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PRINCIPAL INVESTIGATOR: Steven R. Grossman, MD, PhD

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23298

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Boxiao Ding, Fang Yuan, Jolene Windle, Steven R. Grossman

E-Mail:steven.grossman@vcuhealth.org

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14. ABSTRACT

The C-terminal binding protein (CtBP) is elevated in epithelial ovarian cancer, especially in high-grade serous ovarian cancer (HGSOC) and has been implicated in cancer malignancy. However, the CtBP dependency in HGSOC is not well documented. Here we report that CtBP represses HGSOC apoptosis through death receptors (DRs) 4/5. CtBP knockdown upregualted DR4/5, and triggered autonomous apoptosis via caspase 8 activation. We further demonstrated that CtBP represses DR4 or/and DR5 in different cell-type contexts. Activation of DR4/5 by CtBP loss sensitized cell susceptibility to TRAIL. CtBP binds at the promoter regions of DR4/5, represses DR4/5 expression, presumably through the recruitment to a regulatory complex. We also found that CtBP1 and CtBP2 coordinate to repress the DR4/5 pro-apoptotic receptors. Collectively, this study identifies CtBP as a potent suppressor of DR4/5 and indicates targeting CtBP as a promising therapeutic strategy for HGSOC.

15. SUBJECT TERMS
high grade serous ovarian cancer; fallopian tube; C-terminal Binding Protein; dehydrogenase

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1. INTRODUCTION

High grade serous ovarian cancer (HGSOC) is the most lethal form of ovarian cancer, and most likely to present at an advanced stage. In cancers such as HGSOC, where few actionable mutations are found, cancer-specific dependencies can be targeted by antagonizing overexpressed/amplified wild type driver oncogenes that regulate critical processes such as cell cycle, metabolism, or transcription/chromatin dynamics.The C-terminal binding protein (CtBP) family of transcriptional co-regulators are frequently overexpressed at the protein level, but not mutated, and are associated with worse outcome and aggressive tumor features in solid tumors, such as colon, breast, and prostate cancer, and especially invasive ovarian cancer, where ~85% of cases exhibit CtBP2 protein overexpression. At a biological level, CtBP-driven transcriptional programs lead to dysregulated cell survival, and drive a cancer stem cell (CSC)/epithelial-mesenchymal transition (EMT) phenotype associated with metastasis. Importantly for both breast and ovarian cancer, CtBP is a key epigenetic repressor of BRCA1, and given the known predisposition to ovarian cancer caused by BRCA1 mutation, CtBP repression of BRCA1 may be a key driver of ovarian oncogenesis. CtBP is not only a putative ovarian oncogene, but also encodes a functional NADH-dependent dehydrogenase required for CtBP's oncogenic activity, where the dehydrogenase active site offers a tractable structural feature for small molecule targeting. CtBP2 oncogenically transforms primary human fibroblasts in cooperation with tumor suppressor inactivation, and CtBP targeting by substrate competitive inhibitors with sub-micromolar affinity has been recently reported. Genetic or pharmacological inhibition of CtBP have been shown to: a) reverse CtBP's oncogenic transcriptional program, b) exhibit cancer cell selective cytotoxicity, and c) potently limit tumor growth in xenograft and genetic animal models, such as the *Apc*^{min} mouse. **CtBP is therefore a candidate for a tumor-selective molecular target in HGSOC** due to its: 1) potent oncogenic activity 2) tumor-specific overexpression and activation; 3) targetable catalytic domain. We therefore hypothesize that a subset of HGSOC is uniquely dependent on or "addicted" to CtBP due to the high frequency of its overexpression relative to normal tissue, and known ability to drive a CSC phenotype, which is a key feature of the therapeutic resistance profile in HGSOC. Thus, based on published data, and our own preliminary data showing that basal-like breast cancer xenografts (which have genomic/epigenomic similarity, including BRCA1 deficiency, to HGSOC) do not form tumors if CtBP2 is deleted by CRISPR techniques, we propose to establish the role of CtBP1/2 in HGSOC and their possibility as biomarkers and therapeutic targets through the following Aims: **(1)** Establish CtBP as a transforming oncogene and dependency in ovarian cancer and normal fallopian tube epithelial cells; **(2)** Characterize the role of CtBP overexpression in human ovarian carcinogenesis and validate pharmacologic inhibition of CtBP in HGSOC orthotopic xenografts; and **(3)** Develop a fallopian tube-specific transgenic mouse model for CtBP-driven ovarian carcinogenesis.

2. KEYWORDS

high grade serous ovarian cancer; fallopian tube; C-terminal Binding Protein; dehydrogenase

3. ACCOMPLISHMENTS

A. Major Goals

Aim 1. Establish CtBP as a transforming oncogene and dependency in ovarian cancer and normal fallopian tube epithelial cells

a. Test the transforming ability in vitro (3D culture) and in vivo (orthotopic xenograft) of CtBP1/2 in primary human fallopian tube epithelial cells in cooperation with Rb/p53 tumor suppressor inactivation

Milestone(s) Achieved: 1. Demonstrate CtBP1 or 2 transform FTE cells to anchorage independent growth as well as H-RasG12V, 2. Demonstrate CtBP1 or 2 transform FTE cells to grow as orthotopic xenografts in NSG mice as well as H-RasG12V, **Timeline** 12 months

% completion: 50%

b. Analyze ovarian cancer cell lines for CtBP1/2 copy number abnormality (CNA) and protein expression, and correlate CtBP1/2 CNA and protein overexpression with dependence on continued expression of either or both genes for growth and/or viability using CRISPR-mediated CtBP1/2 knockout

Milestone(s) Achieved: 1.CtBP1 and 2 levels established for each ovarian cancer cell line in comparison to FTE cells, 2. CtBP CNA established by FISH in each ovarian cancer cell line, 3. CtBP1/2 CRISPR lines developed, 4. Growth dependency (2D and 3D culture) of each ovarian cancer line on CtBP1 or 2 established by CRISPR, **Timeline** 9 months

% completion: 75%

c.Assess CtBP1/2 dependency of ovarian cancer cell line orthotopic xenografts using CRISPR knockout lines

Milestone(s) Achieved: Luciferase expressing parent ovarian cancer cell lines and CRISPR deleted clones tested vs. parent to establish CtBP dependency in each cell line, **Timeline** 15 months

% completion: 0%

Aim 2. Characterize the role of CtBP overexpression in human ovarian carcinogenesis and validate pharmacologic inhibition of CtBP in HGSOC orthotopic xenografts

a. Analyze an HGSOC case series for CtBP1/2 expression by IHC, and correlate with CSC and EMT markers, overall and progression free survival, and genomic status/CAN

Milestone(s) Achieved: 1. Establish rate of CtBP1 vs. 2 overexpression (relative to normal tissue) in HGSOC,2. Determine correlation between intensity of CtBP1/2 expression and Ki-67 positivity, CSC or EMT marker expression, 3. Correlate CtBP1/2 amplification with CtBP1/2, Ki-67, CSC or EMT marker expression **Timeline** 6 months

% completion: 0%

b. Analyze case series of normal fallopian tube and pre-neoplastic fallopian tube lesions (from risk-reducing surgeries and co-occurrence in HGSOC resections) for CtBP1/2 expression by IHC and genomic status, and determine the earliest that CtBP CNA or overexpression occurs in the neoplastic progression sequence

Milestone(s) Achieved: 1. Establish where in the sequence of ovarian carcinogenesis CtBP1 or 2 protein levels become elevated, 2. Establish where in the sequence of ovarian carcinogenesis CtBP1 or 2 genes are amplified, **Timeline** 9 months

% completion: 0%

c.Compare high vs. low CtBP expressing ovarian cancer cell lines or Ras-transformed vs. CtBP transformed FTE for response to small molecule CtBP inhibition in orthotopic xenografts

Timeline 15-21 months

% completion:0%

Aim 3. Develop a fallopian tube-specific transgenic mouse model for CtBP-driven ovarian carcinogenesis

a. Generate PAX8-Cre/Ctbp2^{LSL} transgenic mice that express Ctbp2 in mouse fallopian tube epithelium

Milestone(s) Achieved: 1. Generate vector for CtBP2 transgene, 2. Generate CtBP2 transgenic founder strains, 3. Breed CtBP2 transgenic strains to Pax8-Cre to establish strain with highest FT-specific overexpression of CtBP2, 4. Perform aging study of Ub-LSL-CtBP2+ / PAX8-Cre+ mice to determine effect of CtBP2 overexpression alone in FTE, **Timeline** 24 months
% completion: 33%

B. Accomplishments toward goals

Aim 1. Establish CtBP as a transforming oncogene and dependency in ovarian cancer and normal fallopian tube epithelial cells.

a. Test the transforming ability in vitro (3D culture) and in vivo (orthotopic xenograft) of CtBP1/2 in primary human fallopian tube epithelial cells in cooperation with Rb/p53 tumor suppressor inactivation

FT282 cells were derived from human fallopian tube secretory epithelium, and immortalized by human TERT and mutant $p53^{R175H}$. To address the basis for CtBP effect on the transformation of normal fallopian tube epithelial cells, we overexpressed CtBP2 in FT282 cells and examined cell proliferation. Ectopic expression of CtBP2 was confirmed by Western blotting (Fig. 10 a), and cell proliferation over 5 days was assessed by trypan blue assay. Growth rate of CtBP2-overexpressed cells was on par with that of vector cells and parental cells (Fig. 10 b). When sparsely seeded cells were cultured for 14 days, both vector and CtBP2-overexpressed cells exhibited similar colony formation (Fig. 10 c). We also performed 3D cell culture with methylcellulose to test whether FT282 cells expressing CtBP2 had acquired the ability to grow in suspension, a key feature of tumorigenic cells. Cells were grown in the medium containing methylcellulose for 21 days, then determined microscopically for colony formation, while FT282/H-Ras^{V12} cells, which were transformed by introducing oncogene H-Ras^{V12}, were used as a positive control. As expected, FT282/ H-Ras^{V12} cells exhibited robust colony formation under these conditions, whereas parental FT282, FT282/vector, and FT282/CtBP2 did not form colonies (Fig. 10 d). These data indicate that CtBP2 is insufficient to transform immortalized FTECs. We asked whether overexpressed CtBP2 could enhance the tumorigenic capacity of FT282/H-Ras^{V12} cells. To this end, we expressed V5-tagged CtBP2 in FT282/H-Ras^{V12} cells (Fig. 10 e). However, no difference in colony formation was observed between FT282/H-Ras^{V12}/vector cells and FT282/H-Ras^{V12}/CtBP2 cells (Fig. 10 f). We further tested the effect of CtBP deficiency on FTECs proliferation. We utilized lenti-shRNA to knock down CtBP in FT282 cells. Fig. 10 g showed the efficiency of shRNAs for CtBP1 and CtBP2. 3D cell culture showed no colony formation in FT282 cells that received shRNA control or shCtBP1/2 (Fig. 10 h). These results together suggested that CtBP by itself does not likely transform immortalized FTECs.

We next tested whether perturbation of CtBP might affect the transformation of FTECs by H-Ras^{V12}. We established stable FT282 cell lines expressing ectopic CtBP2 or empty vector. The stable cell lines were infected with adenovirus mediated H-Ras^{v12} (Fig. 11 a), then were cultured in methylcellulose. H-Ras^{v12} effectively induced colony formation of FT282 cells in the 3D cell culture system, however, both F282/vector/H-Ras^{v12} and F282/CtBP/H-Ras^{v12} cells exhibited similar size and numbers of colonies (Fig. 11 b). We further knocked down CtBP (as shown in Fig. 10 g and Fig. 11 c) and transduced cells with H-Ras^{v12}. We did not

observe the difference in colony formation between the tested groups (Fig. 11 d). These data suggested that CtBP in FTECs unlikely cooperates with H-RasV12 to transform FTECs.

To also test whether CtBP overexpression affects HGSOC cell growth, we established a CtBP2 overexpressed KURAMOCHI cell line. Western blotting assay confirmed the ectopic expression of CtBP2 (Fig. 8a). We examined cell growth ability in 2D and 3D culture. As shown in Fig. 8b and c, CtBP2 overexpression did not halt or enhance cell growth in 2D and 3D culture. We also overexpressed CtBP2 in OVSAHO cells (Fig. 9a). There was no difference in cell growth between parental, vector-expressing and CtBP2-expressing OVSAHO cell lines (Fig. 9b, c and d). These results indicated that ectopic expression of CtBP2 is not required for HGSOC cell growth. Of note, we also observed that CtBP depletion did affect OVSAHO cell growth (Fig. 9).

b. *Analyze ovarian cancer cell lines for CtBP1/2 copy number abnormality (CNA) and protein expression, and correlate CtBP1/2 CNA and protein overexpression with dependence on continued expression of either or both genes for growth and/or viability using CRISPR-mediated CtBP1/2 knockout*

CtBP1/2 loss triggers caspase 8 dependent apoptosis. To address CtBP dependency of HGSOC malignancy, we first knocked down CtBP1 or 2 by lentivirus-based shRNAs in KURAMOCHI cells (Fig. 1a; Table 1), which are genetically representative of HGSOC. We observed a slow but dramatic cell death response when CtBP1 or 2 was depleted by RNAi (Fig. 1b). Annexin V and Propidium Iodide (PI) staining assay revealed that apoptosis underlies CtBP1/2 loss-induced cell death (Fig. 1c). To get insight into the mechanism underlying cell death, we screened proteolytic caspase -3, -7, -9, -8 as well as PARP1. Two shRNAs targeting CtBP1 or CtBP2 were used to exclude the possible nonspecific off-targeting effect of shRNA. We observed markedly elevated levels of cleaved caspases and PARP1, and that both shRNAs showed similar effect on caspase/PARP1 cleavages, indicating that CtBP1/2 knockdown specifically induced activation of caspase cascades (Fig. d). Of note, caspase 8, which is upstream of other caspases and stands at the initiating step of the extrinsic apoptosis pathway, was clearly cleaved, indicating activation of caspase 8 is the critical event of CtBP loss-induced apoptotic cell death. Furthermore, caspase 8 and caspase 3/7 activities were markedly enhanced by CtBP knockdown (Fig. 1E). We also observed that addition of Z-IETD-FMK, a specific caspase 8 inhibitor, completely abrogated cell death in shCtBP1/2 cells (Fig. 1f). We genetically knocked down caspase 8 by siRNA prior to CtBP shRNA infection (Fig. 1g), and analyzed cell viability. As expected, caspase 8 depletion rescued CtBP1/2 shRNA infected cells (Fig. 1h). Taken together, we concluded that CtBP deficiency induces apoptosis via caspase 8 activation.

CtBP loss activates caspase 8 via death receptor 4. Canonical extrinsic apoptosis is initiated by ligands such as FAS, TNFR1, binding to their respective death receptors death receptor 4 (DR4) and DR5. The

apoptotic signal is transduced via receptor oligomerization, and the adaptor protein FADD, which recruits caspase 8, leading to caspase 8 activation by autocatalysis. On the other hand, accumulating studies have pointed out that cell-autonomous activation of death receptors signals activation of caspase 8 in a ligand independent manner. Having shown that CtBP controls apoptosis through caspase 8, we next asked how caspase 8 was activated under the condition of CtBP loss. For this purpose, we examined the expression of death receptors in KURAMOCHI cells receiving shRNAs for CtBP1/2. Interestingly, we found that DR4 was markedly elevated at both protein and mRNA levels in CtBP knockdown cells, whereas TNFR1, FAS and DR5 remained unaltered (Fig. 2a; Table 1). Indeed, KURAMOCHI cells were completely resistant to TRAIL, the ligand of DR4/5. The addition of exogenous TRAIL to cell culture did not sensitize or enhance cell death even though DR4 was already upregulated through CtBP deficiency (data not shown), indicating a possible link between DR4 elevation and caspase 8 activation. We simultaneously depleted DR4 and CtBP1/2 by RNAi (Fig. 2c) and found the inhibition of caspase 8 cleavage as evidenced by diminishing cleaved bands of caspase 8 and reduced caspase 8 activity (Fig. 2c and d). In parallel, CtBP loss-induced cell death was abrogated by DR4 depletion (Fig. 2e). These data collectively suggested that CtBP loss led to autonomous activation of DR4, which activates caspase 8 and consequently the downstream caspase cascade, resulting in cell apoptosis.

CtBP represses DR4 and DR5 depending on cell context. Previous studies have revealed the versatile principles of regulation of DR4/5. Multilevel controls in DR4/5 expression have been found in different types of HGSOC cells. Although we did not observe any change in DR5 expression in KURAMOCHI cells by CtBP knockdown, it is possible that the particular cell context of KURAMOCHI might mask the effect of CtBP on DR5 expression. We assessed the steady state levels of DR4/5 and CtBP1/2 in a panel of HGSOC cell lines. As shown in Fig. 3a, KURAMOCHI cells as well as 3 other cell lines (SKOV3, OVSAHO and CAOV3) had very low DR5 expression, and OVSAHO and A2780 cell lines had no detectable expression of DR4. All of the tested cell lines expressed similar robust levels of CtBP1 and CtBP2. The induction of DR4 was also observed in OVCA429 and SKOV3 cells when CtBP1/2 were knocked down (Fig. 3b, c and d; Table 1). Remarkably, knockdown of CtBP1/2 caused DR5 upregulation in OVCAR429 cells (Fig. 3e and f). However, we did not observe the alteration of DR4/5 in OVSAHO, CAOV3 and Hey cell lines (data not shown). These results together indicated that CtBP1/2 repress DR4/5 in a cell type dependent manner.

CtBP depletion sensitizes cells to TRAIL. We noticed that induction of DR4 and/or DR5 was not accompanied with loss of viability of CtBP knock-down in OVCAR429 and SKOV3 cells. We next tested the apoptotic fate of these cells in response to TRAIL treatment (Table 1). Consistent with previous reports, OVCA429 cells were susceptible to TRAIL. Depletion of CtBP from OVCA429 cells enhanced TRAIL-induced cell death (Fig. 4a). While SKOV3 were resistant to TRAIL, CtBP depletion even sensitized SKOV3 to TRAIL, albeit to a lesser extent (Fig. 4a). In addition, caspase 8 inhibitor, Z-IETD-FMK blocked cell death induced by TRAIL (Fig. 4b). We knocked down DR4/5 along with CtBP1/2 in OVCA429 cells (Fig. 4c), and observed that TRAIL-induced cell death was markedly diminished by siDR5. However, siDR4 did not rescue cell death (Fig. 4c) indicating the predominant role of DR5 in transducing the TRAIL signal in these cells. Collectively, these

results supported the notion that CtBP1/2 control cellular susceptibility to TRAIL via DR4 and DR5 in a cell type dependent manner.

CtBP loss induces senescence. We also found that OVCA429 cells receiving CtBP1/2 shRNA gradually lost proliferation ability and acquired flat and large cell morphology (Fig. 5a). Also, the cytoplasm became positive for β-galactosidase staining (Fig. 5b). These data suggested that CtBP1/2 loss caused senescence in these cells.

CtBP binds to DR4/5 promoters. Considering that CtBP1/2 are transcriptional co-repressors, we sought to perform CHIP assay to determine whether CtBP1/2 bound chromatin at the regulatory regions of DR4/5 genes. Since CtBP1 and CtBP2 share about 78% identical amino acid sequence, we tested the specificity of commercially available rabbit antibodies against CtBP1 (#8684, Cell Signaling Technology) and CtBP2 (#13256, Cell Signaling Technology) on cell lysates prepared from OVSAHO, OVSAHO CRISPR-CtBP1, HCT116 and HCT116 CRISPR-CtBP2 cells. CtBP1 antibody clearly recognized CtBP1 migrating at 48 kDa in OVSAHO cells, but not in OVSAHO CtBP1 KO cells. In addition, immunoprecipitation with anti-CtBP1 antibody pulled down CtBP1 and CtBP2 only in CtBP1 expressing OVSAHO cells, not in CtBP1 KO cells. Accordingly, CtBP2 antibody only recognized CtBP2， not CtBP1 (Fig. 6a). Having confirmed that CtBP1 antibody does not cross-react with CtBP2 and vice versa, we utilized these antibodies and further confirmed that CtBP1 and CtBP2 physically interact with each other in OVCA429 (Fig. 6b), and in OVSAHO as well (Fig. 6a). These antibodies were further validated for CHIP IP assay (Fig. 6c). We utilized these antibodies to pull down CtBP1/2 and tested the CtBP1/2 occupancy at DR4/5 promoters. We designed 6 pairs of qPCR primers, which covered the whole promoter region, as illustrated in Fig. 6d and 6f. Regular CHIP assay was performed and showed extremely weak signal (Fig. 6e and g). Considering that CtBP might form a regulatory complex with other factors, we included the protein-protein crosslinking step using DSG in the two-step CHIP. Under this condition, we proved that both CtBP1 and CtBP2 bind at DR4/5 promoters (Fig. 6d and g), presumably through recruitment by DNA binding transcription factors.

CtBP1 and CtBP2 functionally coordinate to repress DR4/5. We have showed that both CtBP1 and CtBP2 are present at the DR4/5 promoters, and that individually knocking down one or another leads to upregulation of DR4/5. These findings raise a question whether CtBP1/2 depend on each other to repress DR4/5. To address this possibility, we compared CtBP1/2 double knockdown OVCA429 cells against either CtBP1 or CtBP2 single knock down cells and control cells. Western blotting and qPCR showed that DR4/5 proteins as well as mRNAs in CtBP1/2 double knockdown cells were induced to the similar level as that in single CtBP1 or CtBP2 knockdown cells (Fig. 7a and b), indicating that CtBP1 and CtBP2 might work as a functionally identical unit to repress DR4/5. We performed CHIP assay to test whether deficiency of CtBP1 or CtBP2 will dissociate the other one from DR4/5 promoter regions. As shown in Fig.7 c and d, knocking down CtBP1 or CtBP2 did not exclude the occupancy of other one from the promoters. CHIP-reCHIP assay revealed that CtBP1 and CtBP2

present at the same regions of DR4/5 promoters (Fig. 7e). These results supported that CtBP1 and CtBP2 coordinate with each other on the regulation of DR4/5.

Table 1. Summary of the effect CtBP loss on cell fate

	KURAMOCHI	OVSAHO	SKOV3	OVCA429
Apoptosis	+++	–	–	–
Growth Inhibition	+++	–	+	+
Senescence	–	–	+	+++
Response to TRAIL	–	–	+	+++
DR4 status*	+++	–	+++	+++
DR5 status*	+	+	+	+++
p53 status	mt	mt	mt	wt

*As determined by Western blot.

Aim 2. Characterize the role of CtBP overexpression in human ovarian carcinogenesis and validate pharmacologic inhibition of CtBP in HGSOC orthotopic xenografts.

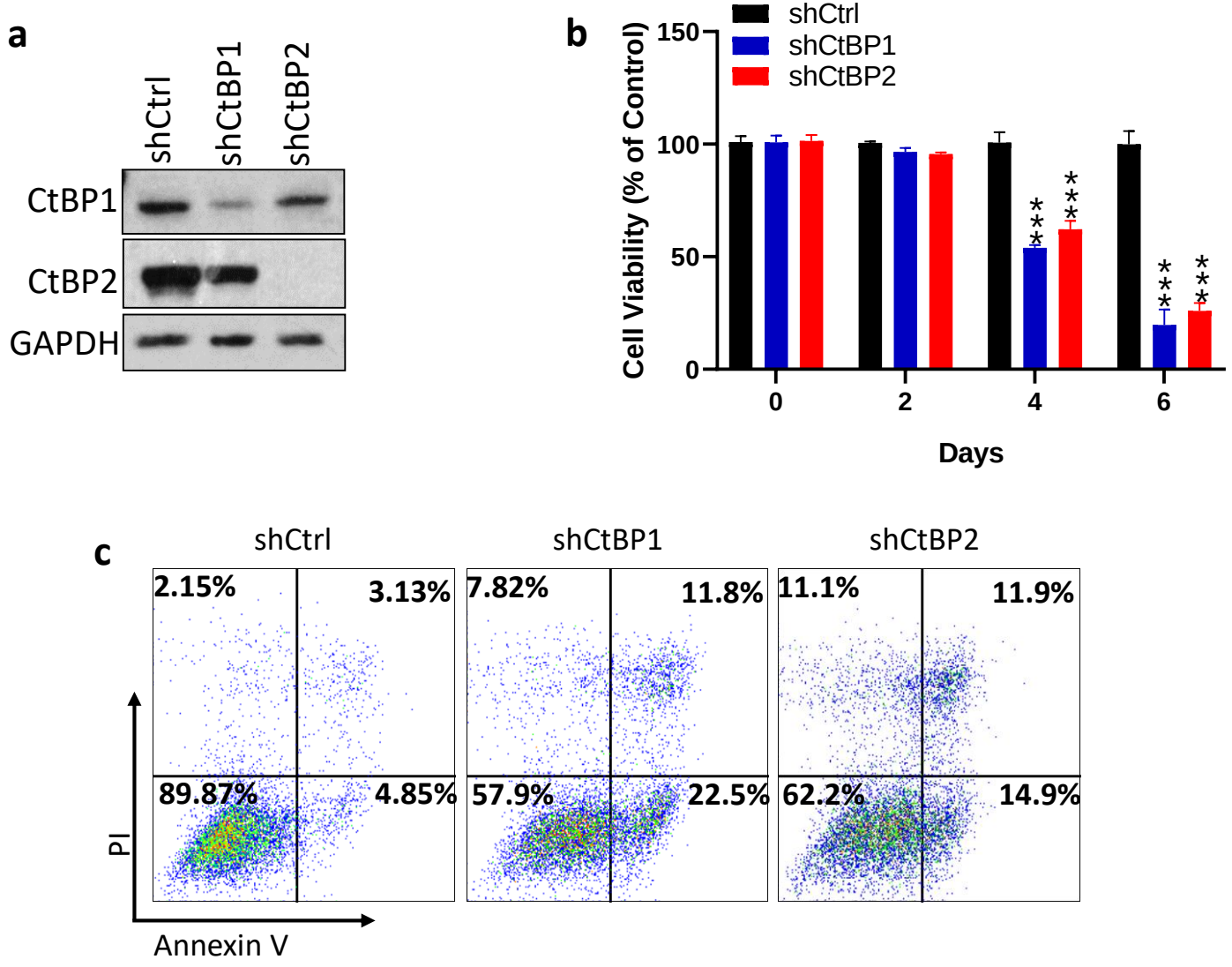
No results are yet available from this Aim.

Aim 3. Develop a fallopian tube-specific transgenic mouse model for CtBP-driven ovarian carcinogenesis.

To create a line of mice that overexpress CtBP2 specifically in fallopian tube epithelial cells, we originally proposed to create a new transgenic mouse line expressing CtBP2 under the control of the ubiquitin promoter, but interrupted by a lox-STOP-lox (LSL) cassette that will be deleted specifically in FTE upon interbreeding of this line to the existing Pax8-Cre line. However, we have slightly modified this approach to place CtBP2 downstream of the strong ubiquitous CAG promoter followed by an LSL. Further, we are introducing the transgene in the ROSA26 locus using a CRISPR-Cas9 approach, which will guarantee that only a single copy of the transgene will be integrated. This will preclude the possibility that integration of multiple transgene copies in a tandem array will result in complicated deletion products following Cre expression. To make these mice, we have established a sizeable breeding colony of Ai14 mice (JAX stock #007914), which carry a CAG-LSL-tdTomato reporter gene knocked into the ROSA26 locus, and these are being used as the source of fertilized eggs for the CRISPR-mediated targeting, with the plan to swap out the tdTomato gene for the CtBP2 gene. We have designed guide RNAs (using the IDT Alt-R system) that target both ends of the tdTomato gene in Ai14, and a megamer (long single strand DNA repair template) (Genewiz) encoding the CtBP2 coding region, flanked by homology arms matching the Ai14 sequences on either side of the excised tdTomato gene. The

VCU TG/KO Core has performed three injection days (on 4/24/19, 5/3/19 and 5/10/19), and pups from the first injection day have recently been born. If we do not obtain correctly targeted mice after several attempts, we will revert to the original plan of creating a standard transgenic mouse.

Fig. 1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100
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2
4
6
Days
***
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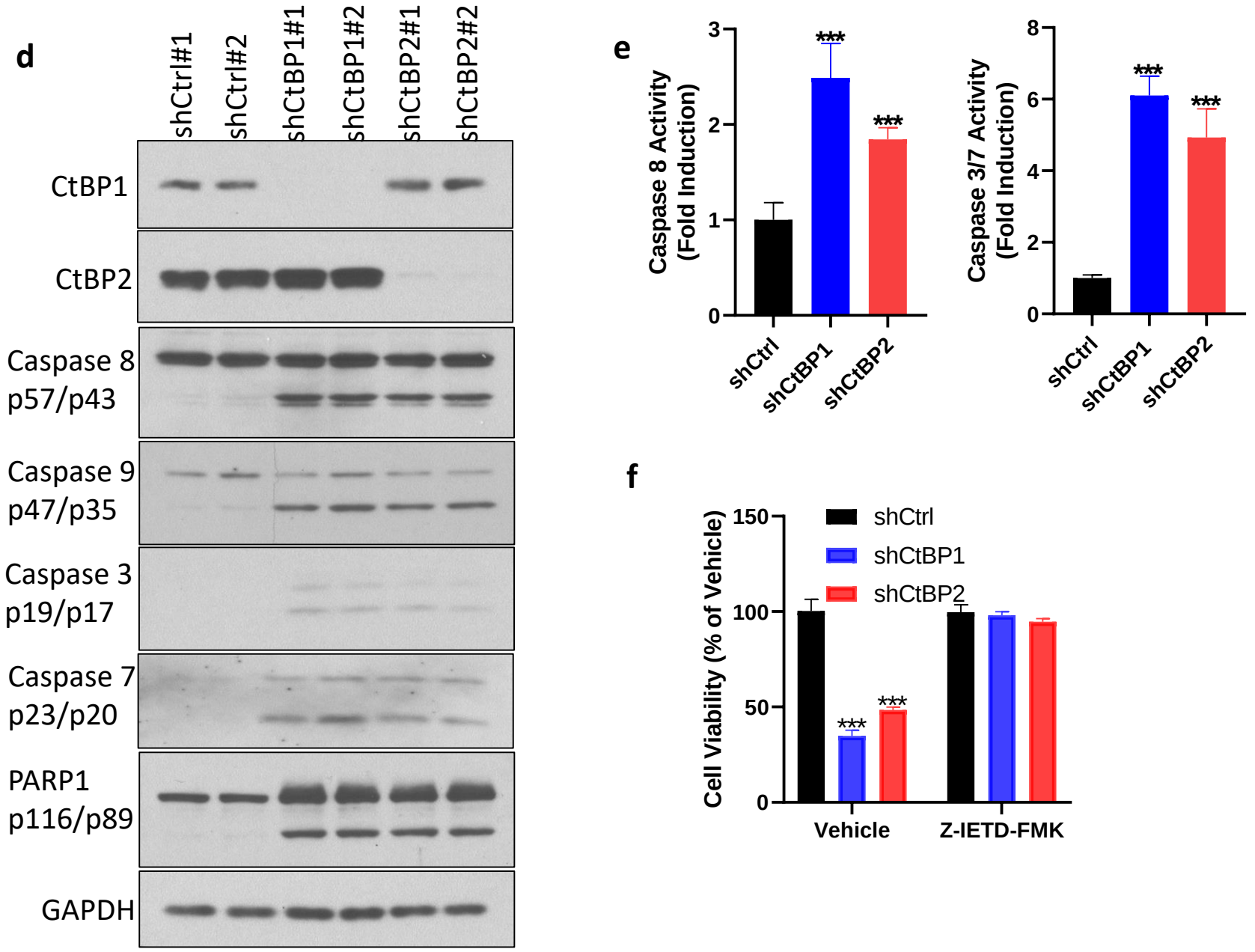
PI
Annexin V

Fig. 1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Cell Viability (% of Vehicle)
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Vehicle
Z-IETD-FMK

Fig. 1

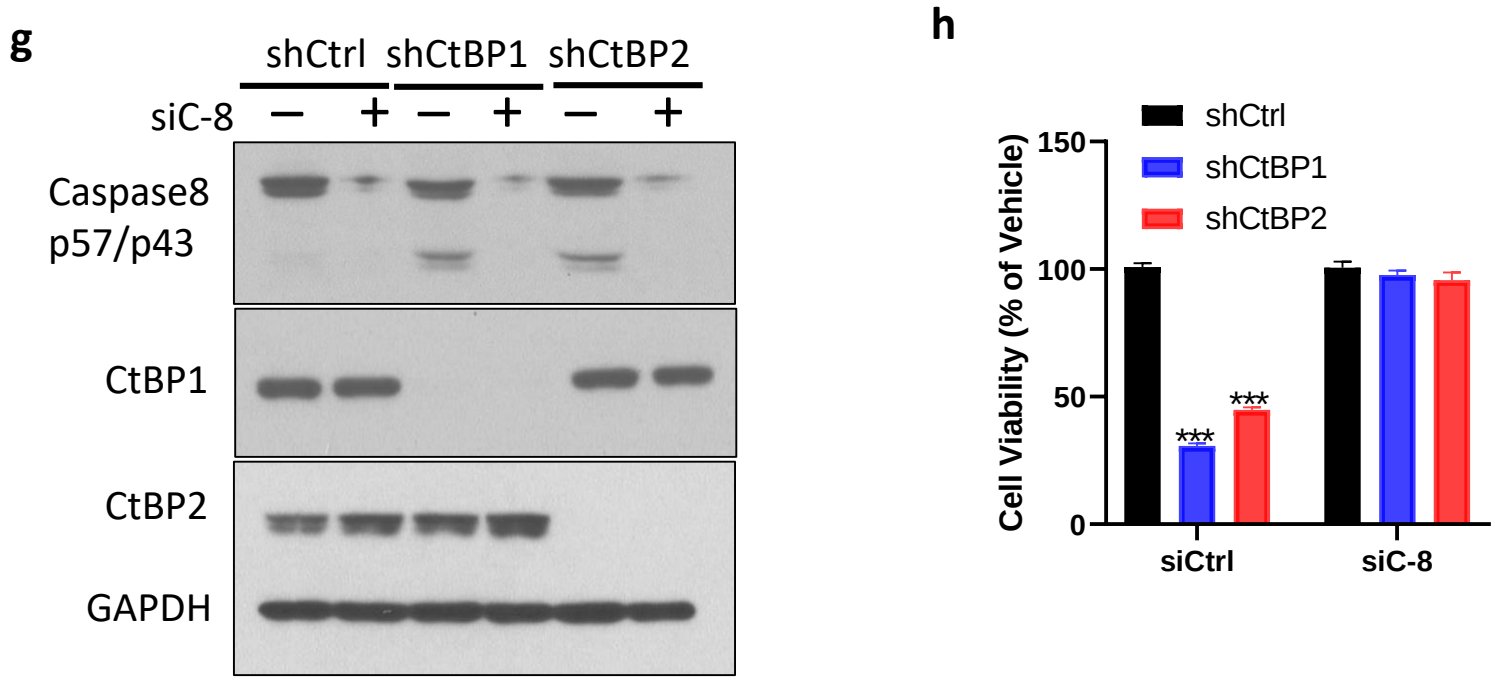


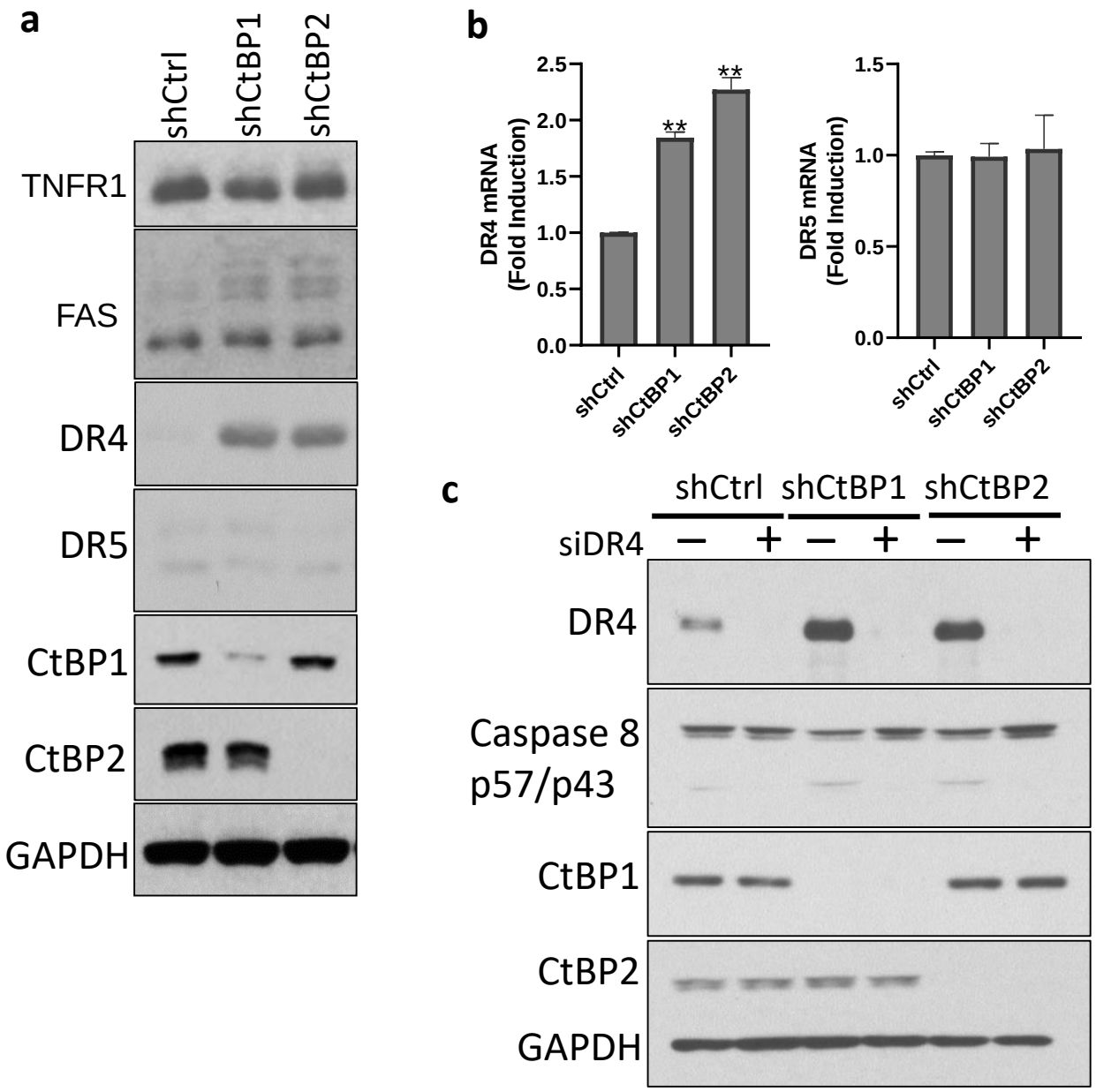
Fig. 1. CtBP loss triggers caspase 8 dependent apoptosis.

a-d. KURAMOCHI cells were infected with indicated shRNAs. CtBP RNAi efficiency was analyzed by Western blotting (a). Cell viability was monitored using a CCK-8 kit. ***p<0.001 as compared with shCtrl group (b). At 4 days post-infection, cell apoptosis was analyzed by the Annexin V and PI staining (c); cleaved caspases and PARP1 were analyzed by Western blotting (d) and caspase 8 and caspase 3/7 activities were monitored (e), ***p<0.001 as compared with shCtrl group.

f. KURAMOCHI cells, infected as indicated, were treated with vehicle or Z-IETD-FMK (20μM) for 6 days, cell viability was monitored, ***p<0.001 as compared with shCtrl group.

g & h. KURAMOCHI cells were transfected with siRNAs followed by infection with shRNAs. Cleaved caspase 8 were examined by Western blotting (g), cell viability was analyzed (h) at 6 days after transfection/infection, ***p<0.001 as compared with shCtrl group.

Fig. 2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DR5
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CtBP2
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2.0
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1.0
0.5
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CtBP2
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Fig. 2

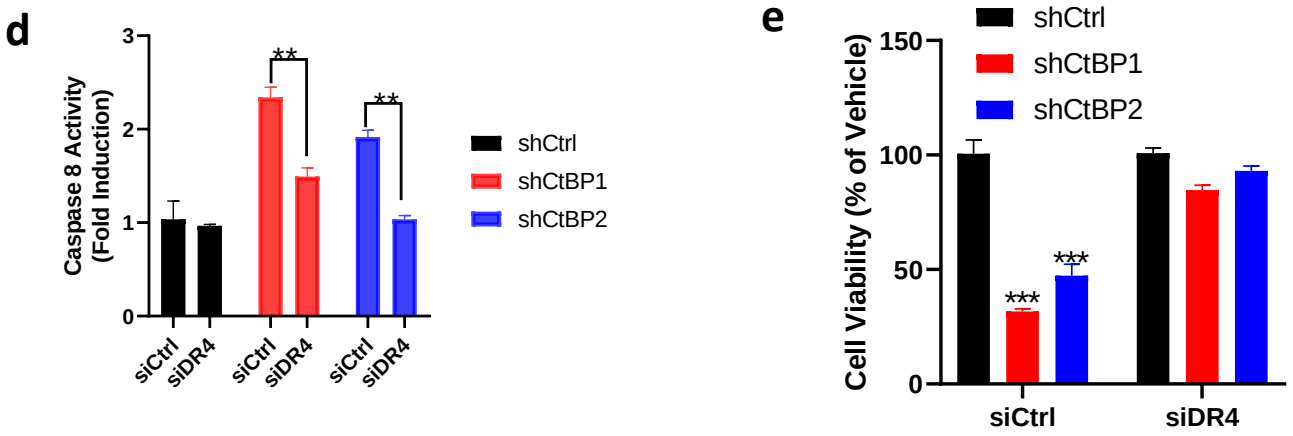
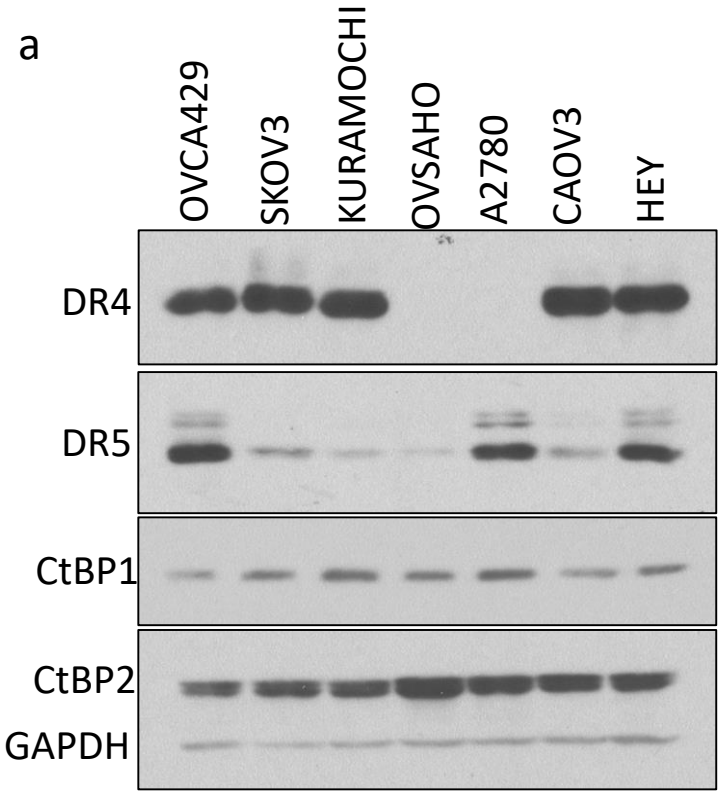


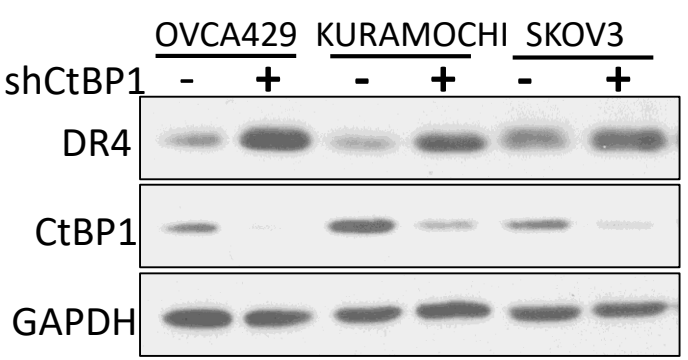
Fig. 2. CtBP loss activates caspase 8 via death receptor 4.

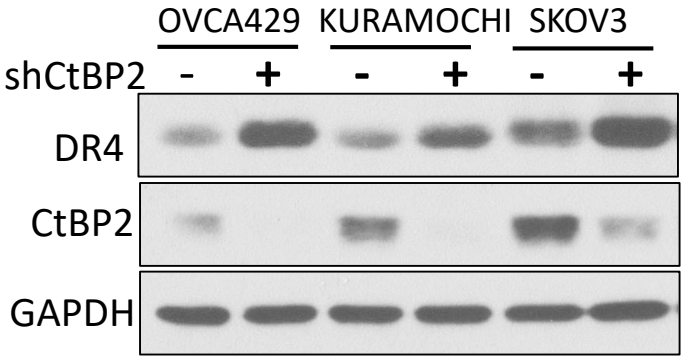
a & b. KURAMOCHI cells were infected with indicated shRNAs. Cell death receptors were examined by Western blotting (a), DR4 and DR5 mRNA levels were examined by qPCR (b), **p<0.01 as compared with shCtrl group.

c-e. KURAMOCHI cells were treated with indicated siRNAs along with shRNAs. Western blotting showed cleaved caspase 8, and knockdown efficiency of DR4, CtBP1/2 (c); caspase 8 activity was examined (d), **p<0.01 as compared with shCtrl group; and cell viability was monitored at 6 days post transfrection/infection (e), ***p<0.001 as compared with shCtrl group.

Fig. 3


a
OVCA429
SKOV3
KURAMOCHI
OVSAHO
A2780
CAOV3
HEY
DR4
DR5
CtBP1
CtBP2
GAPDH


b
OVCA429
KURAMOCHI
SKOV3
shCtBP1
- + - + - +
DR4
CtBP1
GAPDH


c
OVCA429
KURAMOCHI
SKOV3
shCtBP2
- + - + - +
DR4
CtBP2
GAPDH

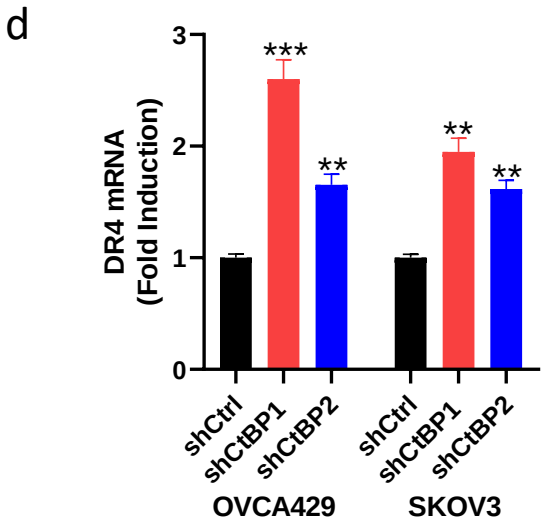

d
DR4 mRNA (Fold Induction)
3
2
1
0
***
**
**
**
shCtrl
shCtBP1
shCtBP2
shCtrl
shCtBP1
shCtBP2
OVCA429
SKOV3

Fig. 3

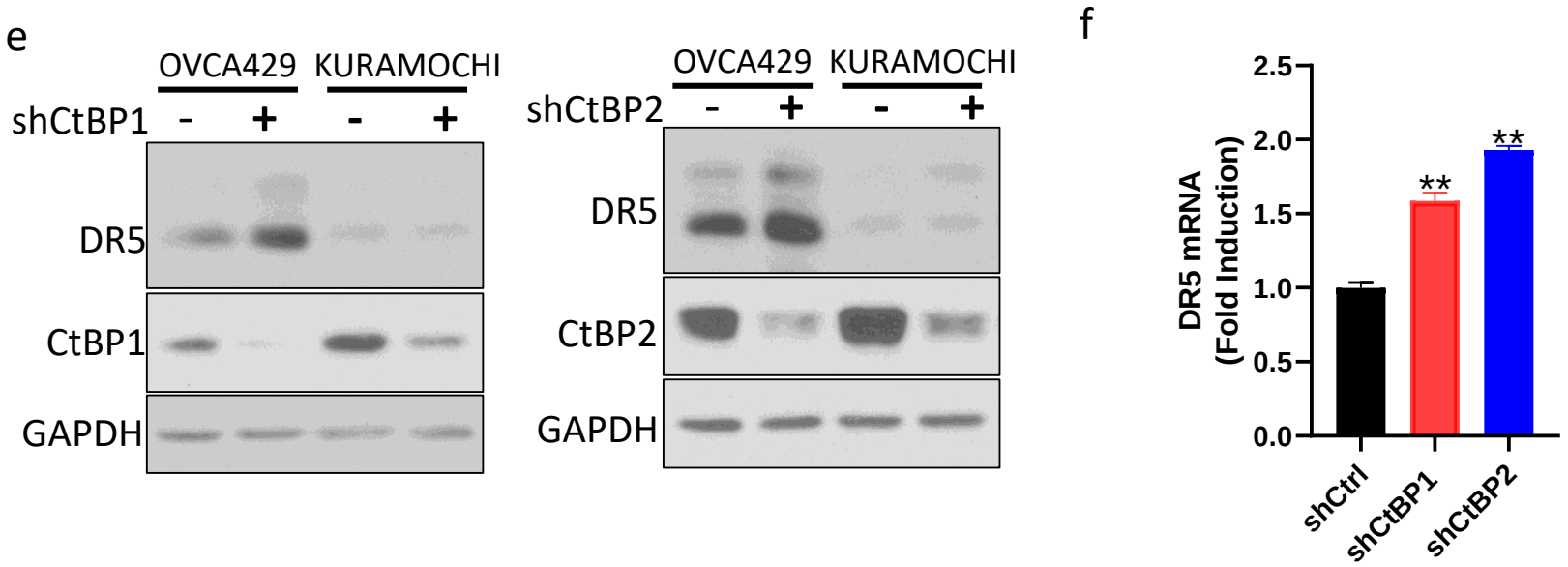


Fig. 3. CtBP represses DR4 and DR5 depending on cell context.

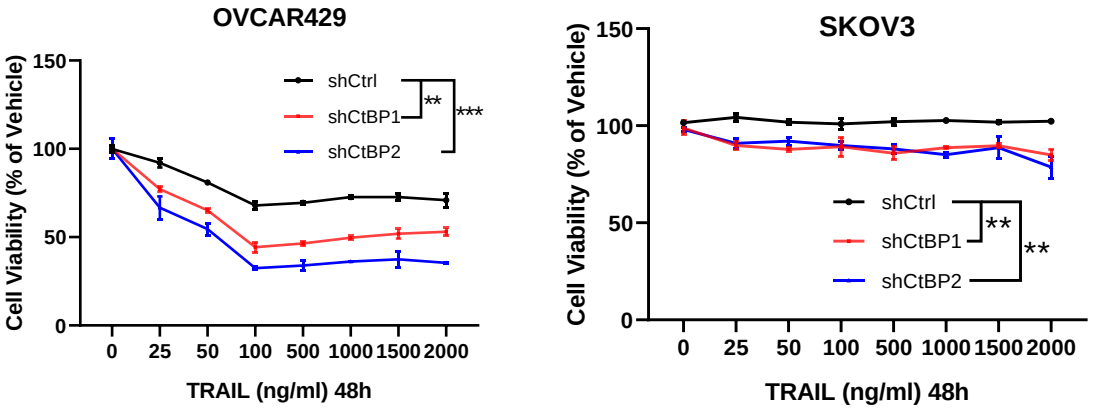
a. DR4 and DR5 protein levels were examined by Western blotting in indicated HGSOC cell lines.

b - d. OVCA429, KURAMOCHI and SKOV3 were infected with indicated shRNAs. DR4 protein was examined by Western blotting (b, c), mRNA level of DR4 was analyzed by qPCR (d), **p<0.01,***p<0.001 as compared with shCtrl group.

e & f. OVCA429 and KURAMOCHI cells were infected as indicated, DR5 protein level was examined by Western blotting (e), DR5 mRNA level in OVCA429 cells was analyzed by qPCR (f), **p<0.001 as compared with shCtrl group.

Fig. 4

a


OVCAR429
Cell Viability (% of Vehicle)
150
100
50
0
shCtrl
shCtBP1
shCtBP2
**
***
0 25 50 100 500 1000 1500 2000
TRAIL (ng/ml) 48h
SKOV3
Cell Viability (% of Vehicle)
150
100
50
0
shCtrl
shCtBP1
shCtBP2
**
**
0 25 50 100 500 1000 1500 2000
TRAIL (ng/ml) 48h

b

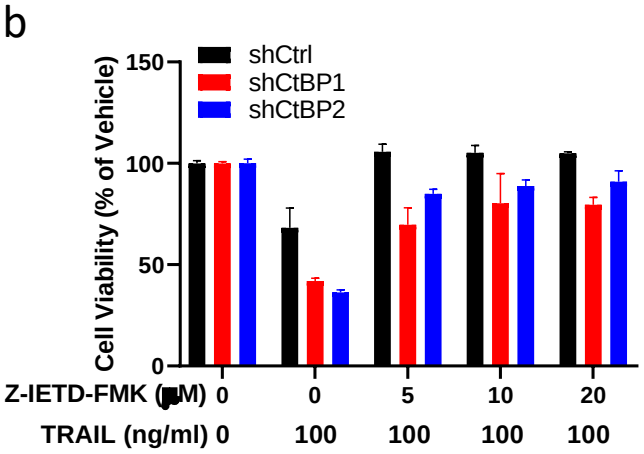
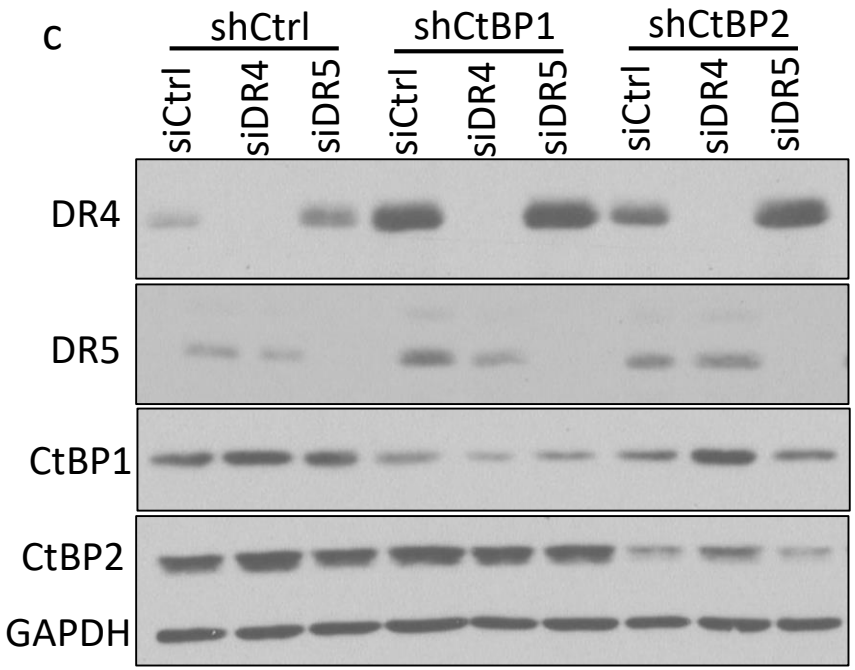

Cell Viability (% of Vehicle)
150
100
50
0
shCtrl
shCtBP1
shCtBP2
Z-IETD-FMK (μM) 0 0 5 10 20
TRAIL (ng/ml) 0 100 100 100 100

Fig. 4



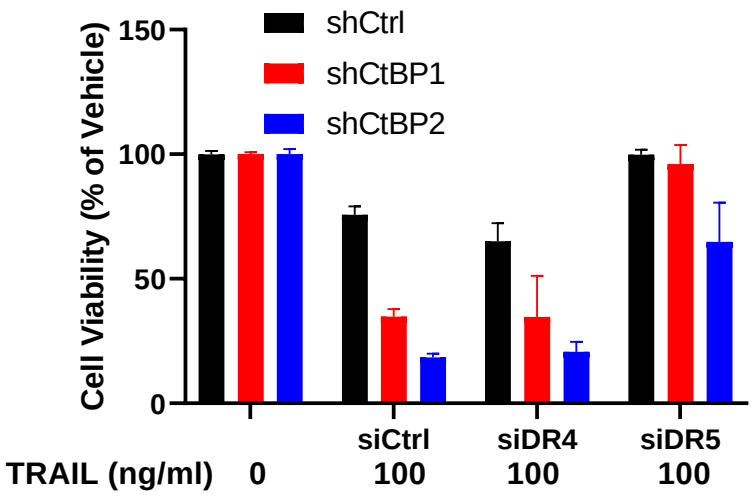


Fig. 4. CtBP depletion sensitizes cells to TRAIL treatment.

a. OVCA429 and SKOV3, received indicated shRNAs infection, were treated with vehicle of TRAIL for 48 hours, cell death was assessed using a CCK-8 kit, **p<0.01, ***p<0.001 as compared with shCtrl group.

b. OVCA429 cells, infected as indicated, were treated with vehicle or a combination of Z-IETD-FMK and TRAIL for 48 hours, cell viability was assessed.

c. OVCA429 cells were transfected with siRNAs overnight, then infected with shRNAs. Right panel: Western blotting showing the knockdown efficiency of indicated protein; Left panel: Cells were treated with TRAIl for 48 hours, cell viability was assessed.

Fig. 5

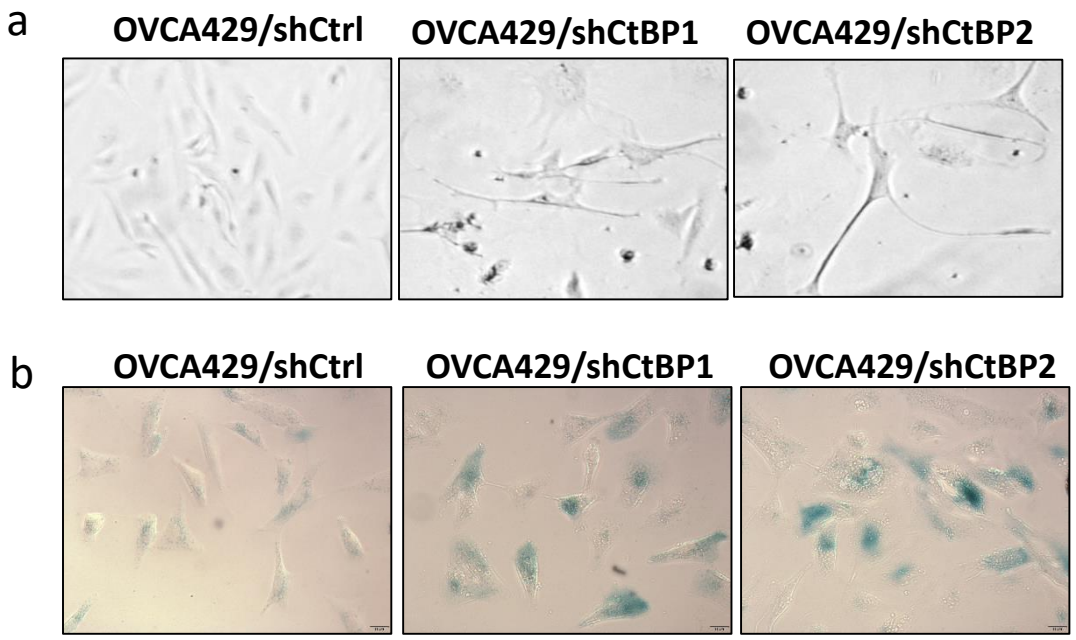


Fig. 5. CtBP loss induces senescence.

a. OVCA429 were infected with indicated shRNAs infection, and were cultured sparsely for 6 days. Cell morphologic changes were monitored by light microscopy.

b. OVCA429 cells were treated as in a. β-Galactosidase staining was performed.

Fig. 6

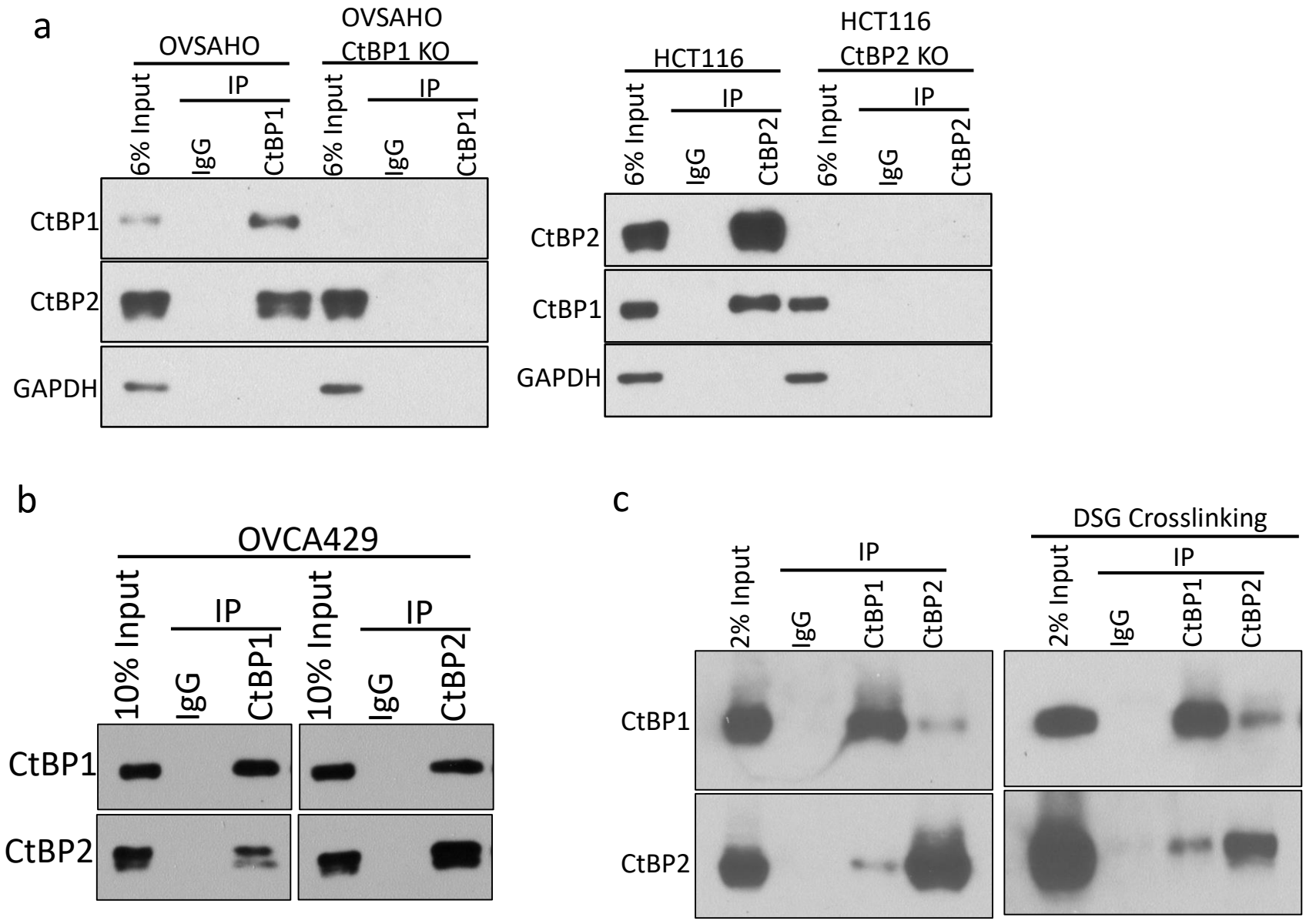
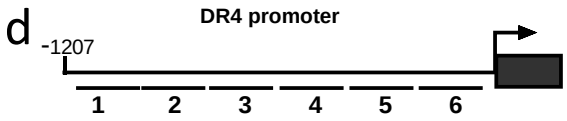
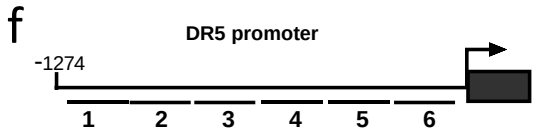
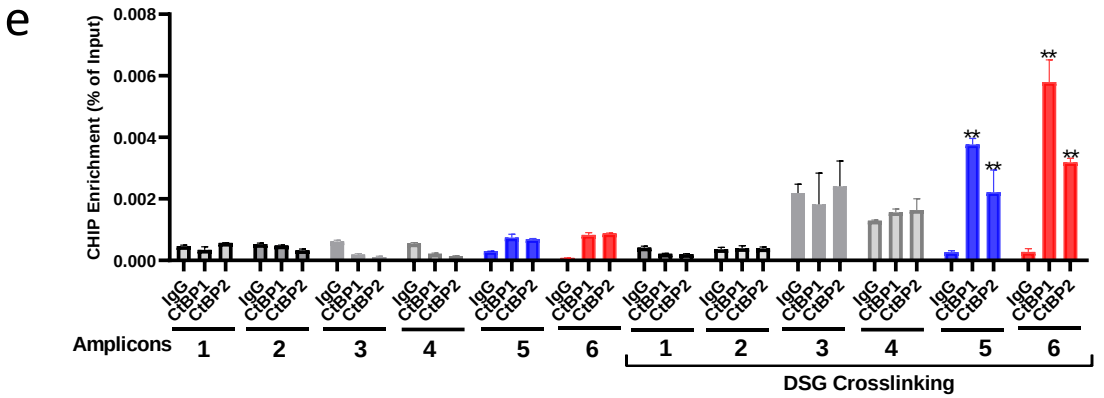

a
OVSAHO
OVSAHO CtBP1 KO
6% Input
IP
IgG
CtBP1
CtBP1
CtBP2
GAPDH
HCT116
HCT116 CtBP2 KO
6% Input
IP
IgG
CtBP2
CtBP2
CtBP1
GAPDH
b
OVCA429
10% Input
IP
IgG
CtBP1
CtBP2
c
DSG Crosslinking
2% Input
IP
IgG
CtBP1
CtBP2

Fig. 6







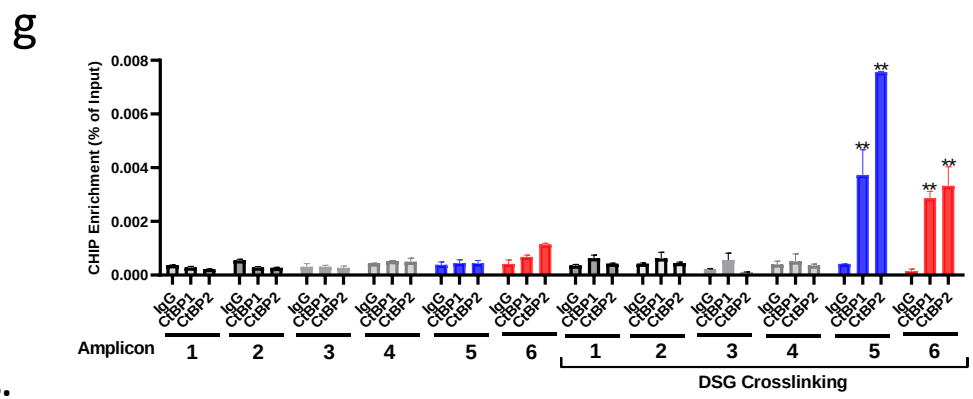


Fig. 6. CtBP binds to DR4/5 promoters.

a. Validating the specificity of CtBP antibodies. IP-western was performed in OVSHO, OVSAHO/CtBP1 KO, HCT116, and HCT116/CtBP2 KO cells.

b. IP-western showing the interaction between CtBP1 and CtBP2.

c. Validating CtBP antibodies used for CHIP assay.

d. Cartoon showing the DR4 promoter. Black bars labeled 1-6 represent the location of PCR amplicons used in CHIP experiments.

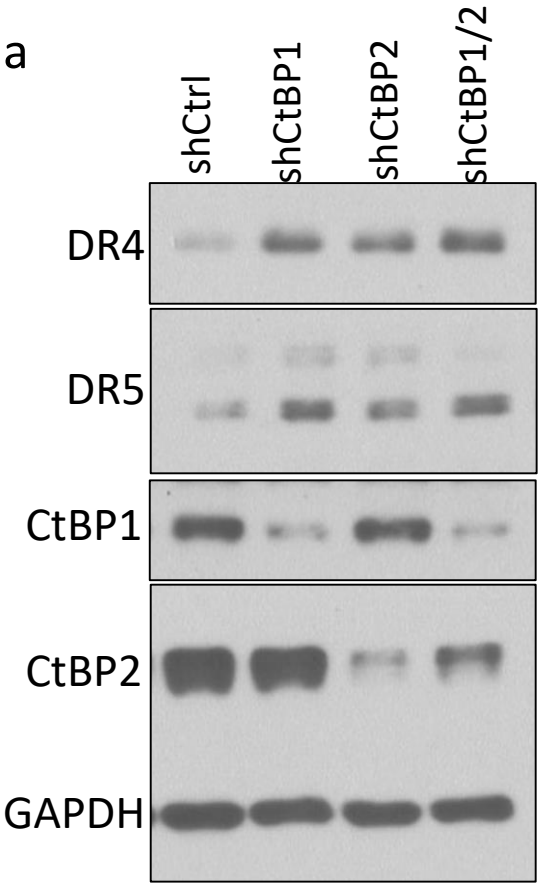
e. CtBP1/2 occupancy at DR4 promoter in OVCA429 cells. CHIP was performed with OVCA429 cells, PCR amplicons from (d) were used for CHIP, **p<0.01 as compared with normal IgG group.

f. Cartoon showing the DR5 promoter. Black bars labeled 1-6 represent the location of PCR amplicons used in CHIP experiments.

g. CtBP1/2 occupancy at DR5 promoter in OVCA429 cells. CHIP was performed with OVCA429 cells, PCR amplicons from (f) were used for CHIP, **p<0.01 as compared with normal IgG group.

Fig. 7

a


shCtrl
shCtBP1
shCtBP2
shCtBP1/2
DR4
DR5
CtBP1
CtBP2
GAPDH

b

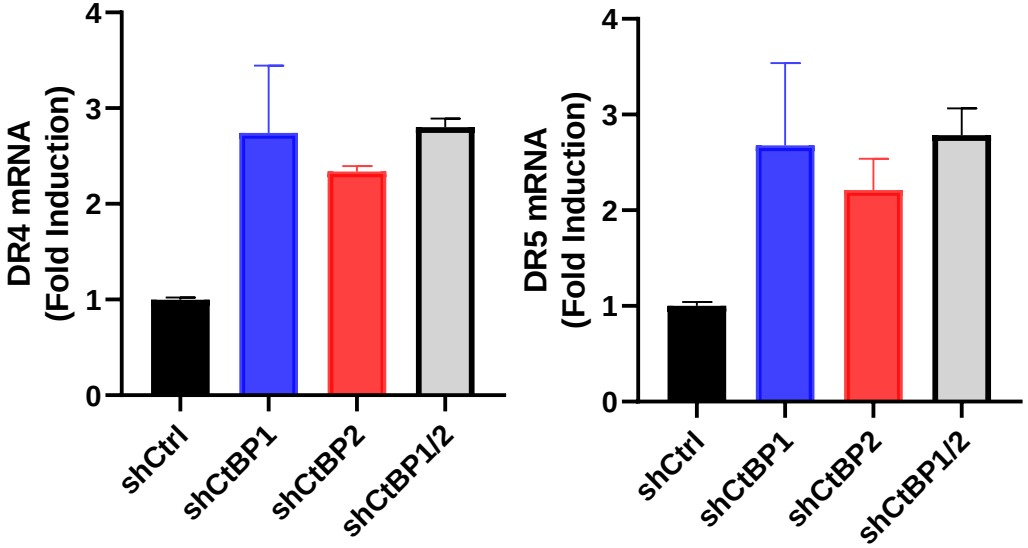
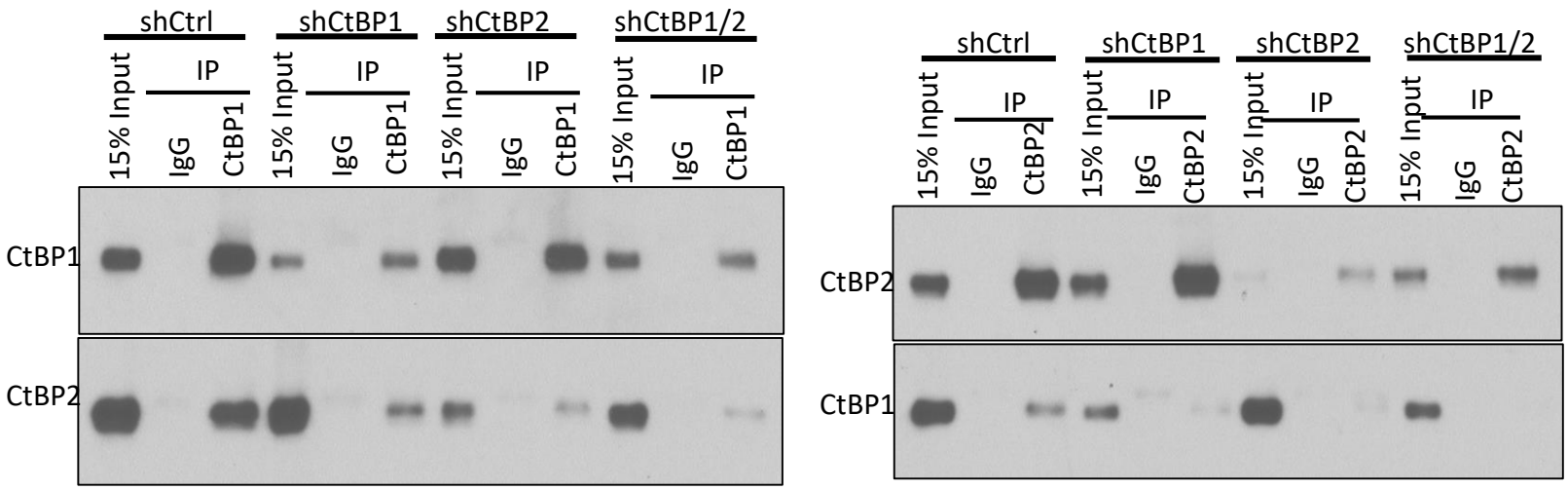

DR4 mRNA (Fold Induction)
0
1
2
3
4
shCtrl
shCtBP1
shCtBP2
shCtBP1/2
DR5 mRNA (Fold Induction)
0
1
2
3
4
shCtrl
shCtBP1
shCtBP2
shCtBP1/2

Fig. 7

c


shCtrl
shCtBP1
shCtBP2
shCtBP1/2
15% Input
IP
IgG
CtBP1
CtBP2
CtBP1
CtBP2

d

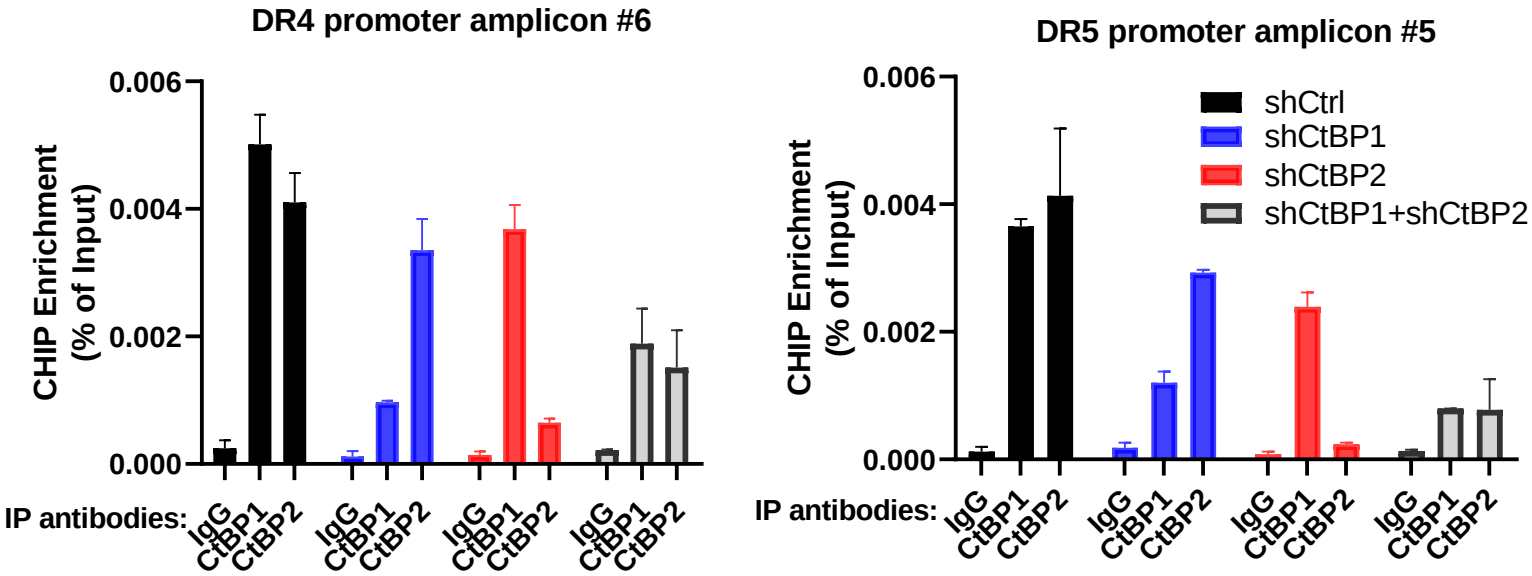

DR4 promoter amplicon #6
DR5 promoter amplicon #5
CHIP Enrichment (% of Input)
0.006
0.004
0.002
0.000
shCtrl
shCtBP1
shCtBP2
shCtBP1+shCtBP2
IP antibodies:
IgG
CtBP1
CtBP2

Fig. 7

e

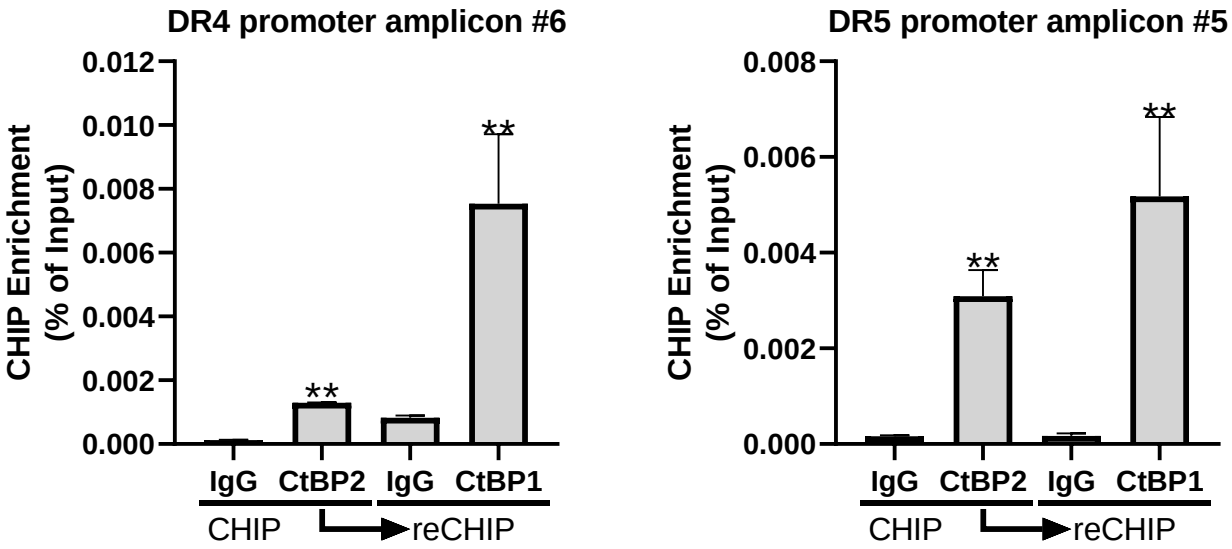


Fig. 7. CtBP1 and CtBP2 coordinate to repress DR4/5.

a. Western blotting showing the protein abundance in OVCA429 cells received indicated shRNAs.

b. Fold induction of DR4/5 mRNAs in OVCA429 cells received indicated shRNAs was assessed by qPCR.

c. Western blotting showing the CtBP abundance in CHIP experiments with OVCA429 cells when infected with indicated shRNAs.

d. CtBP1/2 occupancy at DR4/5 promoters in OVCA429 cells. CHIP was performed with OVCA429 cells when CtBP1/2 was knocked down. Indicated PCR amplicons were used for CHIP.

e. CHIP-reCHIP assay showing the CtBP1/2 occupancy at the same region of DR4/5 promotors. CHIP-reCHIP was performed with OVCA429 cells, **$p<0.01$ as compared with normal IgG group.

Fig. 8

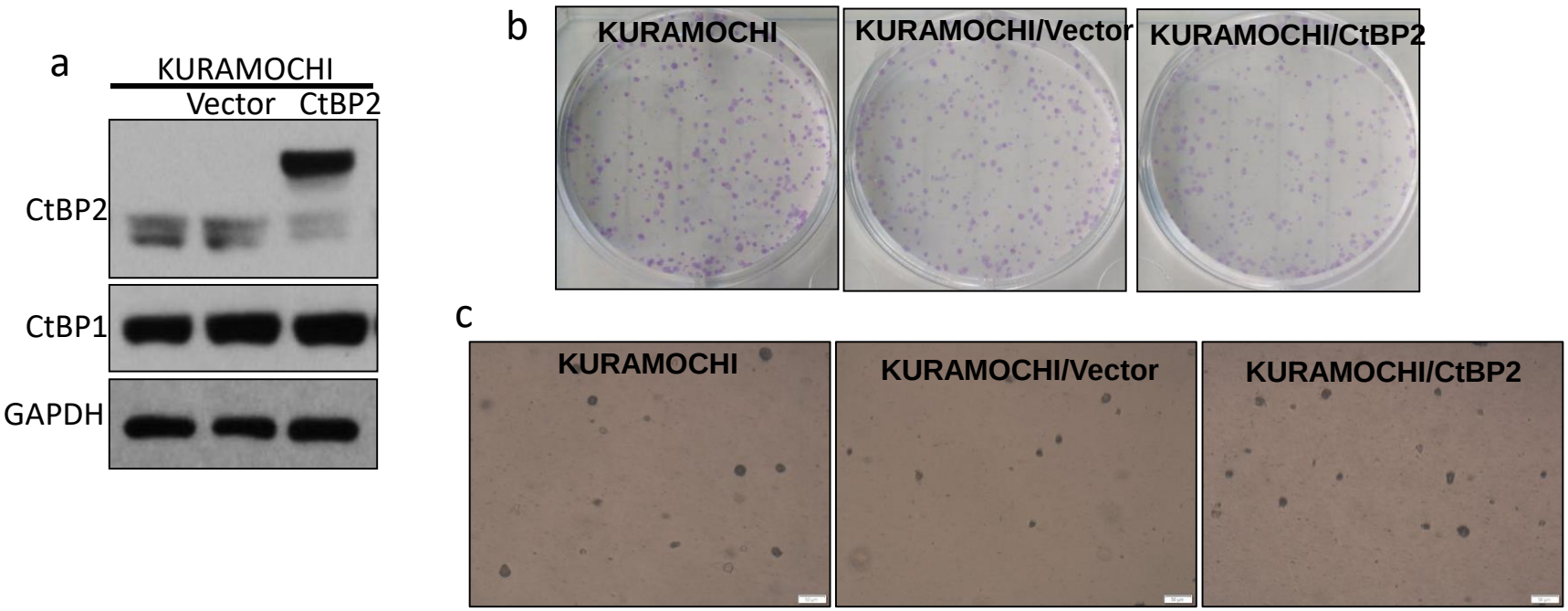


Fig. 8. CtBP2 overexpressed KURAMUCHI cells do not exhibit growth advantage.

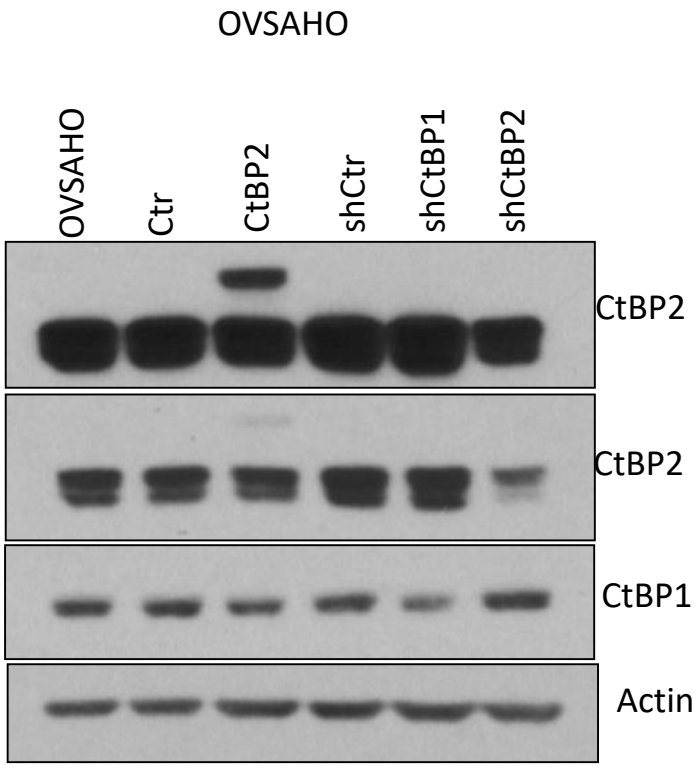
a. Parental, vector-expressing and CtBP2-expressing KURAMOCHI cells were examined by Western blotting.

b. KUAMUCHI cells were sparsely seeded in 6-well-plate and cultured for 14 days, Crystal violet staining were performed.

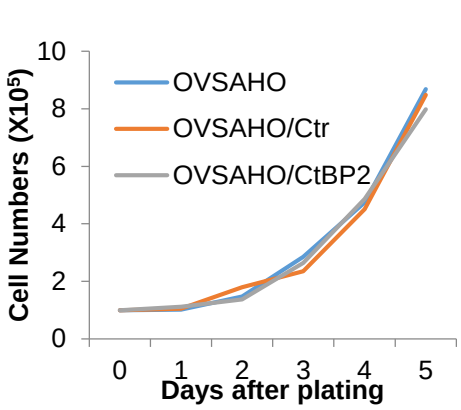
c. KURAMUCHI cells were grown in 3D culture medium for 21 days, colony formation was monitored by microscopy.

Fig. 9

a


OVSAHO
OVSAHO
Ctr
CtBP2
shCtr
shCtBP1
shCtBP2
CtBP2
CtBP2
CtBP1
Actin

b


Cell Numbers (X10⁵)
10
8
6
4
2
0
0
1
2
3
4
5
Days after plating
OVSAHO
OVSAHO/Ctr
OVSAHO/CtBP2

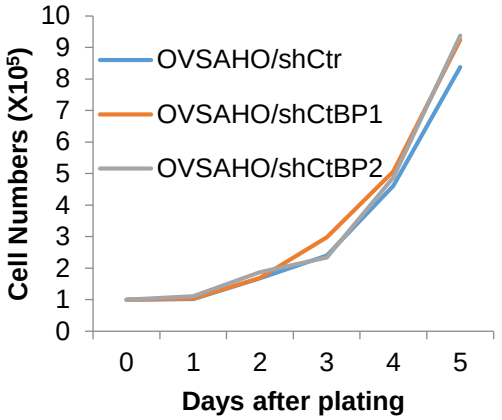
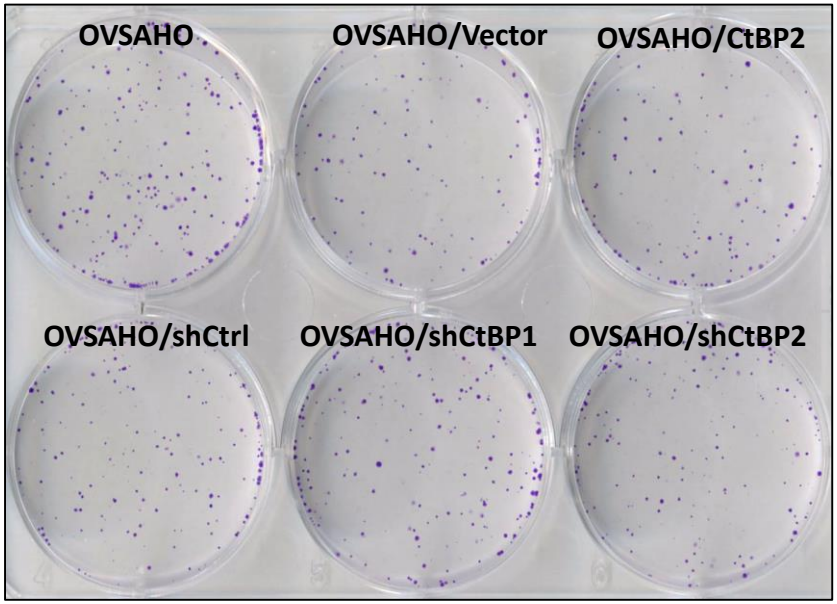

Cell Numbers (X10⁵)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Days after plating
OVSAHO/shCtr
OVSAHO/shCtBP1
OVSAHO/shCtBP2

Fig. 9



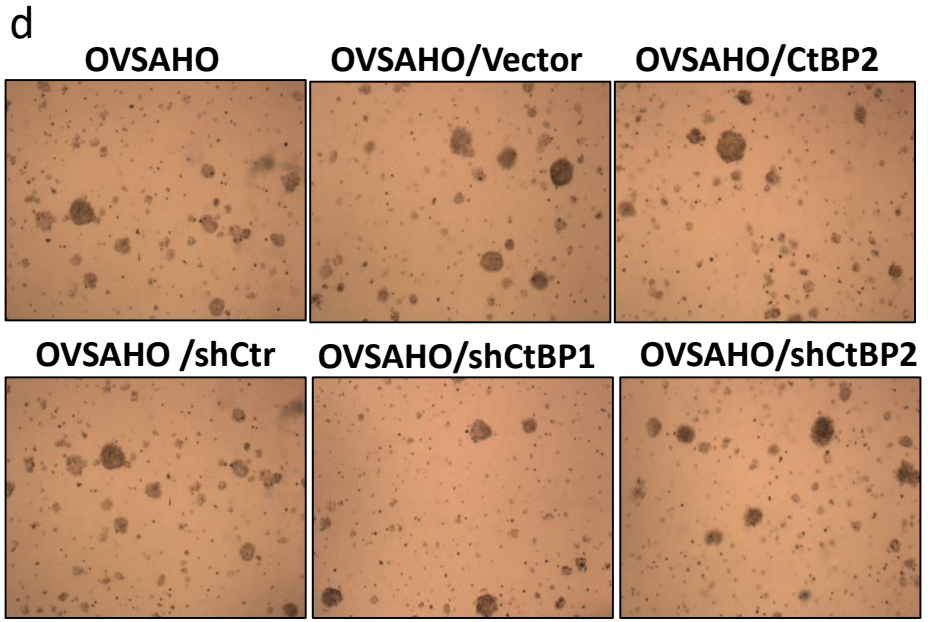


Fig. 9. Genetically alteration of CtBP expression in OVSAHO cells does not perturb cell growth.

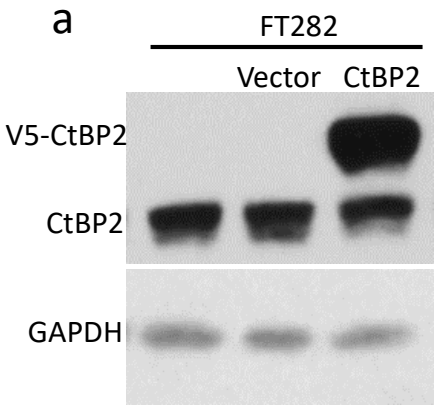
a. CtBP was either overexpressed or knocked down in OVSAHO cells. Western blotting showed the expression of CtBP.

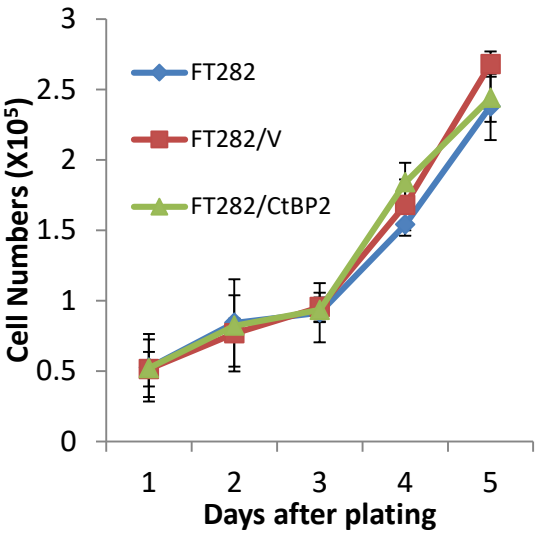
b. Indicated cell growth was monitored over 5 days.

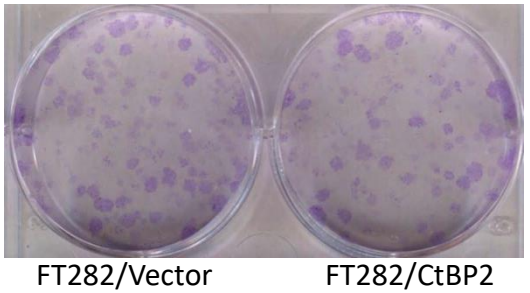
c. Cells were sparsely seeded in 6-well-plate and cultured for 14 days, Crystal violet staining were performed.

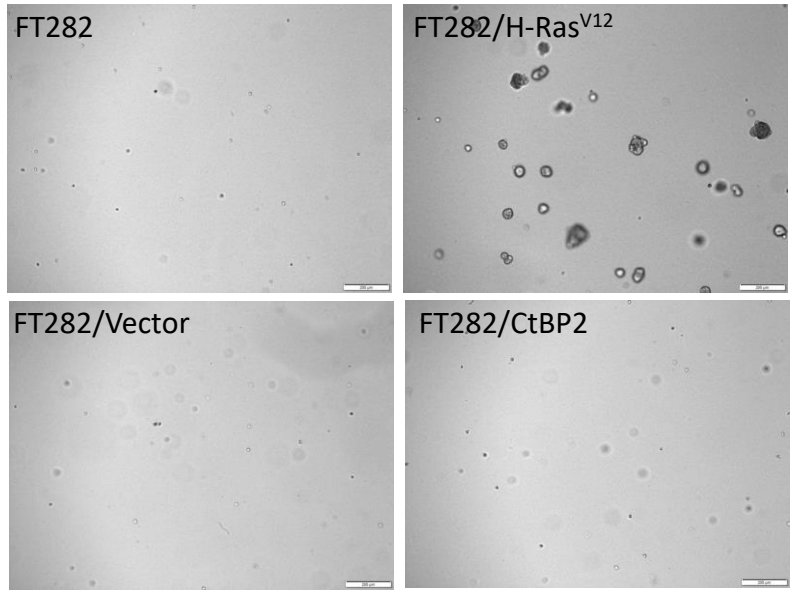
c. Cells were grown in 3D culture medium for 21 days, colony formation was monitored by microscopy.

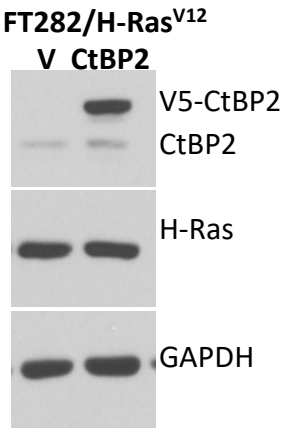
Fig. 10


a
FT282
Vector
CtBP2
V5-CtBP2
CtBP2
GAPDH


b
FT282
FT282/V
FT282/CtBP2
Cell Numbers (X10⁵)
Days after plating
0
0.5
1
1.5
2
2.5
3
1
2
3
4
5


c
FT282/Vector
FT282/CtBP2


d
FT282
FT282/H-RasV12
FT282/Vector
FT282/CtBP2


e
FT282/H-RasV12
V
CtBP2
V5-CtBP2
CtBP2
H-Ras
GAPDH

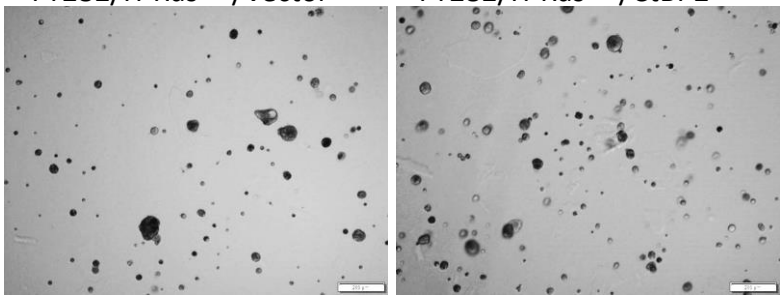
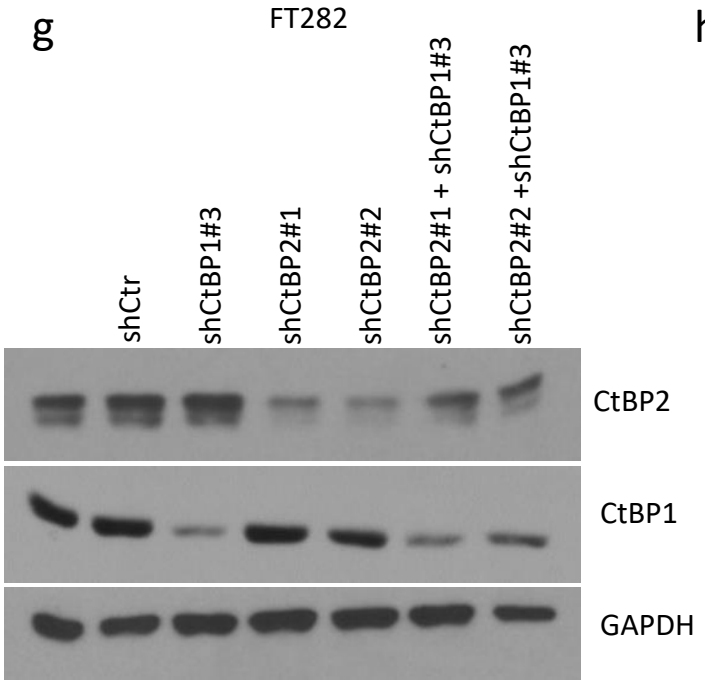

f
FT282/H-RasV12/Vector
FT282/H-RasV12/CtBP2

Fig. 10



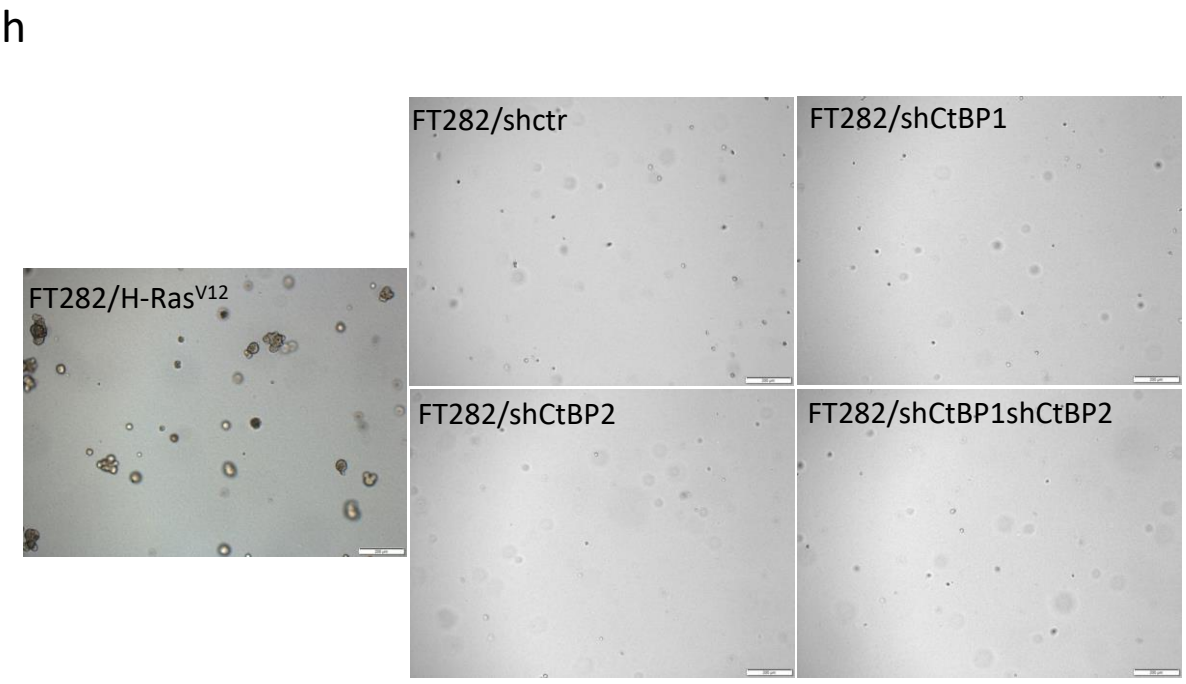


Fig. 10. Effect of CtBP on the proliferation of fallopian tube epithelial cells.

a-d. Immortal FT282 cells were infected with lenti-vector or lenti-V5-tagged CtBP2, ectopic CtBP2 expression was validated by Western blotting (a). Cell proliferation (b) and colony formation (c) were assessed. 3D cell culture with methylcellulose was performed, the transformed FT282/H-Ras^{V12} cell line was used as a positive control. Scale bar= 200μm.

e and f. Transformed FT282/H-Ras^{V12} cells infected as above were analyzed by Western blotting to confirm the over-expressed CtBP2 (e), colony formation was assessed by 3D cell culture (f). Scale bar= 200μm.

g and h. FT282 cells were infected with lenti-shRNA for CtBP1/2. The efficiency of CtBP1/2 RNAi was assessed by Western blotting (g), colony formation (h) was examined by 3D cell culture, FT282/H-Ras^{V12} served as a positive control. Scale bar= 200μm.

Fig. 11

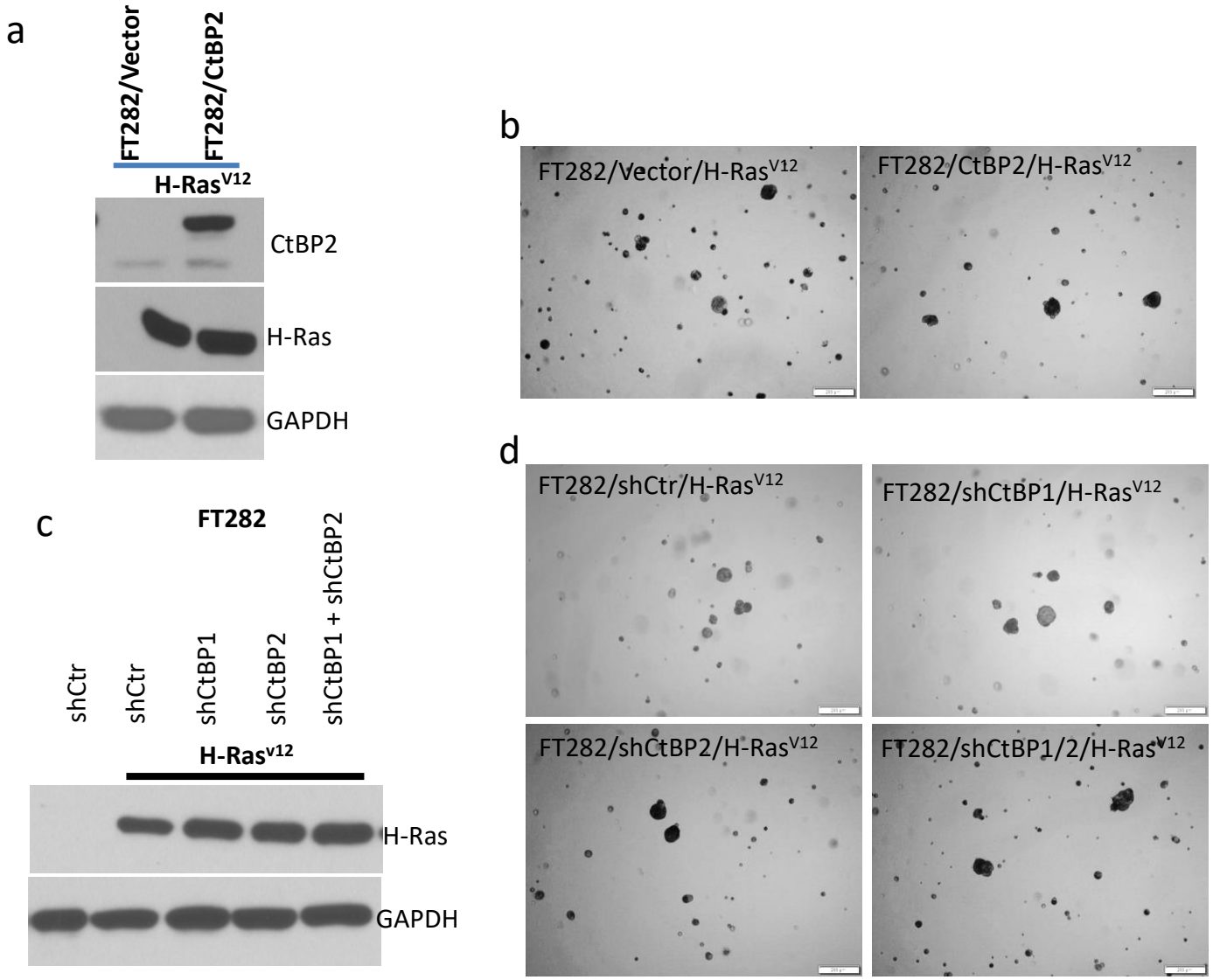


Fig. 11. Effect of CtBP on the transformation of FTECs by H-Ras^{V12}.

a and b. FT282/vector, FT282/CtBP2 stable cells were transduced with H-Ras^{V12}(a). Western blotting confirmed the expression of H-Ras^{V12}, 3D cell culture showed the cell transformation(b). Scale bar=200μm .

c and d. FT282 cells expressed shCtBP were transduced with H-Ras^{V12}(c). Western blotting confirmed the expression of H-Ras^{V12}, 3D cell culture showed the cell transformation(d). Scale bar=200μm.

C. Opportunities for training and professional development

A post-doctoral fellow, Boxiao Ding, performed much of the work described in this report and his training involved close one-on-one mentoring by the PI to assist him in designing, executing and analyzing the experiments performed. Dr. Ding also attended bimonthly cancer biology seminars sponsored by the Cancer Center, and presented at a weekly lab meeting of the PI. He will be submitting his work to an upcoming ovarian cancer meeting and will attend for further professional growth.

D. Dissemination of results to communities of interest

a. Nothing to report

E. Plans for next reporting period

In the next reporting period we plan to:

1) Complete xenograft experiments in Aim 1a
2) Fully complete Aim 1b by testing CNA of the HGSOC cell lines
3) Complete Aim 1c
4) Complete Aim 2
5) Complete Aim 3 by establishing founder strains, testing CtBP2 expression in fallopian tubes, and beginning an aging study

4. IMPACT

A. Impact on development of the principle discipline

Our work has the potential to show that CtBP1 and 2 are key dependencies in HGSOC and thus may be valuable drug targets.

B. Impact on other disciplines

Nothing to report

C. Impact on technology transfer

Nothing to report

D. Impact on society beyond science and technology

Nothing to report

5. CHANGES/PROBLEMS

Aim 1: Due to the lack of growth of most HGSOC lines with transient lentiviral shRNA knockdown of CtBP1 or CtBP2, we have abandoned the CRISPR approach except for OVSAHO cells, as we expect CRISPR knockouts will not grow or select for variants that grow out, and we can successfully achieve high grade knockdown with shRNA for either CtBP1 or CtBP2. The shRNA approach also allows us to easily knock down CtBP1 and 2 simultaneously cells in cell culture.

Aim 3: The construction of the transgenic mouse was updated to take advantage of new CRISPR technology that allows efficient single copy insertion of a CtBP2 transgene into the ROSA locus.

6. PRODUCTS

Nothing to report

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

A. Individuals who have worked on the project one person month the past year:

Name: Steven Grossman, MD, PhD

Project Role: PI

Researcher Identifier: N/A

Person months worked: 0.6

Contribution: Coordinated and supervised all work

Funding support: NIH

Name: Boxiao Ding, PhD

Project Role: Postdoctoral Associate

Researcher Identifier: N/A

Person months worked: 7.2

Contribution: Boxiao performed or supervised the performance of all work in this report

Funding support: N/A

Name: Fang Yuan

Project Role: Laboratory Technician

Researcher Identifier: N/A

Person months worked: 3

Contribution: Fang assisted in the performance of all the work presented in this report.

Funding support: N/A

B. Change in the active other support of the PD/PI or key personnel:

PI:

1. 5R01 CA172660-05 ended 1/31/2019.
2. Added:

1R21CA216685-01A1 (Oh)	04/01/18 to 03/31/20	0.36 calendar
NIH/NCI	$137,500 (direct)	

Therapeutic potential of neutrophil protease inhibitors in colon cancer

The goals of this grant are: 1) Test Aralast (AAT) and novel small peptide

AAT inhibitor as preventive and/or therapeutic interventions for colon cancer; 2) IGFBP-3, NSPs and specific cytokines (TNF-a, IL-6 etc.) as potential diagnostic/prognostic colon cancer biomarkers; and 3) the AAT-NSP-IGFBP-3/IGFBP-3R axis as therapeutic targets using two well-established colon cancer mouse models.

Role: Co-investigator

Overlap: None

C. Other organizations as partners:

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report