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The cellular apoptosis susceptibility *CAS/CSE1L* gene protects ovarian cancer cells from death by suppressing RASSF1C

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ABSTRACT **The cellular apoptosis susceptibility gene *CAS/CSE1L* is overexpressed in cancer, although it was originally identified as a gene that renders cells vulnerable to apoptotic stimuli. CAS/CSE1L has roles in the nucleocytoplasmic recycling of importin-α and in the regulation of gene expression, cell migration, and secretion. We identified CAS/CSE1L as a survival factor for ovarian cancer cells *in vitro* and *in vivo*. In 3/3 ovarian cancer cell lines, CAS/CSE1L was down-modulated by the unorthodox proapoptotic signaling of the MET receptor. *CAS/CSE1L* knockdown with RNA interference committed the ovarian cancer cells to death, but not immortalized normal cells and breast and colon cancer cells. In 70 and 95% of these latter cells, respectively, CAS/CSE1L was localized in the cytoplasm, while it accumulated in the nucleus in >90% of ovarian cancer cells. Nuclear localization depended on AKT, which was constitutively active in ovarian cancer cells. In the nucleus, CAS/CSE1L regulated the expression of the proapoptotic Ras-association domain family 1 gene products RASSF1C and RASSF1A, which mediated death signals evoked by depletion of *CAS/CSE1L*. Our data show that CAS/CSE1L protects ovarian cancer cells from death through transcriptional suppression of a proapoptotic gene and suggest that the localization of CAS/CSE1L dictates its function.—Lorenzato, A., Martino, C., Dani, N., Oligschläger, Y., Ferrero, A. M., Biglia, N., Calogero, R., Olivero, M., Di Renzo, M. F. The cellular apoptosis susceptibility *CAS/CSE1L* gene protects ovarian cancer cells from death by suppressing RASSF1C. *FASEB J.* 26, 000–000 (2012). www.fasebj.org**

Key Words: receptor tyrosine kinases · MET receptor · hepatocyte growth factor · nuclear import

Apoptosis has profound effects on the malignant phenotype. Mutations that activate oncogenes and those that inactivate tumor suppressor genes usually disrupt apoptosis. Our study focuses on the MET tyrosine kinase receptor, which is encoded by the *MET* oncogene and usually activates antiapoptotic pathways, although it can also commit cells to death (1, 2). We have previously demonstrated that activation of the MET receptor by its ligand, hepatocyte growth factor (HGF) sensitizes, rather than protects, ovarian cancer cells to chemotherapeutic agents, both *in vitro* and in an animal model (3, 4). This was a novel finding, as in several instances, HGF can protect a variety of normal and cancer cells from apoptosis promoted by different stimuli (5). Nonetheless, HGF had been shown to either induce death or sarcomas and hepatomas (1). HGF-dependent sensitization to chemotherapeutics is maximal after long-term (up to 48 h) exposure (3) and involves p38MAPK activation (6, 7), which suggests that the HGF effect is coupled to transcriptional regulation of apoptosis-related genes.

To dissect out the molecular mechanisms of the unorthodox proapoptotic signaling of the MET receptor, we carried out the expression profiling of 3 ovarian carcinoma cell lines. These cells show different patterns of mutations and susceptibility to chemotherapeutics, to which they are sensitized by HGF. Here, we show that in these cells, HGF signaling initiates a transcriptional cascade that is characterized by comparable transcriptome profiles. Among the transcripts that are modulated in all of these cell lines, we identified *CAS/CSE1L*, which encodes a protein with a differential localization in ovarian cancer cells, whereby its product shows a specific function. Intriguingly, *CAS/CSE1L* is a Janus molecule, as it was originally identified as a cellular

Abbreviations: CDDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; PTX, paclitaxel; HGF, hepatocyte growth factor; LV, lentiviral

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apoptosis susceptibility gene, although it was also shown to be overexpressed rather than suppressed in several human cancer histotypes, including ovarian (8, 9) and colorectal (10) cancers. Moreover, CAS/CSE1L has a number of apparently unrelated roles, for example in cell secretion (11) and migration (12), in the nucleocytoplasmic recycling of importin-α (13), and in the regulation of p53 target-gene expression (14). Here we show that CAS/CSE1L is an antiapoptotic protein in ovarian cancer cells, and that this role is associated with its nuclear localization and its regulation of the transcription of the proapoptotic *RASSF1* gene.

MATERIALS AND METHODS

Cell lines and reagents

The cell lines were all purchased from American Type Culture Collection (Manassas, VA, USA) and were grown as indicated by the provider. SK-OV-3 cells stably expressing the dominant-negative DNp38MAPK are described elsewhere (6). Cisplatin [*cis*-diamminedichloroplatinum(II) (CDDP)] was obtained from Bristol-Myers Squibb (Rocky Hill, NJ, USA). Pure human recombinant SF1/HGF was purchased from R&D Systems (Minneapolis, MN, USA). AKT-specific inhibitor X was from Calbiochem (Darmstadt, Germany). The AKT^{Myr} retroviral vector and the relevant controls were produced using the retroviral vectors pBabe-Neo-Myr-Flag-AKT1 and the empty vector, respectively, obtained from Addgene (Cambridge, MA, USA), following the manufacturer's instructions.

Microarray preparation and analysis

Expression profiling was performed for 3 independent experiments. Total RNA was purified using the SV total RNA Isolation System (Promega, Madison, WI, USA). The RNAs were then quantified and inspected by bioanalyzer analysis (Agilent Technologies, Waldbrom, Germany). cRNAs were generated and hybridized on Sentrix Bead Chip Bead Map Files Human RefSeq 8 according to the Illumina TotalPrep RNA Amplification Protocol (Illumina, San Diego, CA, USA). The chips were then scanned with the Illumina scanner to generate the digitized image data files. Data were analyzed using the BeadStudio software package including an Illumina gene expression system. Hierarchical clustering (ST, Euclidean distance, average clustering, 5000 jackknife resampling steps) was performed using TMEV 3.1 software (http://www.tigr.org/software/). For the comparison of HGF+CDDP *vs.* nontreated (NT) samples, in each cell line under analysis, the genes were ranked on the basis of their average $\log_2$ fold-change. For any further analysis, we took into consideration only genes characterized by an absolute $\log_2$ fold-change threshold ≥ 1 in all of the comparisons. The same approach was used to identify the genes associated with *CAS/CSE1L* silencing. The microarray data have been deposited in the U.S. National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), with the approved accession numbers GSE24589 and GSE24429.

Transfection

SK-OV-3 cells were transfected with the pCMV6-Entry (C-terminal Myc and DDK tagged)-*RASSF1C* expression vector (Origene, Rockville, MD, USA) using Lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Quantitative RT-PCR

Real-time quantitative PCR was carried out as described elsewhere (7). Primer sequences were VAV, forward: TCTTATCTGCGACTCTGTCTCGG, and reverse: ATCCACAGGAGTGTTTCTGCTTG; SEMA4D, forward: TGACGTGTTGAAGGCCGAG, and reverse: GGCACTGGTACACCCCACTG; CSE1L, forward: TTAATAGTTCAGTGTATGGCGTTGG, and reverse: AGTCTCCAC AGTGTCCGTCAAA; PPFIA1, forward: GGCATTAAGTCCTCCATTGGC, and reverse TCCGTCTCGGAAACACCAG; ELYS, forward: TGTCACTTTTCAAGAACCTGAGAATG, and reverse: CAGCAGATGCAAACTCAAAACATC; NRIP1, forward: GTGCACTTG AGCCATGATGC, and reverse: TCGGACACTGGTAAGGCAGG; RASSF1C, forward: AAATGACCTGGAGCAGCACG, and reverse: GCGAGCTAGCGAGGTTCG; and RASSF1A, forward: GCTGCCCTGAGAAGCTGCT, and reverse: GTTTGCAGGGTCTCCA AGCTTTT.

Western blots and antibodies

Western blotting was carried out as described previously (6). The CAS/CSE1L goat polyclonal antibody and the β-tubulin rabbit polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); vinculin mouse monoclonal antibody and the anti-FLAG mouse monoclonal antibody were from Sigma (St. Louis, MO, USA). Anti-RASSF1 antibody was from Santa Cruz Biotechnology. The anti-phospho-AKT rabbit polyclonal antibody (Ser473, anti-AKT rabbit monoclonal antibody, anti-phospho-P70S6K rabbit polyclonal antibody (Thr389), and anti-P70S6K rabbit monoclonal antibody were from Cell Signaling Technology (Beverly, MA, USA). When indicated, cells were incubated with 10 μM AKT inhibitor X for 24 h. Bound antibodies were detected using the appropriate peroxidase-conjugated secondary antibody and revealed by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

RNA interference

RNA interference was performed using ON-TARGETplus SMARTpool (Dharmacon, Lafayette, CO, USA). In each experiment, the ON-TARGETplus nontargeting pool (Dharmacon) was used as the negative control. The cell lines were plated at 30 to 40% confluency and transfected with the indicated siRNA pools (100 nM) using Oligofectamine (Invitrogen), according to the manufacturer's instructions. Stable interference of *CAS/CSE1L* was achieved using the *CAS/CSE1L*-specific human TRIPZ lentiviral (LV)-inducible shRNA (clone V2THS_113224; Open Biosystems, Huntsville, AL, USA). The LV vectors were produced and used as previously reported (6).

Methylation analysis

The methylation status of the *RASSF1A* promoter was assessed as described by Burbee *et al.* (15). Primer sequences were unmethylated, forward: GGGTTTTGCGAGAGCGCG, and reverse: GCTAACAAACGCGAACCG; and methylated, forward: GGTTTTGTGAGAGTGTGTTTAG, and reverse: CACTAACAAACACAAACCAAAC.

Flow cytometry analysis of apoptosis induction

Apoptosis was measured as staining with APC-conjugated annexin V (Bender MedSystems, Burlingame, CA, USA) and

propidium iodide (Invitrogen), in accordance with the manufacturer's instructions. Caspase-3 activation was determined with a rabbit anti-active caspase-3 primary antibody (BD PharMingen, San Diego, CA, USA) and a PE-conjugated goat anti-rabbit secondary antibody (BD PharMingen).

FLAG detection was performed using a mouse anti-FLAG antibody (Sigma). For intracytoplasmatic staining of FLAG and active caspase-3, the cells had initially been fixed and permeabilized with BD Cytofix/Cytoperm (Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. The samples were analyzed on a CyAN-Adp flow cytometer (Dako, Fort Collins, CO, USA). Data acquisition was performed using the Summit software (Dako).

Immunofluorescence

Cells were plated on glass coverslips and, when indicated, incubated with 10 μM AKT inhibitor X for 24 h. For immunofluorescence, cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton-X100 in PBS for 2 min on ice. Then cells were incubated at room temperature with 1% BSA in PBS for 30 min and with an anti-CAS/CSE1L goat polyclonal antibody (Santa Cruz Biotechnology) diluted 1:200 in PBS containing 1% donkey serum for 1 h. After washing, cells were fluorescently labeled with an Alexa Fluor A488 donkey anti-goat antibody (Molecular Probes, Eugene, OR, USA) diluted 1:400 in PBS containing 1% donkey serum for 1 h. Nuclei were stained with DAPI. F-actin was stained with TRITC-conjugated phalloidin (50 μg/ml). The cells were analyzed using a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heerbrugg, Switzerland).

Tumorigenesis assay

Cells were injected subcutaneously (3×10^6) in immunodeficient $nu^{-/-}$ female mice in a Swiss CD-1 background (Charles River Laboratories, Calco, Italy). All of the animal procedures were approved by the local ethics committee [Comitato Etico per la Sperimentazione Animale (CESA), Candiolo, Italy] and by the Italian Ministry of Health. Treatment of the mice with doxycycline in their drinking water (200 μg/ml) was initiated when the tumors reached an approximate weight of 70 mg to 100 mg, 28 to 30 days after cell injection. Tumor volume (W) was calculated using the formula $W = \{(D \times d^2)/2\}4/3\pi$, where D is the major tumor axis, and d is the minor tumor axis (16).

RESULTS

HGF and CDDP regulate a common set of transcripts in ovarian cancer cell lines

The transcriptome that is regulated by HGF and CDDP was studied in 3 ovarian cancer cell lines that show different genetic profiles: SK-OV-3, TOV-21G, and NIH:OVCAR-3 cells (ref. 17 and http://www.sanger.ac.uk/perl/genetics/CGP/core_line_viewer?action=cell_lines). For example, SK-OV-3 cells harbor *CDKN2A* and *TP53* deletions and a *PIK3CA* mutation; TOV-21G cells have wild-type *TP53* and *FANCF* inactivated by methylation, and NIH:OVCAR-3 cells carry a *TP53* mutation and show *PIK3CA* amplification.

These 3 cell lines all express the receptor for HGF, encoded by the *MET* oncogene (3). They also show different susceptibilities to the chemotherapeutic agent

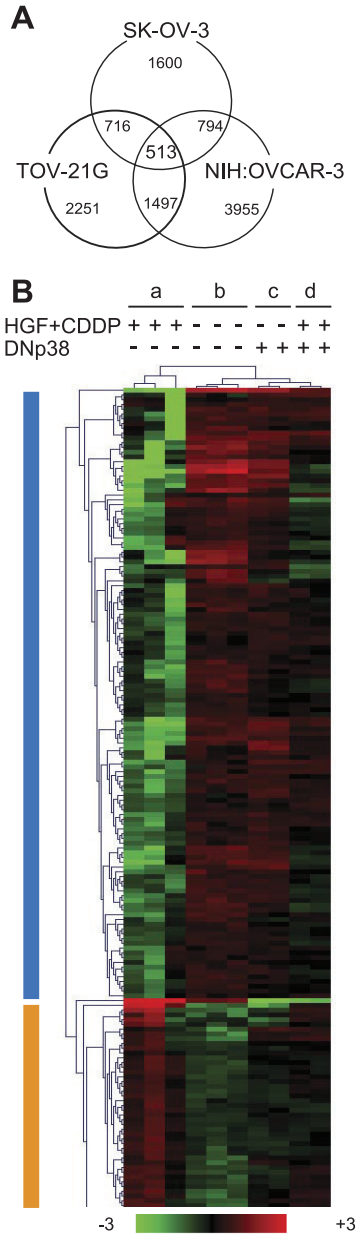

Figure 1. *A*) HGF plus CDDP modulation of the expression of a common set of genes in three ovarian cancer cell lines. Venn diagram of the genes differentially expressed in cells pretreated with 50 ng/ml HGF for 48 h and exposed to CDDP for 12 h (TOV-21G and NIH:OVCAR-3) or 24 h (SK-OV-3). A common set of 513 differentially expressed genes was identified, either increased or decreased by the combination of HGF and CDDP. *B*) Hierarchical clustering of genes differentially expressed in wild-type SK-OV-3 cells treated with HGF and CDDP (*a*) as compared to the untreated cells (*b*), and SK-OV-3 cells transduced to stably express the dominant-negative DNp38MAPK transgene (DNp38), either untreated (*c*) or treated with HGF and CDDP (*d*). Blue and orange clusters (left) indicate genes down-modulated and up-modulated, respectively, in wild-type SK-OV-3 cells treated with HGF plus CDDP. Clustering also shows that the transcriptional response to HGF plus CDDP treatment, as *a vs. b*, is abrogated by DNp38MAPK expression, as *c vs. d*.

CDDP, with the SK-OV-3 cells the most resistant and the TOV-21G cells the most sensitive (18, 19). As we have reported already (3), pretreatment of these cell lines with HGF results in their marked sensitization to the subsequent treatment with CDDP (Supplemental Fig. S1) at concentrations below the CDDP IC_{50} (18, 19) and below the serum concentrations that are obtained during human therapy with CDDP (20). We have established and reported elsewhere the generation by means of LV vectors of SK-OV-3 cells expressing a dominant-negative form of the p38MAPK (DNp38MAPK; ref. 6). Expression of DNp38MAPK almost abolished the apoptosis promoted by CDDP and enhanced by HGF (Supplemental Fig. S1), as we have reported previously (6, 7).

To determine the mechanisms of HGF-dependent cell sensitization to chemotherapeutic agents, the transcriptional response of these 3 ovarian cancer cell lines to HGF and CDDP was studied by microarray-based transcription profiling (data are deposited at GEO: GSE24589).

Figure 1*A* shows that 513 genes were similarly modulated by HGF and CDDP treatment of these 3 cell lines. Most of these genes were consistently down-modulated by the combination of HGF and CDDP (Fig. 1*B*, blue cluster). Some other genes were up-modulated, which indicated that these events are not merely due to cell death (Fig. 1*B*, orange cluster). Quantitative PCR validation of 10 transcripts was consistent with microarray data (Supplemental Fig. S2).

To refine the selection of the transcripts involved in ovarian carcinoma cell sensitization to CDDP, we analyzed the transcriptional variations of these 513 genes by expression of the DNp38 MAPK using microarray analysis. Of the 513 genes, 339 were no longer modulated by the treatment with HGF and CDDP in the ovarian cancer cells expressing the DNp38MAPK (Fig. 1*B*). The 339 genes identified by this analysis are involved in different biological processes, including transcription and translation, signal transduction, cell proliferation, and apoptosis (see GEO: GSE24589).

Selected genes alone regulate survival and sensitization of ovarian cancer cells to CDDP

Some of the top down-modulated genes in cells sensitized to CDDP by HGF were no longer modulated when p38MAPK function was suppressed by expression of the DNp38 MAPK. Among these, a few have already been associated with cell death in cancer, such as *HMGA2* and *PPP2R2C*. We hypothesized that some of the other genes were involved in the control of cell survival on the basis of the literature data. These included *VAV3, SEMA4D, NRIP1, PPFIA1, ELYS*, and *CAS/CSE1L*. The expression of each of these genes was then knocked down in the SK-OV-3 cells. Here we used gene-specific siRNA pools, each made of a mixture of 4 siRNAs targeting one of these mRNAs. Using these siRNA pools allowed reduction of the concentration of each of the individual siRNAs, thus limiting potential off-target effects. As a control, the SK-OV-3 cells were transfected with a pool of nontargeting siRNA sequences. Down-modulation of the mRNA of each gene was measured at 48 h after transfection using qPCR. As shown in **Fig. 2*A***, all of these genes were significantly silenced.

The transient silencing of *SEMA4D*, *ELYS*, or *PPFIA1* did not affect the SK-OV-3 cells (Fig. 2*B*). Conversely, silencing of *VAV3, NRIP1,* or *CAS/CSE1L* resulted in cell death and/or cell sensitization to CDDP (Fig. 2*B*). Silencing of *VAV3* impaired cell survival (Fig. 2*B*) *per se* but did not increase the sensitivity of the SK-OV-3 cells

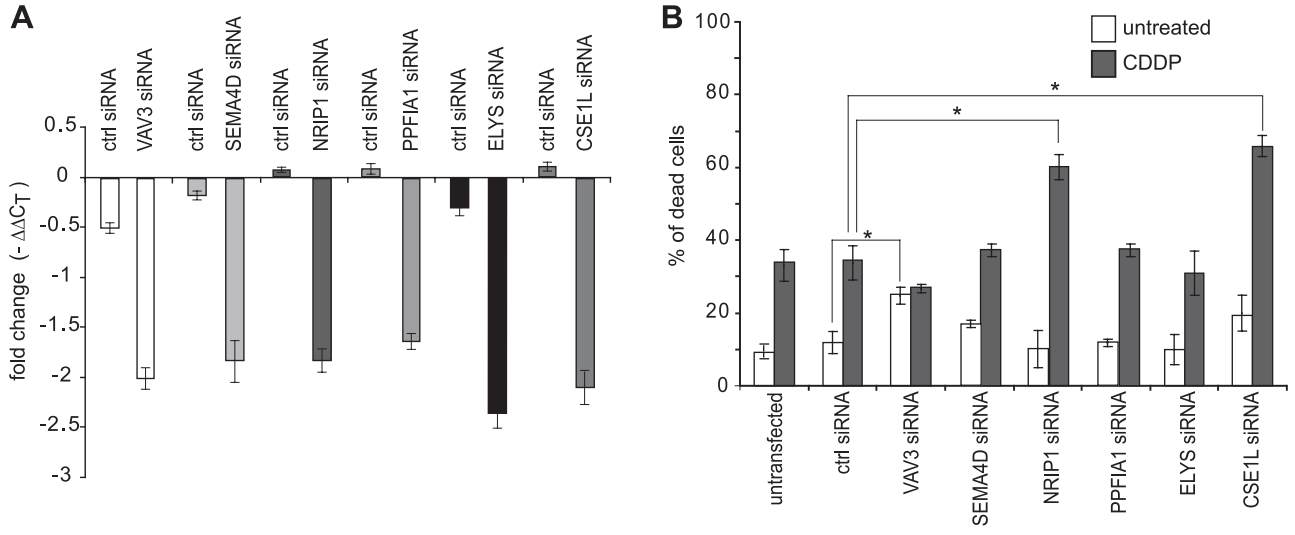

Figure 2. *A*) Individual silencing of the genes down-modulated by HGF and CDDP in microarrays of all ovarian cancer cell lines was carried out in SK-OV-3 cells. Cells were transfected with the nontargeting siRNA pool (ctrl siRNA) or gene-specific siRNA pools (as indicated), which were all effective in silencing. Mean fold-change in expression of the target gene in transfected cells *vs.* untransfected cells was calculated using the formula $-\Delta\Delta C_T = \{(C_T, \text{target} - C_T, \text{cyclophilin A})\ \text{transfected cells} - (C_T, \text{target} - C_T, \text{cyclophilin A})\ \text{untransfected cells}\}$. *B*) Sensitization of these silenced cells to CDDP (20 μM, 24 h). Percentage of dead cells was calculated by adding annexin V-PI to PI-only positive cells, determined using a multiparametric FACS assay. Significance was calculated using the Student's *t* test, by comparing gene-specific silenced cells to cells transfected with the nontargeting siRNA pools. Three independent experiments were evaluated. $^*P < 0.01$.

to CDDP (Fig. 2*B*). Conversely, the transient silencing of *CAS/CSE1L* or *NRIP1* did not affect the SK-OV-3 cells *per se* but did increase the sensitivity to CDDP (Fig. 2*B*).

An antiapoptotic role of *CAS/CSE1L* was unexpected, as it is the human homologue of the yeast chromosome-segregation protein *cse1*, which is also known as *CAS, i.e.*, the cellular apoptosis susceptibility gene (21, 22). However, our finding was compatible with the knowledge that *CAS/CSE1L* is overexpressed in ovarian cancer (8, 9, 23), and this suggested that *CAS/CSE1L* might have a specific role in ovarian cancer cells.

CAS/CSE1L knockdown selectively commits ovarian cancer cells to death *in vitro* and *in vivo*

Consistent expression of CAS/CSE1L was seen in the SK-OV-3, TOV-21G, and NIH:OVCAR-3 ovarian cancer cell lines (**Fig. 3**), as well as in ovarian cancer samples (data not shown), as previously reported (23). We also performed *in silico* analysis of published data (GEO: GSE3149) that was made available by Bild *et al.* (24); in microarray transcription profiles of 153 ovarian cancer samples, CAS/CSE1L is prevalently located within the top-ranked signal intensities.

To study *CAS/CSE1L* function in depth, this gene was silenced not only in the SK-OV-3 cells but also in the other ovarian cancer cell lines, *i.e.*, the NIH:OVCAR-3 and TOV-21G cells, the MCF-7 breast cancer cell line, and the HT-29 colon cancer cell line. These last 2 cell lines have been used previously to study the function of *CAS/CSE1L* (12, 14, 21, 22). We also used the human nontransformed HK-2 cell line (immortalized proximal tubule epithelial cells) and the MCF-10A cell line (mammary nontumorigenic epithelial cells) as controls. The silencing of *CAS/CSE1L* abrogated the expression at both the RNA (not shown) and protein levels in all of these cell lines (Figs. 3*A, D, F* and **4*A*, *C*, *E*, *G***).

In the NIH:OVCAR-3 and SK-OV-3 cells, the silencing of *CAS/CSE1L* increased their sensitivity to exposure to CDDP at concentrations below its IC_{50} (Fig. 3*B, D*). The broad effect of *CAS/CSE1L* silencing was shown by the sensitization to the microtubule binding agent paclitaxel (Fig. 3*C*). The TOV-21G cells also showed basal susceptibility to *CAS/CSE1L* silencing, with a marked increase in cell death after CDDP treatment at a concentration below its IC_{50} (Fig. 3*F*). This was not unexpected, as TOV-21G cells are more responsive to CDDP than SK-OV-3 and NIH:OVCAR-3 cells. Cell sensitization to CDDP was not associated with alterations in the cell cycle of the NIH:OVCAR-3 and SK-OV-3 cells (Supplemental Fig. S3). Conversely, the analysis of the cell cycle of the silenced TOV-21G cells showed that 20 to 30% of the cells were in the sub-G_0 region; *i.e.*, they were undergoing apoptosis (Supplemental Fig. S3). In all of the ovarian cancer cells where *CAS/CSE1L* was silenced, CDDP treatment resulted in

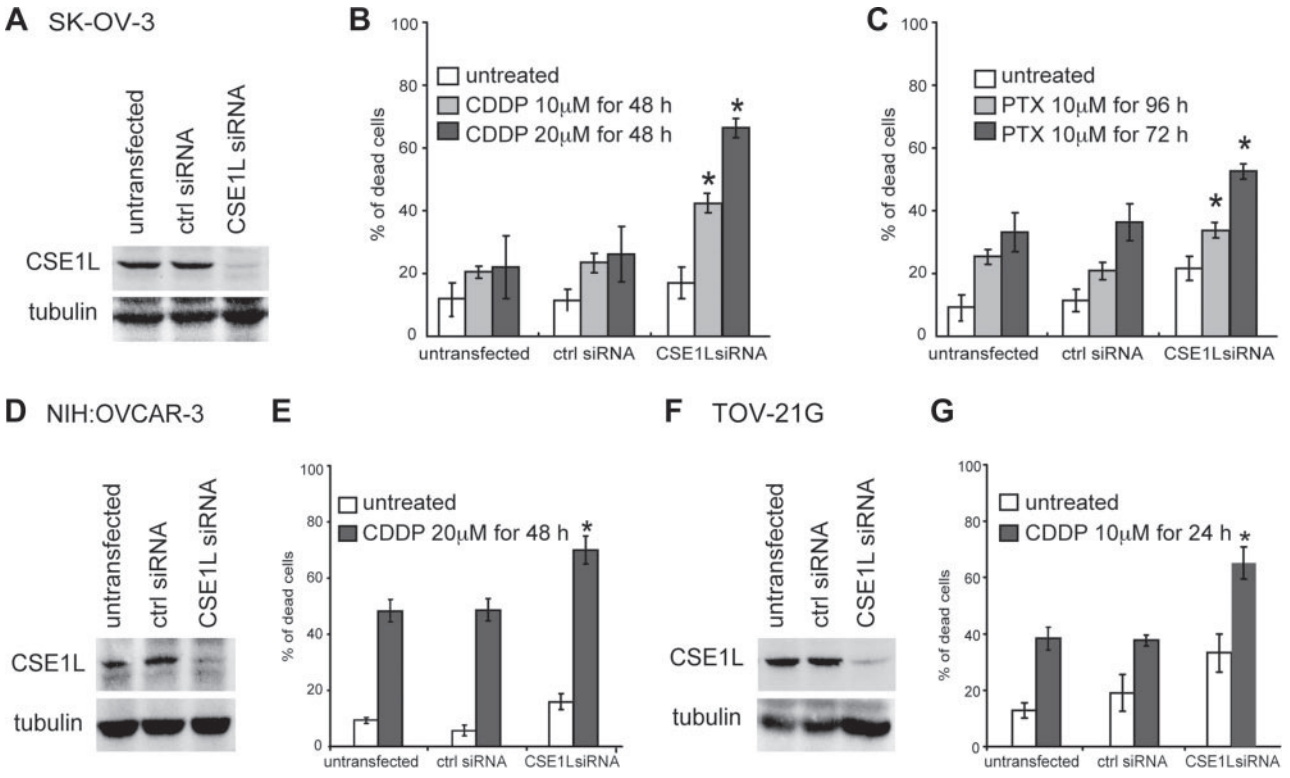

Figure 3. *A, D, F*) Silencing of *CAS/CSE1L* expression with specific siRNAs (CSE1L siRNA) in SK-OV-3 (*A*), NIH:OVCAR-3 (*D*), and TOV-21G (*F*) cells, as confirmed using a polyclonal antibody that recognizes the C-term of CAS/CSE1L in Western blot analysis. Cells were also transfected with the nontargeting siRNA pool as control (ctrl siRNA). Blots were reprobed for β-tubulin to confirm equal loading. *B, C, E, G*) Sensitization to treatments with CDDP of SK-OV-3 cells (*B*), NIH:OVCAR-3 (*E*) and TOV-21G (*G*) and with paclitaxel of SK-OV-3 cells (PTX; *C*) at the indicated doses and times, as assessed using multiparametric FACS assays. Percentage of dead cells was calculated by adding annexin V-PI- to PI-only-positive cells. Significance was calculated using the Student's *t* test, by comparing *CAS/CSE1L* silenced cells to cells transfected with ctrl siRNA. $*P < 0.01$.

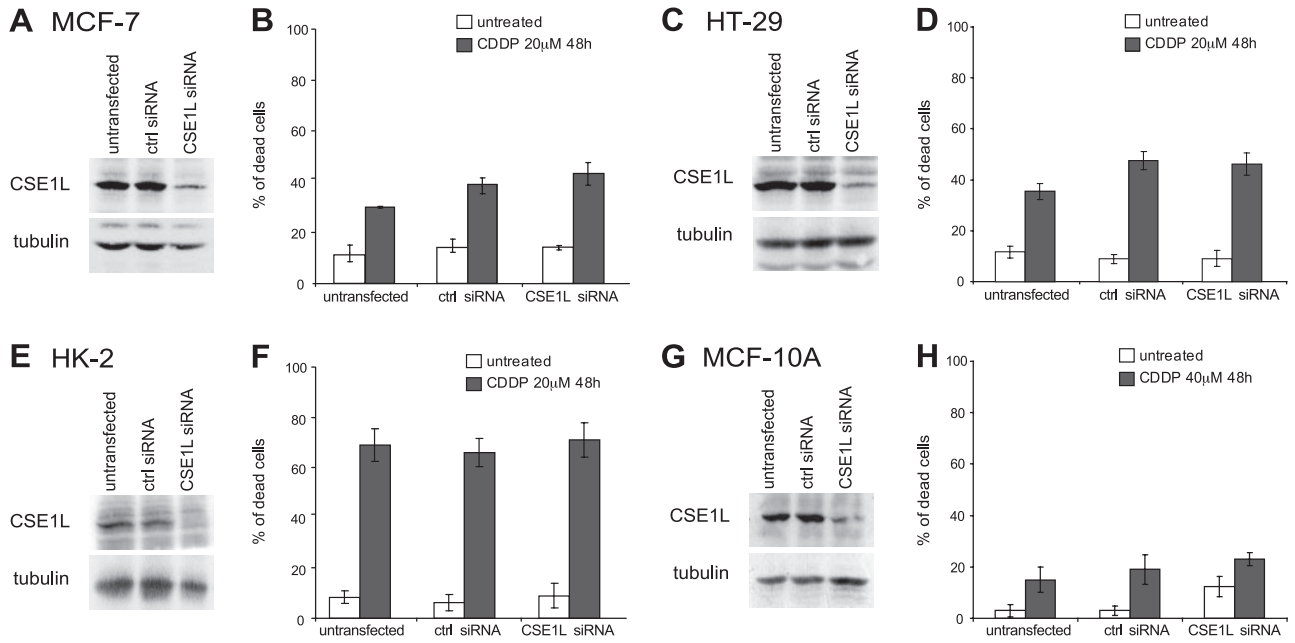

Figure 4. Silencing of *CAS/CSE1L* expression with specific siRNAs (CSE1L siRNA) in MCF-7 breast cancer cells (*A*, *B*), HT-29 colon cancer cells (*C*, *D*), HK-2 normal kidney cells (*E*, *F*), and MCF-10A nontransformed breast cells (*G*, *H*), transfected with the nontargeting siRNA pool (ctrl siRNA) or the *CSE1L*-specific siRNA pool (CSE1L siRNA), and treated with CDDP where indicated. Percentages of dead cells were calculated by adding annexin V-PI- to PI-only-positive cells, determined using a multiparametric FACS assay (*B, D, F, H*).

accumulation of annexin V and active caspase-3 (Supplemental Fig. S4). Altogether, these data show that *CAS/CSE1L* silencing predisposes these cells to an apoptotic type of cell death. The nonovarian MCF-7 and HT-29 cancer cells and the nontransformed HK-2 and MCF-10A cells were not affected by this *CAS/CSE1L* silencing and were not sensitized to CDDP (Fig. 4*B, D, F, H*).

The antiapoptotic role of *CAS/CSE1L* in ovarian cancer cells was confirmed by its stable silencing in the SK-OV-3 cells. This was achieved by transduction of the SK-OV-3 cells with a shRNA under the control of a tet-inducible promoter (**Fig. 5*A***). *In vitro*, this stable, long-term *CAS/CSE1L* suppression resulted in both a reduced proliferation rate and basal cell death (Fig. 5*B, C*). More important, *in vivo*, SK-OV-3 cells expressing *CAS/CSE1L*-specific shRNA did not grow as xenografts (Fig. 5*D*).

CAS/CSE1L protein is localized in the nucleus of ovarian cancer cells, and this is due to AKT activation

The CAS/CSE1L protein has been reported to have different roles; for example, in cell migration and cell secretion (11, 12, 25), in the nucleocytoplasmic recycling of importin-α (13) and in regulation of p53 target-gene expression in the nucleus (14). These roles involve localization of CAS/CSE1L either in the cytoplasm or in the nucleus. We found that CAS/CSE1L

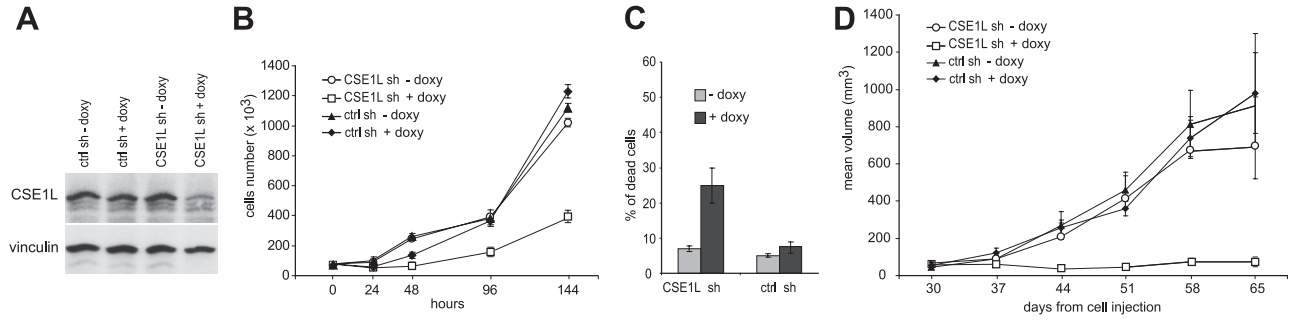

Figure 5. *A*) SK-OV-3 cells were transduced to express a *CAS/CSE1L*-specific shRNA (CSE1L shRNA) or control shRNA (ctrl) under the control of a tet-on promoter, whereby doxycycline (doxy)-induced shRNA expression and *CAS/CSE1L* silencing. Silencing was evaluated as in Fig. 3. *B*) Stable long-term *CAS/CSE1L* silencing impairs cell proliferation *in vitro*, as measured by counting cells of 3 culture plates in 3 independent experiments. *C*) Stable long-term *CAS/CSE1L* silencing impaired cell survival *in vitro*. Percentage of dead cells was calculated by adding annexin V-PI- to PI-only-positive cells, as determined using a multiparametric FACS assay, 72 h after the addition of doxycycline. *D*) Stable *CAS/CSE1L* silencing impaired the growth of SK-OV-3 cells *in vivo*, as a xenograft model in nude mice. Mice were injected with 3×10^6 cells s.c. After tumors became palpable (30 d after cell injection), doxycycline was added to drinking water.

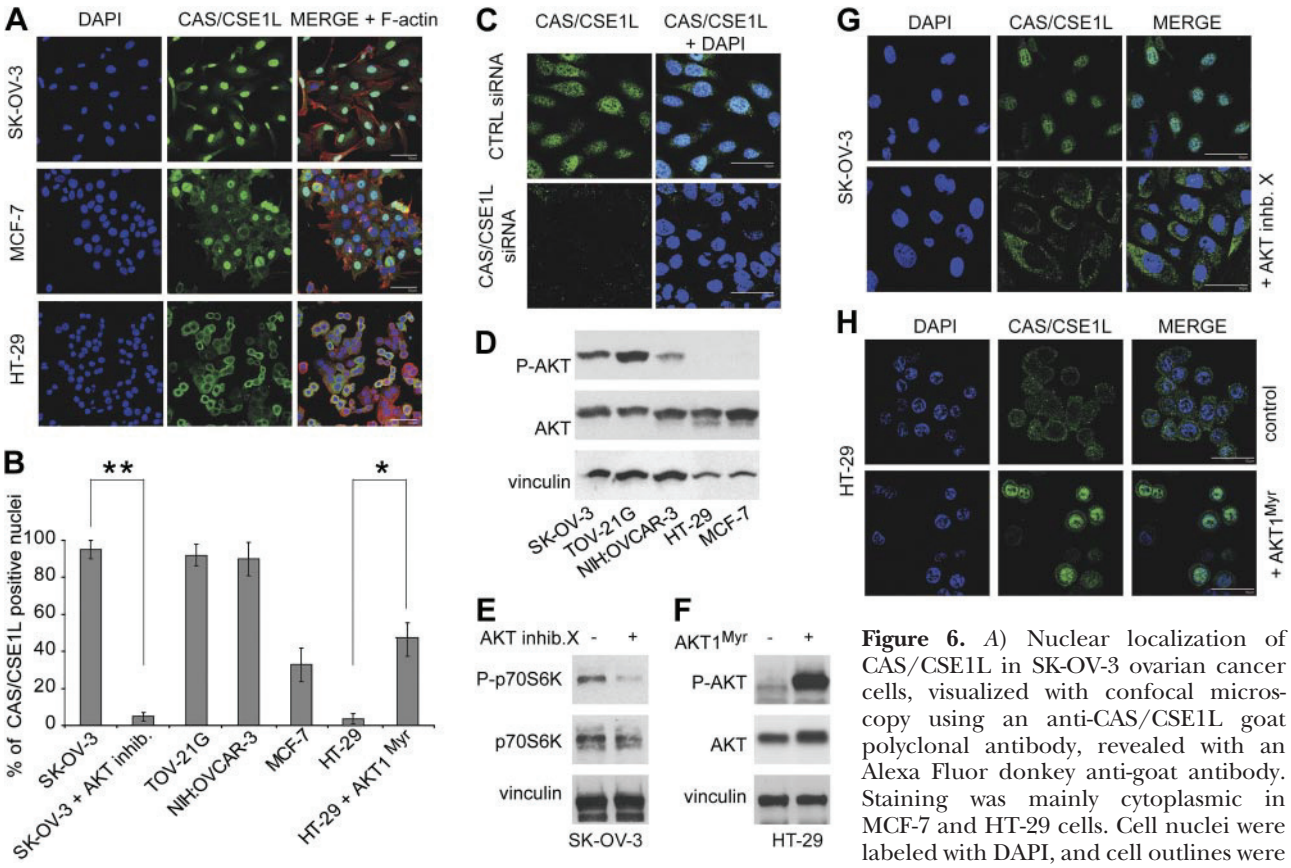

Figure 6. *A*) Nuclear localization of CAS/CSE1L in SK-OV-3 ovarian cancer cells, visualized with confocal microscopy using an anti-CAS/CSE1L goat polyclonal antibody, revealed with an Alexa Fluor donkey anti-goat antibody. Staining was mainly cytoplasmic in MCF-7 and HT-29 cells. Cell nuclei were labeled with DAPI, and cell outlines were revealed with TRITC-conjugated phalloidin, which binds F-actin. *B*) Percentages of CAS/CSE1L-positive nuclei, according to cell line, calculated by counting 5 microscopic fields of 2 different glass coverslips. $*P < 0.001$, $**P < 0.0001$; Student's *t* test. *C*) Immunostaining with anti-CAS/CSE1L antibody of SK-OV-3 cells where *CAS/CSE1L* is silenced showed negative labeling and thus staining specificity. DAPI staining shows nuclei. *D*) AKT basal activation in the 3 ovarian cancer cell lines, as shown by its constitutive phosphorylation at Ser473. No activity was detected in MCF-7 and HT-29 cells. Phosphorylated AKT and total AKT were visualized with Western blotting of total protein extracts. *E*) Biochemical AKT inhibitor X impaired phosphorylation of the AKT substrate p70S6K, shown with the Western blotting of total protein extracts. *F*) Transduction of HT-29 cells with a retroviral vector carrying myristoylated AKT1 ($AKT1^{Myr}$) promoted the expression of a constitutively active, *i.e.*, phosphorylated, AKT. Western blots were reprobed with an anti-vinculin antibody to show equal loading (*C–E*). *G*) Confocal microscopy with the anti-CAS/CSE1L antibody shows that inhibition of AKT phosphorylation with inhibitor X resulted in translocation of CAS/CSE1L from the nucleus to the cytoplasm of SK-OV-3 cells. *H*) Expression of constitutively active AKT (AKT^{Myr}) in HT-29 cells resulted in translocation of CAS/CSE1L from the cytoplasm to the nucleus. Control cells were infected with an empty retroviral vector. DAPI staining shows nuclei (*G*, *H*).

mainly and consistently localizes in the nucleus of the ovarian cancer cell lines (**Fig. 6*A, B***). Conversely, CAS/CSE1L was localized in the cytoplasm in the majority of the MCF-7 cells, and almost exclusively in the cytoplasm of the HT-29 cells (Fig. 6*A*, *B*). The localization of CAS/CSE1L in the latter cell lines is similar to that already reported (12, 21).

It has been suggested that nuclear localization of CAS/CSE1L might be regulated by the activation of the PI3K/AKT pathway (26). We found constitutive phosphorylation of AKT, which is the marker of its activation, in the ovarian cancer cell lines, but not in the MCF-7 and HT-29 cells (Fig. 6*C*), as already reported (27, 28). Treatment of the SK-OV-3 cells with the specific biochemical AKT inhibitor X (Fig. 6*D*) resulted in CAS/CSE1L translocation out of the nucleus (Fig. 6*F*), although it did not change the localization of CAS/CSE1L in HT-29 cells (data not shown). In agreement with our working hypothesis, the expression of an active, myristoylated form of AKT1 in HT-29 cells (Fig. 6*E*) resulted in translocation of CAS/CSE1L into the nucleus (Fig. 6*G*).

CAS/CSE1L silencing results in up-regulation of RASSF1 gene expression

The nuclear localization of CAS/CSE1L in ovarian cancer cells suggested that it might regulate transcription. Therefore, using microarrays, we evaluated the expression profiles of the SK-OV-3 and TOV-21G cells when *CAS/CSE1L* was suppressed (data are deposited at GEO: GSE24429). Among the genes affected by *CAS/CSE1L* silencing, we found a remarkable up-regulation of the *RASSF1* gene in both of these ovarian cancer cell lines. An in-depth, qPCR-based analysis of *RASSF1* isoforms demonstrated that in *CAS/CSE1L*-silenced TOV-21G cells the expression levels of *RASSF1A* and *RASSF1C* were increased (**Fig. 7*A***), while in the SK-OV-3

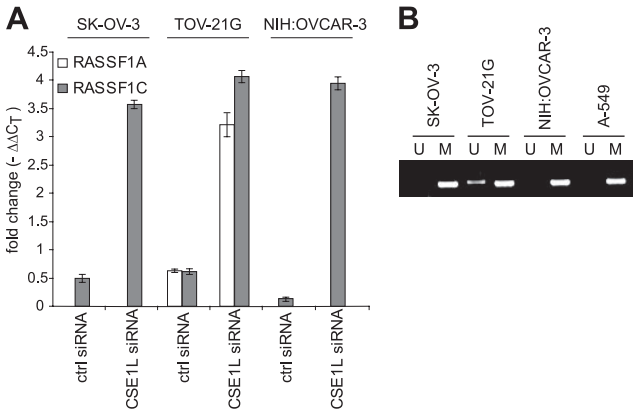

Figure 7. *A*) Quantitative PCR for *RASSF1A* and *RASSF1C* isoforms in ovarian cancer cell lines after *CAS/CSE1L* silencing. Silencing induced expression of *RASSF1C* in all the ovarian cancer cell lines, while expression of *RASSF1A* mRNA was induced only in TOV-21G cells. *B*) Methylation-specific PCR of the *RASSF1A* promoter demonstrated that in TOV-21G cells, the promoter is not fully methylated (U, unmethylated), while it is fully methylated (M) in the other ovarian cancer cell lines and in the A549 lung carcinoma cell line, used as a control, as methylation and lack of *RASSF1A* expression are known for these cells (49).

and NIH:OVCAR-3 cells, where *RASSF1A* expression is stably suppressed by promoter methylation (Fig. 7*B*), *CAS/CSE1L* silencing was followed by increased expression of *RASSF1C* only (Fig. 7*A*). Interestingly, previous microarray data (GSE24589; see above) have shown that expression of the *RASSF1* gene was not changed after treatment of cells with HGF, although it was up-regulated as above after treatment with CDDP.

In agreement with these data obtained in the cell lines, *in silico* analysis of the above-mentioned data (GEO: GSE3143) of Bild *et al.* (24) showed that the *RASSF1* gene and the *CAS/CSE1L* gene were oppositely expressed in the 153 ovarian cancer samples, with the signal intensity for the *RASSF1* gene lower in samples where the *CAS/CSE1L* signal intensity was higher.

In ovarian cancer cells, RASSF1C overexpression alone increased the sensitivity of cells to CDDP and mediates sensitization due to CAS/CSE1L silencing

As *RASSF1A* expression is not inducible in all ovarian cancer cell lines, we inferred that RASSF1C alone might sensitize cells to CDDP. A very low level of endogenous expression of *RASSF1C* was detected in all of these ovarian cancer cell lines, using qPCR (data not shown). Transfection of ovarian cancer cells with *RASSF1C* cDNA increased its expression (**Fig. 8*A***), and this alone increased the sensitivity of ovarian cancer cells to CDDP (Fig. 8*B*).

To understand whether CAS/CSE1L-mediated regulation of cell survival is hierarchically associated to *RASSF1* gene expression, we knocked out both *RASSF1A* and *RASSF1C* using RNA interference in *CAS/CSE1L*-silenced ovarian cancer cells (Fig. 8*C*, *E*). *RASSF1* gene silencing alone did not affect these cells, but it did reverse the effects of *CAS/CSE1L* silencing, *i.e.*, it reversed the cell sensitization to CDDP in both the SK-OV-3 (Fig. 8*D*) and TOV-21G (Fig. 8*F*) ovarian cancer cells.

DISCUSSION

In the present study, we have shown that the CAS/CSE1L protein has specific and unexpected functions in ovarian cancer cells, as it can protect these cells from apoptosis. This role is associated with the localization of CAS/CSE1L in the nucleus, and with the suppression of the proapoptotic *RASSF1* gene.

The *CAS/CSE1L* gene encodes a protein with a multifaceted role. *CAS/CSE1L* is the human homologue of the yeast *cse1* gene (29), which was also identified as *CAS*, a cellular apoptosis susceptibility gene in breast (30) and colon (22) cancer cells. Conversely, we show here that *CAS/CSE1L* is one of the transcriptional targets that is down-modulated by the unorthodox proapoptotic signaling of the MET receptor in ovarian cancer cells. Then, we demonstrated that *CAS/CSE1L* silencing commits ovarian cancer cells to death *in vitro* and *in vivo*. These findings match with the knowledge that CAS/CSE1L is overexpressed in ovarian cancer (23) and that its expression is related to adverse patient outcome (8, 9). Moreover, this is also in agreement with the finding that the mouse homologue of *cse1* is essential for early embryonic growth and development (31).

Other apparently unrelated functions have been attributed to CAS/CSE1L. It has also been implicated as exportin2 in the nuclear-cytoplasmic reshuffling of importin-α (13), as a microtubule-associated protein in cell proliferation (21) and migration and invasion of cancer cells (12), where it also stimulates cell shedding (10), and as a chromatin-binding protein in regulating expression of p53 target genes (14). In zebrafish, CAS/CSE1L is involved in fluid secretion (11). These functions have been demonstrated in different experimental settings, and they are likely to be associated with either the cytoplasmic or nuclear accumulation of CAS/CSE1L.

We show here that CAS/CSE1L is consistently accumulated in the nucleus of ovarian cancer cells, and that this localization depends on the constitutive activation of AKT in these cells. Interestingly, when AKT is expressed and regulated physiologically, CAS/CSE1L is mainly accumulated in the cytoplasm; this has been demonstrated by other studies (32, 33), as is also the case in the MCF7 and HT-29 cells here. Indeed, these latter cells harbor activating mutations of *PI3K*, which, however, do not result in enhanced AKT activation and which trigger other oncogenic pathways (27, 28). Here, we show that in the HT-29 cells, enforced expression of a constitutively active AKT results in the translocation of CAS/CSE1L to the nucleus. Moreover, in ovarian cancer cells, AKT inhibition results in the translocation of CAS/CSE1L from the nucleus to the cytoplasm. Altogether, these data show that nuclear localization of CAS/CSE1L is dynamically regulated by AKT. We can hypothesize that AKT controls CAS/CSE1L through Ran, which forms a complex with importin-α and CAS. It has been demonstrated that AKT modulates the

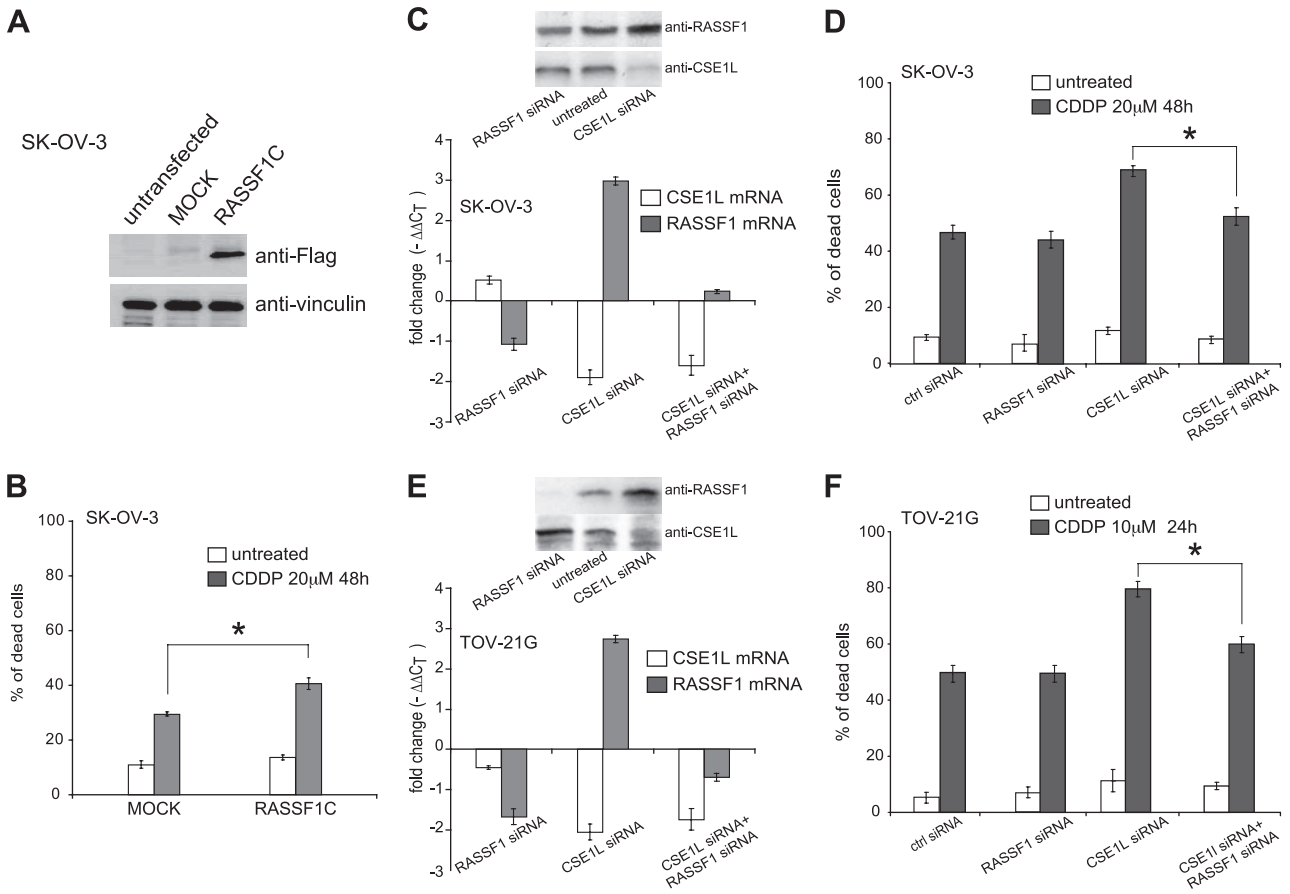

Figure 8. *A*) SK-OV-3 cells were transfected for expression of Flag-conjugated *RASSF1C* cDNA. Western blotting with anti-Flag antibody shows expression of the cDNA. *B*) SK-OV-3 cells expressing *RASSF1C* are sensitized to CDDP. Percentage of dead cells was assessed as in Fig. 2. $*P < 0.01$; Student's *t* test. *C*) Silencing of *CAS/CSE1L* or *RASSF1* or both in SK-OV-3 cells. Mean fold change in expression of the target gene in transfected cells *vs.* untransfected cells was calculated using the formula $-\Delta\Delta C_T$, as in Fig. 2. Inset: Western blot analysis of RASSF1 and CAS/CSE1L proteins after silencing. *D*) *RASSF1* silencing reversed the sensitization to CDDP caused by *CAS/CSE1L* suppression. Percentages of dead cells and significance as in *B*. *E*) Silencing of *CAS/CSE1L* or *RASSF1* or both in TOV-21G cells. Mean fold changes in expression were calculated as in *C*. Inset: Western blot analysis of RASSF1 and CAS/CSE1L proteins after silencing. *F*) *RASSF1* silencing reversed the sensitization to CDDP caused by *CAS/CSE1L* suppression. Percentages of dead cells and significance as in *B*.

nucleocytoplasmic gradient of Ran, and thus its nuclear transport, by interacting with, and phosphorylating, Ran-binding protein 3 (RanBP3) and thus regulating its function (34). More important, as the PI3K/AKT/mTOR pathway is a druggable target, these data show that CAS/CSE1L might be displaced, with its antiapoptotic function inhibited by inhibitors of this pathway.

CAS/CSE1L has frequently been shown to be increased in human ovarian cancer samples, but it has been localized in the cytoplasm or in the nucleus, and in both of these cellular compartments (8, 9). As CAS/CSE1L localization is dynamically regulated by the PI3K/AKT pathway, a different localization might reflect the different mutation patterns of the tumors studied, as ~50% of ovarian cancers show activation of this pathway (35).

In general, the well-documented role of CAS/CSE1L in nucleocytoplasmic reshuffling of importin-α (13) might explain how CAS/CSE1L regulates the cell cycle and gene expression (25). CAS/CSE1L controls the compartment-specific concentration of importin-α, and thus the nucleocytoplasmic concentrations of transcription factors, apoptotic proteins, and microtubule-organizing proteins. Moreover, it was shown recently that the nuclear accumulation of importin-α2 is associated with the transcriptional regulation of several genes, among which there is *STK35*, in HeLa cells (36). Interestingly, CAS/CSE1L silencing did not affect the expression of *STK35* in ovarian cancer cells (data not shown; see GEO: GSE24429), while importin-α2 overexpression and nuclear accumulation did not affect *RASSF1* expression (see GEO: GSE25303), which suggests a cell-specific effect. The control of gene expression by CAS/CSE1L accumulation in the nucleus might also be more direct, as CAS/CSE1L has been found associated to chromatin in mammalian cells (14). In addition, it has been reported that in yeast, nuclear transport factors are associated with discrete sets of active genes and can regulate their activities (37), and that, in *Drosophila*, nuclear-pore components directly regulate gene expression (38).

It was previously shown that CAS/CSE1L can regulate the expression of select p53 target genes, including antiapoptotic and proapoptotic genes (14). In ovarian cancer

cells, the increased expression of the proapoptotic *RASSF1* gene is the prevalent outcome of CAS/CSE1L down-modulation, irrespective of p53 proficiency. This might be explained by a dose effect. While transient down-modulation of CAS/CSE1L in breast cancer cells induces expression of p53-dependent proapoptotic and antiapoptotic genes, its consistent depletion results in death of MCF-7 (14), HT-116 (14), and HeLa (39) cells, as we demonstrated in ovarian cancer cells.

The expression of both the RASSF1A and RASSF1C isoforms, which derive from the use of two different promoters on the *RASSF1* gene, was increased in *CAS/CSE1L*-silenced ovarian cancer cells: RASSF1A was only induced in cells where its promoter is not methylated, while RASSF1C was induced in all of the ovarian cancer cell lines. The *RASSF* tumor-suppressor genes include 10 members. Although these were originally identified as genes that encode scaffold proteins that might link RAS to tumor-suppressor pathways (40), they also show RAS-independent tumor-suppressor activities (41). Both RASSF1A and RASSF1C are ubiquitously expressed in normal human tissues, and they promote apoptosis when overexpressed (42). Silencing of *RASSF1A* by promoter methylation is one of the most frequent epigenetic inactivation events detected in human cancers, which includes ovarian carcinomas (40, 43). However, deletion of *rassf1A* in transgenic mice resulted in a detectable but modest increased susceptibility to cancerogenesis (44), probably because the *rassf1C* isoform was left unaltered. *RASSF1C* expression is also suppressed by an unknown mechanism in most ovarian cancer cell lines (42). Here, we show that the expression of both *RASSF1A* and *RASSF1C* is downstream to CAS/CSE1L, that *RASSF1C* expression can be induced by *CAS/CSE1L* silencing in all ovarian cancer cell lines, and that this results in cell sensitization to CDDP.

We show that up-regulation of *RASSF1C* also sensitized p53-defective cells to CDDP. This is in agreement with the finding that RASSF1A promotes stabilization of both p53 (45) and p73 (46). As p73 is rarely mutated in cancer cells, activation of members of the RASSF1 protein family might restore susceptibility to DNA-damaging agents even in p53-null cells. In ovarian cancer cell lines, RASSF1C showed proapoptotic activity also in the absence of a *RAS*-activating mutation, as in the SK-OV-3 cells; this might be of particular importance, as *RAS* mutations are rarely found in ovarian cancer cell lines and carcinomas, but in borderline tumors (http://www.sanger.ac.uk/search?db=cosmic&t=ovary). However, increased RAS expression can also be envisaged in ovarian carcinomas. In ~25% of ovarian carcinomas, a *KRAS* variant has been found (47), which does not bind the *let-7* microRNA that decreases *KRAS* levels in *in vitro* assays (48).

In summary, the present study shows not only that CAS/CSE1L has an unexpected antiapoptotic role in ovarian cancer cells but also that its baseline expression suppresses the proapoptotic genes of the *RASSF1* family, among which is *RASSF1C*, the expression of which can be reactivated. Therefore, ovarian cancer cells might reexpress *RASSF1C*, which can enhance cell responses to DNA damage, even when *RASSF1A* is stably silenced, and thus also commit p53-deficient cells to death. FJ

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