ASBEL were subjected to immunoblotting analysis with antibodies against the indicated proteins. α-tubulin was used as a loading control. (c) (Left) qRT-PCR analysis of ANA/BTG3 expression in JHOC5 cells transfected with either the sense or antisense ASBEL expression plasmid. (Right) Cell lysates were subjected to immunoblotting analysis with antibodies against the indicated proteins. α-tubulin was used as a loading control. (d) qRT-PCR analysis of ASBEL and ANA/BTG3 expression in JHOC5 cells infected with a lentivirus expressing an shRNA targeting ANA/BTG3. (e) JHOC5 cells that had been infected with a lentivirus expressing shRNA targeting ANA/BTG3 was transfected with siRNA targeting ASBEL and their viability was assessed.

* P < 0.05.

Figure 3 | ASBEL downregulates ANA/BTG3 protein, but not mRNA expression. (a) qRT-PCR analysis of ASBEL and ANA/BTG3 expression in ovarian cancer cell lines transfected with siRNA targeting ASBEL. Prior to fold-change calculation, the values were normalized to the signal generated from GAPDH mRNA. (b) Cell lysates from ovarian cancer cell lines transfected with siRNA targeting ASBEL were subjected to immunoblotting analysis with antibodies against the indicated proteins. α-tubulin was used as a loading control. (c) (Left) qRT-PCR analysis of ANA/BTG3 expression in JHOC5 cells transfected with either the sense or antisense ASBEL expression plasmid. (Right) Cell lysates were subjected to immunoblotting analysis with antibodies against the indicated proteins. α-tubulin was used as a loading control. (d) qRT-PCR analysis of ASBEL and ANA/BTG3 expression in JHOC5 cells infected with a lentivirus expressing an shRNA targeting ANA/BTG3. (e) JHOC5 cells that had been infected with a lentivirus expressing shRNA targeting ANA/BTG3 was transfected with siRNA targeting ASBEL and their viability was assessed.

formation with their antisense transcript5,6,10. However, ASBEL does not affect ANA/BTG3 mRNA expression but rather regulates the levels of ANA/BTG3 protein. Furthermore, our subcellular fractionation and RT-PCR analyses revealed that ASBEL forms duplexes with ANA/BTG3 mRNA in the nucleus and suppresses its cytoplasmic transportation (Fig. 5). Although some ncRNAs such as the nuclear-retained ncRNA MALAT1 are known to regulate alternative splicing3, our results showed that ASBEL does not affect splicing of ANA/BTG3 mRNA. The mechanism by which ASBEL inhibits cytoplasmic transportation of ASBEL-ANA/BTG3 RNA duplexes remains to be elucidated. Investigation of the normal function of ASBEL and regulation of its activity is also underway in our laboratories.

Methods

Cell culture. OVISCE, JHOC5, JHOC9, OV1063, and 2008 cells were cultured in RPMI1640 supplemented with 10% bovine serum. 293FT cells were cultured in DMEM supplemented with 10% bovine serum.

Antibodies. Anti-FLAG (F3165) antibody was obtained from Sigma. Anti-Lamin A/C (612162) antibody was obtained from BD Biosciences. Anti-α-tubulin (CP-06) antibody was obtained from CALIBOCHEM. Anti-ANA/BTG3 antibody was prepared as described previously9.

Strand-specific RT-PCR. RNA isolated using TRIzol (BIOLINE) was treated with DNase I (TAKARA), and reverse transcribed using SuperScript III (Invitrogen) in the presence of strand-specific primers (2 pmol) (Supplementary Table S1) or oligo(dT) (500 ng) at 35 °C. PCR amplification was performed using the indicated primers.

Northern blot. Total RNA was isolated using TRIzol (BIOLINE) and was purified using Dynabeads Oligo (dT) magnetic beads (Invitrogen). Poly(A)+ enriched RNA was boiled in formaldehyde containing buffer, separated by gel electrophoresis with a 2% denaturing formaldehyde gel in 1× MOPS buffer, transferred to Hybond N+ (Amersham) in 20× SSC (3 M NaCl, 0.3 M Na3 citrate). Membranes were cross-linked by ultraviolet irradiation and hybridized overnight at 60 °C with 32P-CTP-labelled DNA probes in Hybridization Buffer (7% SDS, 0.5 M Sodium Phosphate, pH7.2, 1 mM EDTA). The membranes were washed with NB Wash Buffer A (1× SSC, 0.1% SDS) for 10 min at 25 °C and then twice with NB Wash Buffer B (2× SSC, 0.5% SDS) for 20 min at 60 °C. Signals were visualized on a phosphorimager (Typhoon FLA 7000, GE Healthcare). DNA probes were labelled with 32P-CTP using the Megaprime DNA Labelling System (GE Healthcare). Primers used for generating probes are shown in Supplementary Table S2 online.

qRT-PCR analysis. Total RNA was isolated using the Total RNA Isolation kit (MACHEREY-NAGEL) and treated with DNase I (TAKARA). One microgram of RNA was reverse transcribed using PrimeScript RT Master Mix (TAKARA, RR036A). qRT-PCR analysis of cDNA was performed on a LightCycler 480 (Roche Applied Science) using Syber Green PCR mastermix (Applied Biosystems). Primers used for qRT-PCR are shown in Supplementary Table S4 online.

RNA interference. Stealth siRNA duplexes targeting ANA/BTG3 were purchased from Invitrogen. siRNA duplexes targeting ASBEL were purchased from Exiqon. Cells were transfected with RNA duplexes using Lipofectamine RNAmax (Invitrogen). The sequences of siRNAs are shown in Supplementary Table S4 online. Validated Stealth negative control RNAi duplex with Medium GC content #2 (Invitrogen) or siRNA negative control (Exiqon, S20C-0600) was used as a control.

Constructs and transfection. An oligonucleotide coding for an RNA hairpin that binds bacteriophage pp7 coat protein3,4 was cloned as an NheI-HindIII fragment in pcDNA3.1(+). FLAG tag and the nuclear localization signal from the SV40 large T-antigen was added to the 5’ end of the pp7 coat protein cDNA [a gift from D. S. Peabody (University of New Mexico School of Medicine)] by PCR and cloned into pcDNA3.1(+). ASBEL, ASBEL-DEL-1 (1 to 926), ASBEL-DEL-2 (1 to 1179), ASBEL-DEL-3 (1279 to 2312) were amplified by PCR using corresponding specific primers and cloned into pcDNA3.1(+) – pp7-5’. A fragment containing the ANA/BTG3 5’ UTR was amplified by PCR from the JHOC5 genome and was subcloned with the ANA/BTG3 ORF into pcDNA3.1(+). Primers used for construction are shown in Supplementary Table S5 online. Plasmids were transfected into cells using polyethyleneimine ‘MAX’ (PEI, Polysciences, Inc. Cat. 24765).

Immunoblotting. Cells (5 × 104) were lysed for 20 min with lysis buffer (0.5% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl pH7.5, 2 mM EDTA, 50 mM sodium fluoride) containing protease inhibitors. After centrifugation at 23,100 × g for 20 min at 4 °C, samples were resolved by SDS-PAGE and then transferred to PVDF membranes (Immobilon-P, Millipore) and analyzed by immunoblotting using HRP-conjugated secondary antibodies. Membranes were blocked with 5% skimmed milk in TBS plus Tween 20 at 4 °C overnight before probing with antibodies. Visualization was performed using the Enhanced Chemiluminescence Plus Western Blotting Detection System (GE Healthcare) and LAS-4000 (EPUV mini Lucensscent Imaging analyzer (GE Healthcare)).

Apoptosis. Phosphatidylserine (PS) exposure at the cell surface was detected using the Annexin V–Biotin Apoptosis Detection Kit (MBL) and Streptavidin–APC conjugates (S888, Invitrogen) according to the manufacturer’s instruction.

MTT assay. One week after lentivirus infection, cells were treated with C3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Calbiochem) for 4 h. Cell viability was determined by measuring the absorbance at 570 nm using a
ASBEL forms duplexes with ANA/BTG3 mRNA and prevents its nuclear-cytoplasmic transportation. (a) Schematic representation of the genomic organization of the region containing ASBEL and ANA/BTG3 and the ASBEL mutant constructs used in RIP assays. (b, c) RIP assay. Lysates prepared from JHOC5 cells transfected with the wild type, antisense (Antisense-ASBEL) or mutant pp7-ASBEL (ASBEL fused to a pp7 coat protein-binding sequence), ANA/BTG3 mRNA and FLAG-tagged pp7 coat protein expression constructs were subjected to immunoprecipitation with anti-FLAG antibody followed by RT-PCR analysis to detect ANA/BTG3 mRNA. Four mutants shown in (a) were used in (c). The apparent immunoprecipitation efficiency for a specific RNA was calculated by dividing the amount of RT-PCR product obtained in the immunoprecipitated sample by the amount obtained from the input RNA. HPRT1 was used as a negative control. (d) Subcellular distribution of ANA/BTG3 mRNA and ANA/BTG3 protein. JHOC5 cells transfected with siRNA targeting ASBEL or control siRNA were subjected to subcellular fractionation. (Left) The amounts of the indicated RNAs in each fraction were evaluated by RT-PCR analysis. U1 was used as a loading control. (Right) The cytoplasmic fraction was subjected to immunoblotting analysis with antibodies against the indicated proteins. α-tubulin was used as a loading control.

The ncRNA ASBEL is required for tumorigenicity of ovarian cancer. ASBEL forms duplexes with ANA/BTG3 mRNA in the nucleus and suppresses its nuclear-cytoplasmic transportation. ASBEL-mediated inhibition of ANA/BTG3 protein expression may be important for the tumorigenicity of ovarian cancer.
Mithras LB 940 (Berthold). The absorbance values of formazan were calculated as a percentage of the control wells.

**CellTiter-Glo**. Cell viability was determined indirectly by measuring the intracellular levels of ATP using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). Luminescence was measured on a Mithras LB 940 (Berthold).

**Subcutaneous xenograft**. JHOC5 cells infected with a lentivirus expressing an shRNA targeting ASBEL were injected stereotactically into 6-week-old nude mice (BALB/cCaI-nu/nu, CLEA Japan). All animal experimental protocols were performed in accordance with the guidelines of the Animal Ethics Committee of the University of Tokyo.

**RIP assay**. Cells growing in 6-well dishes were lysed in 0.5 ml of 0.5% Lysis buffer (50 mM HEPES pH 7.5, 150 mM KCl, 0.5% NP40, 2 mM EDTA, 1 mM NaF) containing protease inhibitors and RNase Inhibitor (Promega), and centrifuged at 13,000 r.p.m. for 10 min. The supernatant were incubated with anti-FLAG antibody for 3 h at 4°C with gentle rotation. Thirty microliters of Protein G Dynabeads (Invitrogen) were added and then incubated for 1 h at 4°C with gentle rotation. The beads were washed thrice with Wash buffer (50 mM HEPES pH 7.5, 150 mM KCl, 0.05% NP40) containing RNase Inhibitor (Promega) and then twice with PBS containing RNase Inhibitor (Promega). RNA was extracted using the Total RNA Isolation kit (MACHEREY-NAGEL) and qRT-PCR was performed as described above. Primers for qRT-PCR are shown in Supplementary Table S3 online.

**Subcellular fractionation**. Cells were washed and harvested in PBS. The pellet was resuspended in 1 packed cell volume of Hypotonic Buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂). After incubation on ice for 10 min, cells were disrupted by 10 passages through a 25-gauge needle. Cells were centrifuged for 10 min at 1,000 g at 4°C and the supernatant containing the cytoplasmic fraction was collected by further centrifugation at 17,000 g for 15 min. The remaining pellet was washed twice with Hypotonic Buffer, resuspended in Hypertonic Buffer (20 mM HEPES pH 7.5, 420 mM KCl, 1.5 mM MgCl₂, 0.5% NP40) and incubated at 4°C for 30 min with gentle rotation. The supernatant containing the nuclear fraction was collected by centrifugation at 18,000 g for 15 min.

**Polysome preparation**. The JHOC5 cytoplasmic fraction in hypotonic buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 100 μg/mL cycloheximide) was layered onto a 10mL linear sucrose gradient (10%-55% sucrose, 50 mM Tris-HCl pH 7.5, 75 mM KCl, 1.5 mM MgCl₂, 0.5% DTT) containing protease inhibitors and RNase Inhibitor (Promega), and centrifuged in a SW40Ti rotor (Beckman) for 15 h at 25,000 rpm at 4°C. Fractions were collected with a Piston Gradient Fracnation (Biocomp). RNA was extracted using the Total RNA Isolation kit (MACHEREY-NAGEL).

**Author contributions**

S.Y., K.T., A.O. and T.A. conceived the project and designed the experiments. S.Y. and K.T. performed most of the experiments. H.S., E.N., Y.T., M.K., K.O. generated some expression plasmids. H.S., E.N., Y.T., M.K., K.O. and T.A. prepared ovarian cancer specimens. S.Y., K.T., A.O. and T.A. wrote the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports/

**Competing financial interests**

The authors declare no competing financial interests.

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