Elevated CDCP1 predicts poor patient outcome and mediates ovarian clear cell carcinoma by promoting tumor spheroid formation, cell migration and chemoresistance

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ABSTRACT

Hematogenous metastases are rarely present at diagnosis of ovarian clear cell carcinoma (OCC). Instead dissemination of these tumors is characteristically via direct extension of the primary tumor into nearby organs and the spread of exfoliated tumor cells throughout the peritoneum, initially via the peritoneal fluid, and later via ascites that accumulates as a

result of disruption of the lymphatic system. The molecular mechanisms orchestrating these processes are uncertain. In particular, the signaling pathways used by malignant cells to survive the stresses of anchorage-free growth in peritoneal fluid and ascites, and to colonize remote sites, are poorly defined. We demonstrate that the transmembrane glycoprotein CUB-domain-containing protein 1 (CDCP1) has important and inhibitable roles in these processes. *In vitro* assays indicate that CDCP1 mediates formation and survival of OCC spheroids, as well as cell migration and chemoresistance. Disruption of CDCP1 via silencing and antibody-mediated inhibition markedly reduce the ability of TOV21G OCC cells to form intraperitoneal tumors and induce accumulation of ascites in mice. Mechanistically our data suggest that CDCP1 effects are mediated via a novel mechanism of Akt activation. Immunohistochemical analysis also suggested that CDCP1 is functionally important in OCC, with its expression elevated in 90% of 198 OCC tumours and increased CDCP1 expression correlating with poor patient disease free and overall survival. This analysis also showed that CDCP1 is largely restricted to the surface of malignant cells where it is accessible to therapeutic antibodies. Importantly, antibody-mediated blockade of CDCP1 *in vivo* significantly increased the anti-tumour efficacy of carboplatin, the chemotherapy most commonly used to treat OCC. In summary, our data indicate that CDCP1 is important in progression of OCC and that targeting pathways mediated by this protein may be useful for management of OCC, potentially in combination with chemotherapies and agents targeting the Akt pathway.

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy with ~140,000 deaths each year worldwide (1). Ovarian clear cell carcinoma (OCC) is an aggressive form of this malignancy (2) that in Western societies accounts for 5-13% of all ovarian cancers and up to 25% in Asian populations (3, 4). Although stage I patients generally do well, later OCC stages have poorer prognosis than any of the other three major ovarian cancer histotypes; serous, mucinous and endometrioid. These later stage OCC tumors are characterisically resistant to conventional platinum-based chemotherapy, and less than 10% of patients with recurrent disease respond to second line agents (5, 6). Currently, drugs targeting defined signaling pathways are also not available for treatment of OCC patients.

A feature that distinguishes EOC from most other solid malignancies is its mode of metastasis. In contrast with these other tumor types, which generally employ the vasculature to spread to distant sites, hematogenous metastases are rarely present at diagnosis of EOC (7, 8). Instead EOC metastasis occurs via intraperitineal dissemination, a process characterized by direct extension of the primary tumor into nearby organs and the spread of exfoliated tumor cells throughout the peritoneum, initially via the peritoneal fluid, and later via ascites that accumulates as a result of disruption of the lymphatic system. During these processes survival of malignant cells is promoted via non-adherent growth as spheroids. These multi-cellular structures also facilitate attachment to, and invasion into mesothelium of abdominal organs and the omentum (7, 8). Currently, the molecular mechanisms orchestrating these processes are uncertain; in particular, the signaling pathways used by malignant cells to survive anchorage-free stress and colonize remote sites are poorly defined. Understanding these events at the molecular level is likely to provide new opportunities to therapeutically target ovarian cancer (2).

CUB-domain containing protein 1 (CDCP1) is a transmembrane glycoprotein that is widely expressed in epithelial cells as both full-length 135 kDa and proteolytically processed 70 kDa forms (9). While its physiological function is unknown, CDCP1 knock-out mice have no obvious reproductive, developmental or survival abnormalities (10, 11), suggesting that targeting this protein in disease settings, including cancer, may be well tolerated. In several tumor types that commonly metastasize via vascular routes, including lung, kidney, pancreas and colon cancer, elevated or cell surface expression of CDCP1 is associated with poor patient outcome (12-17). Consistent with a role in hematogenous metastasis, in animal models of vascular dissemination, survival of cancer cells undergoing extravasation is markedly enhanced by a mechanism involving serine protease cleavage to generate 70 kDa CDCP1. This initiates pro-survival signaling via focal adhesion kinase-1 (FAK) and PI3 kinase (PI3K) dependent Akt activation resulting in suppression of PARP1-mediated apoptosis (18, 19).

Here we have examined the function and expression of CDCP1 in OCC. Our data indicate that CDCP1 promotes OCC dissemination via a novel non-cleavage mediated mechanism that involves activation of Akt to promote formation and survival of tumor spheroids, as well as cell

migration and chemoresistance. In addition, we demonstrate that silencing CDCP1 and blocking its function using a monoclonal antibody are effective at reducing progression of OCC in a mouse model. Importantly, our immunohistochemical analysis of CDCP1 in normal ovary and fallopian tube and a large cohort of OCC cases, showed that its elevated expression correlates with poor patient outcome.

RESULTS

CDCP1 expression is elevated in OCC and correlates with poor outcome

CDCP1 protein expression was evaluated by immunohistochemistry using three TMAs containing OCC samples from a total of 207 patients, and a TMA containing normal tissue from 25 women with benign gynecological conditions whose samples showed no evidence of disease. From the OCC TMAs, cores for 9 cases were lost during staining leaving 198 evaluable cases, of which 178 (90%) expressed CDCP1 at varying intensity (Figure 1a). In all 178 OCC expressing cases, it was striking that CDCP1 expression was almost exclusive to the surface of malignant cells. CDCP1 signal was high in 20 (10%), moderate in 78 (39%) and weak in 80 cases (40%). It was not detected in stroma of malignant cases (Figure 1a) or in the epithelium or stroma of the 25 normal ovaries examined (Figure 1b). Survival data was available for 134 OCC cases with 41 of these dying of OCC, 26 dying of other causes and 67 alive at last follow-up. Kaplan Meier analysis revealed that both disease free (DFS) and overall (OS) survival were much poorer in cases expressing CDCP1 compared with non-expressers (Figure 1c and d).

CDCP1 mediates migration and non-adherent growth as spheroids of OCC cells in vitro

As elevated CDCP1 expression predicts poor OCC patient outcome, we examined its function in this malignancy. Western blot analysis to identify cell lines suitable for *in vitro* and *in vivo* assays showed that CDCP1 is expressed in each of the 11 OCC lines examined, with lowest levels in KK cells, and proteolytic processed 70 kDa CDCP1 in OVTOKO, RMG-I and JHOC-7 cells with this cleavage product either not present or apparent at low levels in the remaining cell lines (Figure 2a). Based on this analysis TOV21G, KOC7C, OVTOKO, and RMG-I cells were selected for reduction of CDCP1 expression, and KK cells for over-expression. Stable lentiviral-mediated shRNA silencing reduced CDCP1 levels by >90% in TOV21G, KOC7C (Figure 2b), OVTOKO, and RMG-I (Supplementary Figure S1a) cells. To increase CDCP1 expression, KK

cells were stably transduced with a doxycycline (dox)-inducible expression construct generating KK-WT cells. Dox treatment (1 µg/ml) for 72 h increased CDCP1 to levels seen in cells expressing this protein at high endogenous levels (Figure 2b).

As pro-survival signaling involving proteolytic conversion of CDCP1 to 70 kDa requires Akt signaling (18, 20), we assessed whether activation of this kinase is altered in the three cell lines modulated for CDCP1 expression. As shown in Figure 2b, Western blot analysis showed that modulation of CDCP1 was sufficient to robustly impact levels of pAkt in these OCC cell lines, and this was independent of cleavage of CDCP1. The level of Akt activation was directly proportional to the level of expression of CDCP1. Similar data have previously been reported for poorly metastatic melanoma A375 cells (21). For this cell line, CDCP1 over-expression induced Akt activation via a mechanism that was dependent on phosphorylation of tyrosine 734 (Y734) of full-length 135 kDa CDCP1 (21). To examine whether altered Akt activation in our OCC cells is also dependent on CDCP1-Y734, we generated a second dox-inducible KK line able to express phosphorylation defective CDCP1-Y734F (designated KK-Y734). As shown in Figure 2c, dox treatment induced expression of both CDCP1 and CDCP1-Y734F within 6 h and pAkt-S473 levels were consistently elevated in both KK-WT and KK-Y734F cells by 24 h. This novel result indicates that pY734 is not required for CDCP1-induced activation of Akt in KK cells. Consistently, Western blot analysis showed that CDCP1 endogeously expressed by TOV21G and KOC7C cells and induced in KK-WT cells is not phosphorylated at Y734 (Figure 2d).

The generated cell lines were used to examine the role of CDCP1 in processes important in progression of OCC: proliferation, migration and spheroid formation and growth. As shown in Figure 2e, proliferation of each cell line over 96 h was unchanged by altered CDCP1 expression. In contrast, stable silencing of CDCP1 reduced TOV21G and KOC7C cell migration in a Transwell system over 48 h by 65% and 73%, respectively (Figure 2f). Consistently, elevated CDCP1 expression increased KK-WT cell migration 2.9 fold in this *in vitro* system (Figure 2f). The effect of modulated CDCP1 expression on OCC spheroids was assessed after growing cells in low adhesion plates for 72 h. Silencing of CDCP1 reduced the number of spheroids formed by TOV21G and KOC7C cells by 57% and 55% respectively, while elevated expression resulted in ~2.2 fold more KK-WT cell spheroids (Figure 2g). Of note, the function of CDCP1 in migration

and spheroid formation is independent of pY734, as induction of CDCP1-Y734F in KK-Y734F cells triggered similar increases in these processes as KK-WT cells (Figure 2f and g), correlating with the levels of pAkt seen in Figure 2c. In addition, the function of CDCP1 in migration and spheroid formation appears to be independent of proteolytic cleavage of CDCP1. Silencing of CDCP1 in OVTOKO and RMG-I cells, which express significant levels of cleaved CDCP1, reduced migration and spheroid formation without altering prolifertion (Supplementary Figure S1b) simmilar to TOV21G and KOC7C cells which almost exclusively express full-length CDCP1 (Figure 2).

We also examined whether manipulation of CDCP1 expression altered the epithelial and mesenchymal characteristics of OCC cells, as transitions between these states are thought to be important in ovarian cancer (22). However, Western blot analysis indicated no change in expression of the epithelial marker EpCAM or the mesenchymal marker vimentin (Supplementary Figure S2a). Consistently, modulating CDCP1 levels did not alter cell morphology (Supplementary Figure S2b and c).

CDCP1 promotes OCC tumor growth and accummulation of ascites in a mouse model

We employed two approaches to examine the role of CDCP1 in OCC *in vivo*. In the first, we examined the impact of modulating CDCP1 expression on the ability of TOV21G and KK cells to form i.p. tumours in immune compromised NSG mice. Immediately before sacrifice on day 24, tumor burden assessed by bioluminescence imaging (BLI) was markedly lower in mice carrying TOV21G cells silenced for CDCP1 (TOV21G-shCDCP1) compared with scramble control (TOV21G-Scr) cells (Figure 3a and b). At sacrifice there was a 2.5 fold increase in the number of tumor nodules >8 mm^3 in mice injected with TOV21G-Scr compared with TOV21G-shCDCP1 cells, while there was no difference in the size of nodules (Figure 3c). Consistent with reduced tumour burden, ascites volume was >50% lower in the TOV21G-shCDCP1 mouse group (Figure 3d). Western blot analysis showed that levels of CDCP1 in cultured cells prior to injection were maintained in TOV21G-shCDCP1 and TOV21G-Scr xenograft tumors (Figure 3e). It was not possible to examine Akt activation in these tumours by Western blot analysis as pAkt-S473 was masked by the albumin signal which is a similar molecular weight. Accordingly, Akt activation was examined immunohistochemically which showed that levels were markedly

higher in TOV21G-Scr tumors compared with TOV21G-shCDCP1 tumors (Figure 3f)consistent with data from cultured cells (Figure 2b). Data from KK cells also indicated that CDCP1 promotes OCC *in vivo*. BLI showed that 6 weeks after i.p. injection, bioluminesence was 4-5 fold higher in mice carrying KK-WT cell xenografts and fed a dox containing diet to induce CDCP1 expression, than control mice (Figure 3g and h).

In the second approach we compared i.p. growth in mice of populations of TOV21G cells expressing CDCP1 at either low ($CDCP1^{low}$) or high ($CDCP1^{high}$) levels. These populations were selected by fluorescence activated cell sorting (FACs) based on cell surface expression using the anti-CDCP1 monoclonal antibody 41-2 (19). After this selection process the populations were expanded under adherent cell culture conditions for 8 weeks to generate sufficient numbers of cells for mouse assays. Both adherent $CDCP1^{low}$ and $CDCP1^{high}$ cells populations had the same morphology as parental TOV21G cells (Figure 4a). After *in vitro* expansion, to re-establish conditions mimicing growth in ascites the cell populations were replated into low attachment plates for 7 days then examined. Western blot and flow cytometry analysis, assessing the molecular phenotype of these populations, indicated about 5 fold higher levels of total CDCP1 expressed by $CDCP1^{high}$ TOV21G cells (Figure 4b) and ~10 fold more CDCP1 on the surface of these cells compared with $CDCP1^{low}$ cells (Figure 4c). Western blot data also confirmed increased activation of Akt in $CDCP1^{high}$ in comparison with $CDCP1^{low}$ and parental TOV21G cells while there was no change in Src phosphorylation or the mesenchymal marker vimentin (Figure 4b). From samples obtained from mice at sacrifice on day 24, it was apparent that $CDCP1^{high}$ cells were much more tumourigenic *in vivo* than $CDCP1^{low}$ cells, forming many more nodules on adipose tissue and the peritoneal membrane, and occasionally on ovary, liver and spleen. Tumour nodules on the mesenteric membrane attached to the jejunum and ileum are shown in Figure 4d with $CDCP1^{high}$ cells generating 2.5 fold more tumour nodules >8 mm^3 (Figure 4e) and 5.8 fold more ascites than $CDCP1^{low}$ cells (Figure 4f).

CDCP1 is upregulated in spheroid forming cells in vitro and promotes tumorigenicity of sphere forming cells in vivo

Multiple lines of evidence indicate that growth of OCC cells as spheroids promotes anchorage-independent survival and facilitates the seeding of cancer cells on peritoneal organs and

structures (7, 8). We noted above that modulation of CDCP1 has a marked impact on the ability of OCC cells to form spheres under non-adherent conditions (Figure 2g and Supplementary Figure S1d). To explore whether the role of CDCP1 in sphere formation promotes OCC, we selected populations of OCC cells that retain the ability to grow as spheres in long-term cell culture conditions by growing TOV21G and KOC7C cells in ultra-low attachment plates in complete media. Between 7 and 14 days after plating spheroid forming cells (SFCs) and non-SFCs (NSFCs) were separated by differential centrifugation. To expand these subpopulations for downstream assays, SFCs and NSFCs were plated into adherent culture vessels and grown for 8 weeks. The cells were then replated for 7 days in ultra-low attachment plates which demonstrated that SFC and NSFC phenotypes were maintained (Figure 5a). Consistent with CDCP1 having a role in sphere formation, Western blot analysis showed that CDCP1 levels were markedly higher in SFCs compared with NSFCs (Figure 5b, non-adhered). In addition, analysis of Akt, Src and ERK pathways that are important in CDCP1-mediated signaling (18, 20, 23-25), demonstrated that each is activated in SFCs compared with NSFCs (Figure 5b, non-adhered). Interestingly, when both SFCs and NSFCs were replated onto cell culture dishes for adherent growth, levels of CDCP1 and activation of Akt, Src and ERK returned to basal levels (Figure 5b, adhered).

To examine the importance of CDCP1-mediated sphere formation in OCC, we first assessed whether TOV21G SFCs are more tumorigenic than NSFCs, by injecting non-enzymatically dissociated single cell suspensions of each cell population i.p. into mice. At sacrifice both TOV21G^{SFCs} and TOV21G^{NSFCs} had formed tumors throughout the peritoneal cavity and also caused the accumulation of ascites, with TOV21G^{SFCs} forming 3.3 fold more tumour nodules >8 mm^3 (Figure 5c) and inducing 4.2 fold more ascites than TOV21G^{NSFCs} (Figure 5d). To directly examine the importance of CDCP1 in tumorigenicity of OCC spheroids in mice, we examined the effect of the function blocking anti-CDCP1 antibody 10D7 (18-20) on tumorigenicity of TOV21G^{SFCs}. At sacrifice 24 days after injection, 10D7 treated TOV21G^{SFCs} had formed many fewer i.p. tumor nodules than isotype matched IgG treated cells (Figure 5e). Specifically, in comparison with control IgG, the anti-CDCP1 antibody reduced the number of TOV21G^{SFC} tumor nodules >8 mm^3 by 54% (Figure 5f) and the volume of ascites by 57% (Figure 5g).

Targeting CDCP1 increases OCC chemosensitivity

Carboplatin is the most active ovarian cancer chemotherapy and is generally used in combination with paclitaxel (26). Resistance to these agents is a major OCC treatment problem (2). As our data indicate that CDCP1 mediates pro-survival signaling in OCC, we were interested in whether it also has a role in resistance of this malignancy to chemotherapy. This was examined by measuring the response *in vitro* to increasing carboplatin (0-100 μM) and paclitaxel (0-30 nM) (27-29) of TOV21G-Scr versus TOV21G-shCDCP1, TOV21G^{low} versus TOV21G^{high}, and KK versus KK-WT and KK-Y734F cells. Cells were cultured under adherent conditions and viability was measured at 72 h. TOV21G-Scr cells were about twice as resistant to carboplatin and paclitaxel as TOV21G-shCDCP1 cells (Figure 6a and b) and, consistently, that TOV21G^{high} cells were twice as resistant to both carboplatin and paclitaxel as TOV21G^{low} cells (Figure 6c and d). Also consistent with these data, dox treated KK-WT and KK-Y734F cells were both twice as resistant to these agents as control dox treated cells (Figure 6e and f).

To examine mechanistically how CDCP1 mediates OCC resistance to carboplatin and paclitaxel, we performed Western blot analyses on lysates from 3 OCC cell lines that had been modulated for CDCP1 expression and treated with these chemotherapeutic agents. We examined CDCP1 expression as well as activation of pathways previously shown to be regulated by CDCP1 including pAkt-S473/Akt, p-Src-Y416/Src and pERK/ERK (18, 23, 25, 30). Of note, the response to carboplatin and paclitaxel of TOV21G and KOC7C cells, which endogenously express CDCP1 at medium to high levels, was to increase CDCP1 levels within 3 h of treatment and this was accompanied by a transient increase in pAkt levels (Scramble lanes, Supplementary Figure S3a and b). For both of these lines the presence of the CDCP1 silencing construct shCDCP1 was sufficient to overcome this induction of CDCP1 and pAkt caused by chemotherapy (shCDCP1 lanes, Supplementary Figure S3a and b).

Analogous data were obtained from KK cells which express CDCP1 at low levels. As can be seen in Supplementary Figure S3c and d, both carboplatin and paclitaxel induced CDCP1 expression in parental KK cells and KK-WT cells by 3 to 6 h after the start of chemotherapy, and this was accompanied by increased pAkt levels. In KK-WT treated with dox to induce CDCP1 expression, we did not observe a further increase in CDCP1 in response to chemotherapy,

although a transient increase in pAkt levels was seen at 3 (Supplementary Figure S3c and d). Interestingly, dox treated KK-Y734F cells showed the same responses, further supporting data from Figure 2c showing that CDCP1-mediated Akt activation in OCC is unlike the CDCP1-Y734-dependent activation reported in other cancer settings (18, 23, 25, 30). Activation of Src and ERK was not seen consistently in these OCC cell lines in response to chemotherapy, although we did note increased pERK levels over the time course of carboplatin treatment of dox treated KK-WT and -Y734F cells (Supplementary Figure S3).

These data indicate that CDCP1 has a role in chemotherapy resistance and that CDCP1-mediated activation of Akt in OCC in response to chemotherapeutic agents occurs via a novel mechanism independent of Src and ERK activation. To directly address whether blockade of CDCP1 is a useful strategy to increase OCC susceptibility to standard chemotherapy, we compared the impact against mouse i.p. xenografts of 10D7 and carboplatin as monotherapies, and these as combined agents. Twice weekly 10D7 treatments started on day 3, and weekly carboplatin treatments started on day 7. At sacrifice, combined 10D7 and carboplatin was much more efficacious at reducing tumour burden than either monotherapy. Specifically, weekly BLI showed that combining 10D7 and carboplatin significantly reduced tumor growth within the experimental period compared with the controls treated with IgG, 10D7 only, or carboplatin plus IgG (Figure 6g). At sacrifice at day 24, mice treated with combined 10D7 and carboplatin showed 7.9, 3.1, and 3.7 fold fewer tumor nodules >8 mm^3 than IgG, 10D7, and carboplatin plus IgG group, respectively (Figure 6h). Almost no ascites was present in mice that received combined 10D7/carboplatin (Figure 6i).

DISCUSSION

Our data indicate for the first time that the transmembrane glycoprotein CDCP1 is an important and inhibitable mediator of OCC. Significantly, its elevated expression indicates poorer suvival of OCC patients, and our finding that CDCP1 is expressed by the vast majority of OCC tumors but not by normal ovaries, is encouraging that this protein could be selectively targeted in OCC without impacting on normal cells and structures present within the tumor environment. CDCP1 has previously been shown to protect cancer cells that metastasize via the vasculature (18-20), and our data are the first to show that this protein is critical in processes essential for the

peritonel metastasis characteristic of OCC. In particular, for this distinct type of metastasis it is a key mediator of formation and growth of the tumour spheroids that promote the anchorage-independent survival, and seeding of cancer cells on peritoneal organs and structures, that is essential for intraperitoneal dissemination of OCC. In addition, it promotes migration and chemoresistance of OCC cells. An important finding is that the anti-CDCP1 antibody 10D7 increases OCC responsiveness to chemotherapy *in vitro* and *in vivo* suggesting that combining anti-CDCP1 therapies with agents currently used in patients could be useful against OCC.

The importance of elevated CDCP1 expression in OCC was indicated by Kaplan-Meier analysis which showed that patients expressing this protein have much poorer DFS and OS. These patient data were supported by *in vitro* assays and experiments in mice. Data from 4 OCC cell lines silenced for CDCP1 expression and another over-expressing this protein, demonstrated that CDCP1 mediates migration and spheroid formation *in vitro*, processes which are important for OCC *in vivo* (7, 8). Consistently, silencing of CDCP1 in TOV21G cells resulted in markedly lower tumor burden and accumulation of ascites in mice. In addition, TOV21G cells selected for high CDCP1 expression were able to induce 2.5 fold greater tumor burden and 5.8 fold more ascites in mice than TOV21G cells expressing this protein at ~10 fold lower levels. In addition, induced expression of CDCP1 markedly increased the tumorigenicity of KK cells *in vivo*. These findings indicate important roles for CDCP1 as a pro-tumorigenic factor in OCC.

Our observation that CDCP1 is not expressed by normal ovarian epithelium was somewhat surprising as it has previously been shown to be widely expressed by other normal epithelia including epithelium of the colon (24, 31), breast, lung (31), skin (32, 33), pancreatic ducts, lung and prostate (34). The widespread expression outside the ovary may indicate that efforts to target CDCP1 in OCC would require its local rather than systemic delivery. Also of relevance to being able to target CDCP1 in OCC patients, our immunohistochemical analysis showed that CDCP1 in malignant cells it is largely restricted to the surface where it would be accessible to function blocking anti-CDCP1 antibodies which we (18, 19) and others (35) have shown are effective at inducing cell death *in vivo*. Interestingly, we have recently shown that EGFR signaling promotes recycling of CDCP1 to the surface of serous ovarian cancer cell lines (36), however the

mechanism which induces predominant location of CDCP1 on the surface of OCC cells in patients remains to be defined.

Based on data from two different experimental approaches, we show that altered CDCP1 expression is sufficient to modulate Akt activation in OCC cell lines. Our first approach involved modulation of CDCP1 expression via lenti-viral mediated silencing and over-expression. We observed that silencing of CDCP1 in TOV21G, KOC7C, OVTOKO and RMG-I cells markedly reduced pAkt-S473 levels and, consistently, its over-expression in KK cells resulted in robust activation of this pathway which was independent of p-CDCP1-Y734 (Figure 2c). These CDCP1-mediated changes in Akt activation *in vitro* were accompanied by corresponding changes in migration and spheroid formation (Figures 2f and g), which are cellular processes essential for OCC, and chemo-resistance (Figure 6) which is a characteristic feature of many advanced OCC tumors. The impact on Akt signaling *in vitro* of altered CDCP1 expression, was also apparent *in vivo*. Intraperitoneal tumors recovered from mice carrying TOV21G-shCDCP1 cell tumors showed much higher levels of pAkt-S473 than TOV21G-Scr cell tumors (Figure 3e).

In our second approach we selected SFC and NSFC populations of TOV21G and KOC7C cells that were able to maintain long term spheroid-forming capacity. We noted that in both cell lines, CDCP1 expression was markedly induced in SFCs in comparison with NSFCs. Consistent with data from silencing and over-expression assays, the increased CDCP1 expression seen in SFCs was accompanied by activation of Akt. Interestingly, Src and ERK activation was also seen in SFCs compared with NSFCs. Consistent with these data it has been reported that several mediators of CDCP1 signaling, including integrin β1 (37), Akt (38) and ERK (39), also mediate formation of ovarian cancer cell spheroids. We confirmed the importance of elevated CDCP1 by showing that a function blocking anti-CDCP1 antibody that we have previously used to inhibit vascular metastasis (18), caused a marked reduction in the ability of SFCs to form i.p. tumours and accumulation of ascites *in vivo*.

Currently, we have limited information on the mechansim by which altered expression of CDCP1 impacts activation of Akt in OCC. Previous reports have shown that CDCP1 mediated hematogenous metastasis in mouse and chicken embryo models is dependent on activation of

Akt (18, 20). In these *in vivo* models, proteolytic conversion of 135 kDa CDCP1 to 70 kDa p-CDCP1-Y734 initiated docking of this cleaved and phosphorylated form of CDCP1 to β1 integrin, and the FAK and PI3K-dependent activation of Akt critical for cell survival during extravasation (18-20). Our data indicate a novel mechanism of CDCP1 mediated activation of Akt in OCC that is distinct to the serine protease mediated converion of 135 kDa to 70 kDa p-CDCP1-Y734 that promotes hematogenous metastasis via pAkt. Rather than proteolysis of CDCP1 being the initiator of Akt activation, increased CDCP1 expression was sufficient to induce pAkt-S473, and this was independent of both Src and ERK signalling. These data suggest that the increased activation of Src and ERK seen by us in SFCs (Figure 5b), occurred in parrallel with CDCP1 activation of Akt, rather than being involved in the relay of signals between and CDCP1 and Akt. In addition, two of our findings demonstrate that CDCP1 activation of Akt signaling in OCC is not dependent on Y734 of CDCP1. First, in KK cells the phosphorylation defective mutant CDCP1-Y734F was able to induce phosphorylation of Akt-S473 as robustly as wildtype CDCP1 (Figure 2c), even when these cells were under chemotherapeutic challenge (Supplementary Figure S3). In addition, the increased migration, spheroid formation and chemoresistance of KK cells, mediated by elevated expression of CDCP1, was unaffected by loss of Y734 of CDCP1 (Figure 2f-g, and 6e-f). It was also interesting that Western blot analysis showed consistently that within 3 to 6 h the OCC chemotherapies carboplatin and paclitaxel induce expression of CDCP1 in OCC cell lines and that this is accompanied by phosphorylation of Akt without altering Src and ERK activation (Supplementary Figure S3). Understanding the mechanisms by which CDCP1 expression is increased and how it signals to promote OCC may provide additional opportunities to treat this cancer.

In conclusion, our findings implicate CDCP1 as a pro-tumorigenic factor in OCC by promoting migration, spheroid growth and chemoresistance. It is not expressed in normal ovary but is elevated in the vast majority of OCC patients and its elevated expression correlates with poor patient outcome. Our data indicate that blockade of CDCP1 reduces tumor burden and accumulation of ascites, particularly when used in combination with carboplatin, suggesting that targeting OCC with anti-CDCP1 agents and chemotherapy or drugs blocking other signalling pathways may be of benefit to patients.

MATERIALS AND METHODS

Antibodies and reagents, in vitro assays and Western blot and statistical analysis

Antibodies, reagents, ovarian samples, immunohistochemistry, cell culture, luciferase labeling of cells, modulation of CDCP1 expression, *in vitro* assays, cell and tissue lysis, mouse xenografts, and Western blot and statistical analyses are described in Supplementary Materials and Methods.

CONFLICT OF INTEREST

JDH is an inventor on a patent describing CDCP1 as an anti-cancer target. All other authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. CDCP1 expression in OCC and normal ovary. (**a**) Immunohistochemistry of CDCP1 in OCC. Images are representative of negative, weak, moderate and strong CDCP1 staining. (**b**) Representative image of CDCP1 in normal ovary. (**c**) and (**d**) Kaplan–Meier analysis of DFS and OS, respectively. Cases were segregated into CDCP1 expressers (+) and non-expressersn (-).

Figure 2. CDCP1 mediates *in vitro* migration and spheroid growth of OCC cells. (**a**) Western blot analysis of 11 OCC cell lines. Arrows, CDCP1 at 70 and 135 kDa. (**b**) Western blot analysis of CDCP1 silenced (shCDCP1) and scramble control TOV21G and KOC7C cells, and parental KK cells and KK cells induced (Dox 1 µg/ml, 72 h) to express CDCP1. (**c**) Western blot analysis of parental KK cells and KK cells induced to express CDCP1 or CDCP1-Y734F for the indicated times. Expression of pAkt-S473, Akt and CDCP1 relative to GAPDH is displayed graphically. (**d**) Western blot analysis of KK, KOC7C and TOV21G cells for phosphorylated CDCP1-Y734, total CDCP1 and GAPDH. Lysates from HCT116 cells were used as the control for phospho-CDCP1-Y734. (**e-g**) Effect of altered expression of CDCP1 and CDCP1-Y734F on proliferation (e), migration (f) and spheroid formation (g). NS, not significant.

Figure 3. CDCP1 regulates OCC tumor growth and accumulation of ascites *in vivo*. (**a**) BLI of female NSG mice (n=6/group) injected i.p. with luciferase labelled TOV21G cells ($1x10^6$) stably

transduced with a shCDCP1 or scramble control (shScr) immediately before sacrifice on day 24. The luciferase activity of cells *in vitro* before implantation is shown on the left. (**b**) Total photon flux of TOV21G xenografts before harvest at day 24. (**c**) Number of tumor nodules >8 mm^3 at sacrifice. (**d**) Ascites volume at sacrifice. (**e**) Western blot analysis of TOV21G-Scramble and TOV21G-shCDCP1 cells grown *in vitro*, and tumor nodules harvested from the respective mouse xenografts. Five nodules randomly selected from the 6 mice/experimental group were loaded de-identified onto the gel before re-identification after CDCP1 signal was detected. Arrows, 70 and 135 kDa CDCP1. (**f**) Representative images of CDCP1 and pAkt-S473 immunohistochemistry of mouse xenografts. (**g-h**) Mice fed a normal mouse diet (-dox) or a dox containing diet (600 mg/kg in diet; +dox), were injected i.p. with parental KK (n=4) or KK-WT (n=12) cells ($2x10^6$). (**g**) BLI 6 weeks after i.p. injection immediately before sacrifice. Pre-implantation luciferase activity of parental KK and KK-WT cells grown *in vitro* is shown at the top. (**h**) Weekly photon flux values from mouse groups were plotted against time.

Figure 4. CDCP1 promotes OCC progression *in vivo*. Subpopulations of TOV21G cells were obtained by FACs based on cell surface CDCP1 expression then expanded *in vitro* as adherent cultures for 8 weeks before i.p. injection into NSG mice. (**a**) Morphology of $CDCP1^{low}$ and $CDCP1^{high}$ TOV21G cells after 8 weeks of adherent growth. Scale bar: 200 µm. (**b**) Western blot analysis of lysates from $CDCP1^{low}$ and $CDCP1^{high}$ TOV21G cells for CDCP1, activation of Akt and Src, and EMT markers at week 0 and 8 after FACs. Lysates were after FACs (Week 0) and after 8 weeks of adherent growth (Week 8). (**c**) Flow cytometry analysis of TOV21G-$CDCP1^{low}$ (blue) and -$CDCP1^{high}$ (red) cells. Orange, unstained parental TOV21G cells. (**d**) Female NSG mice (n=12/group) were injected i.p. with TOV21G-$CDCP1^{low}$ or -$CDCP1^{high}$ cells ($2x10^6$). At sacrifice 24 days after injection, $CDCP1^{low}$ cells had formed many fewer i.p. tumors than $CDCP1^{high}$ cells. Representative images of the mesenteric membrane of 6 mice/group are shown. *, tumor nodule. (**e**) Tumor nodules >8 mm^3 at sacrifice. (**f**) Ascites at sacrifice.

Figure 5. CDCP1 is upregulated in spheroid forming cells *in vitro* and promotes tumorigenicity of sphere forming cells *in vivo*. (**a**) Phase contrast microscope images of TOV21G and KOC7C SFCs and NSFCs. scale bar: 100 µm. (**b**) Western blot analysis of SFCs and NSFCs show upregulation of CDCP1 and activation of signalling pathways. (**c, d**) Female NSG mice

(n=6/group) were injected i.p. with TOV21G^{NSFCs} or TOV21G^{SFCs} (1x10^6). At sacrifice 24 days after injection, TOV21G^{SFCs} were much more tumorigenic than TOV21G^{NSFCs} showing more tumor nodules >8 mm^3 in size (c) and larger ascites volume (d). (**e-g**) Female NSG mice (n=5/group) were injected i.p. with TOV21G^{SFCs} (1x10^6) and anti-CDCP1 monoclonal antibody 10D7 or isotype matched control IgG (100 µg). Antibody treatment was repeated twice a week until sacrifice. At sacrifice 24 days after injection, TOV21G^{SFCs} co-injected with 10D7 had formed many fewer i.p. tumor nodules. Representative images of the mesenteric membrane from the mice in each group are shown. *, tumor nodule. TOV21G^{SFCs} co-injected with 10D7 formed fewer tumor nodules >8 mm^3 (f) and smaller ascites volume (g).

Figure 6. Targeting CDCP1 increases OCC chemosensitivity. Cell viability in response to 72 h of treatment of TOV21G-Scr and TOV21G-shCDCP1 (**a, b**), TOV21G-CDCP1^{low} and TOV21G-CDCP1^{high} (**c, d**), and dox treated KK, KK-WT and KK-Y734 cells (**e, f**), over the indicated concentration ranges of carboplatin (a, c, e) and paclitaxel (b, d, f). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. NA, not applicable. (**g-i**) Female NSG mice injected with luciferase-labelled TOV21G cells (1x10^6/mouse) were treated with isotype IgG, carboplatin and IgG, 10D7, or carboplatin and 10D7. Doses were: IgG and 10D7 at 25 mg/kg/dose commencing 3 days after cell injections then continuing twice weekly; carboplatin 15 mg/kg/dose commencing 7 days after cell injections then continuing weekly. Tumor growth was monitored weekly by BLI and total photon flux was plotted against time (**g**). At harvest tumors >8 mm^3 were counted (**h**) and ascites volume measured (**i**).

Figure 1

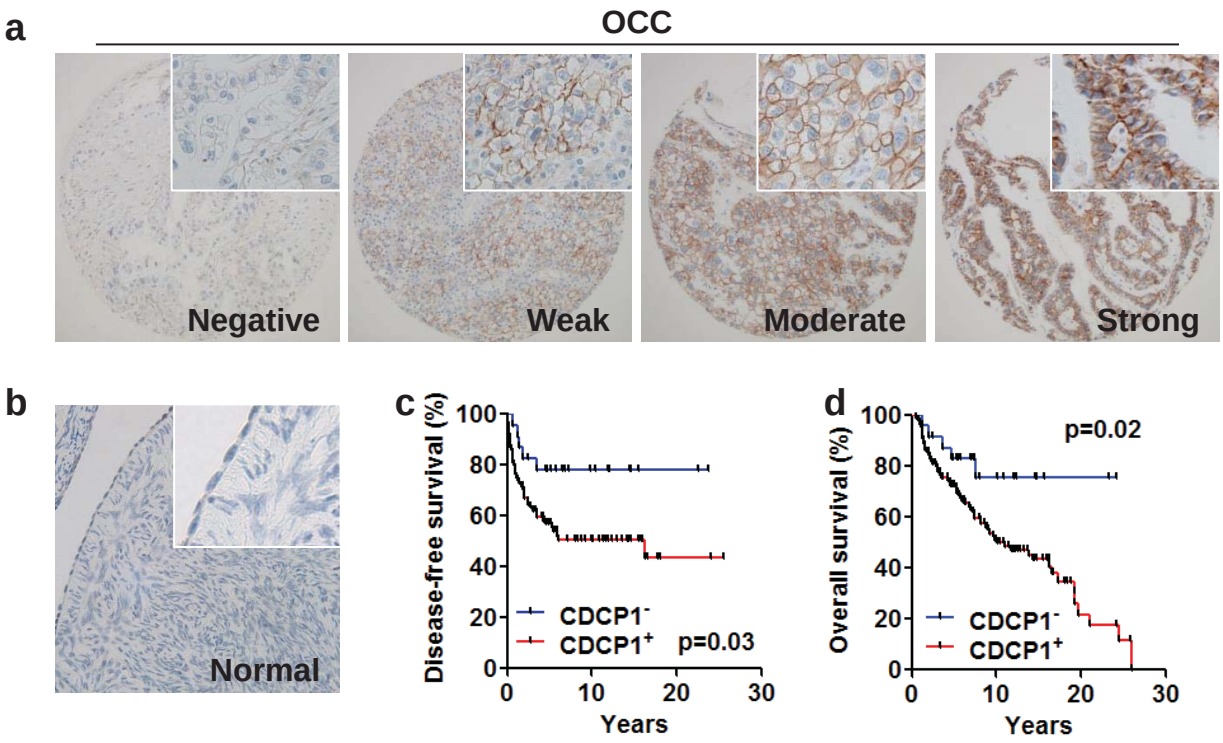
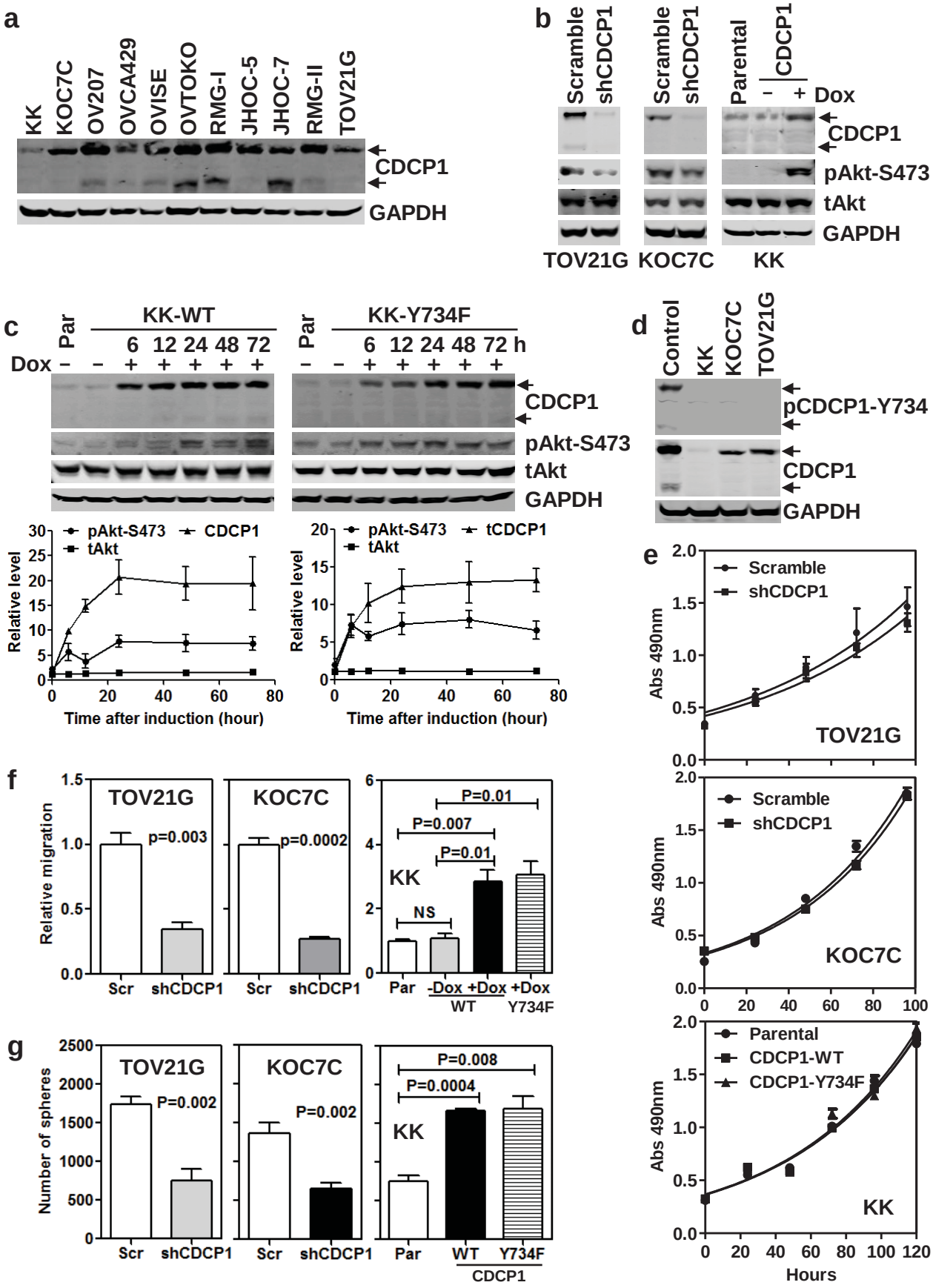
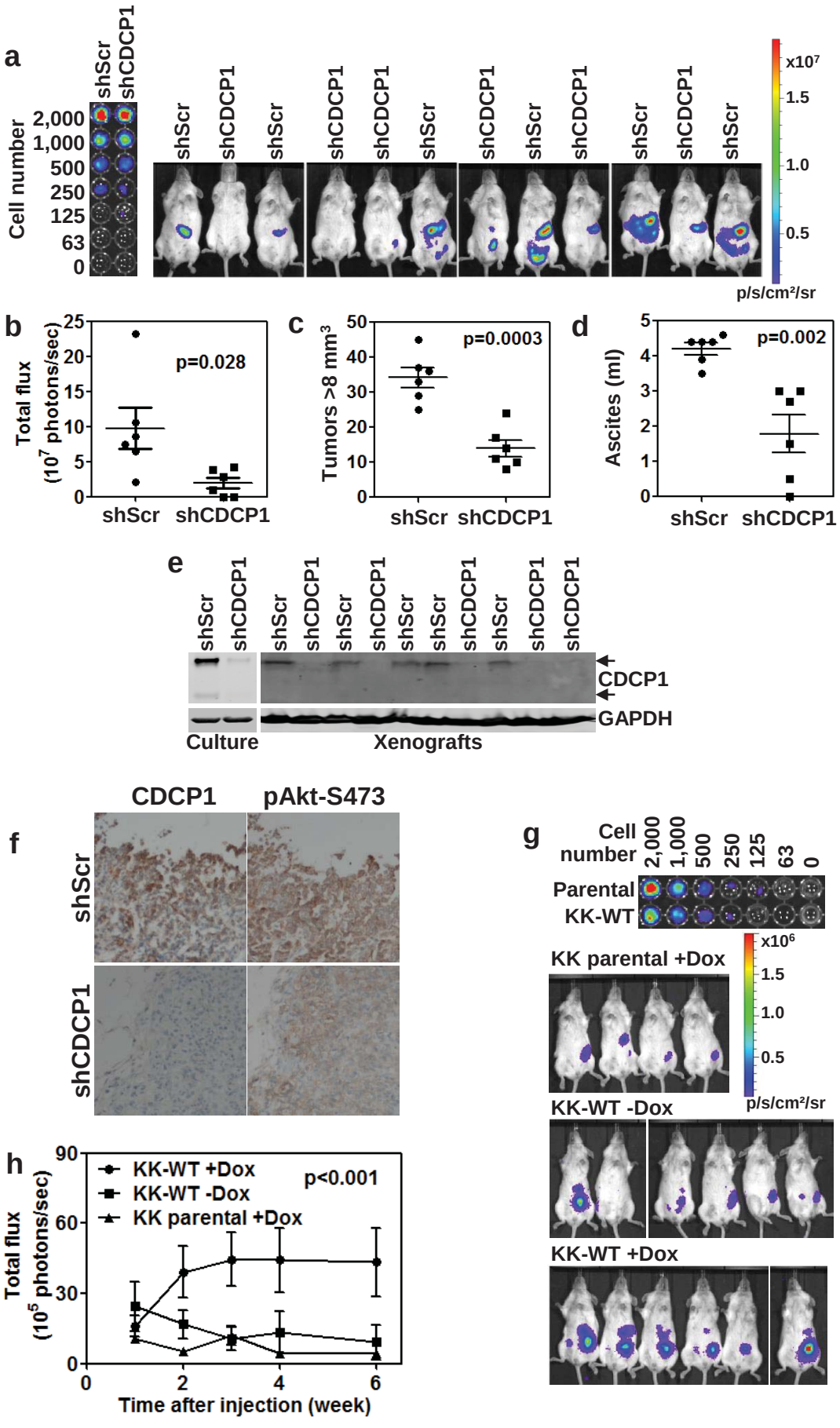

a
OCC
Negative
Weak
Moderate
Strong
b
Normal
c
Disease-free survival (%)
100
80
60
40
20
0
0
10
20
30
Years
CDCP1-
CDCP1+
p=0.03
d
Overall survival (%)
p=0.02
CDCP1-
CDCP1+
Years

Figure 2


a
KK
KOC7C
OV207
OVCA429
OVISE
OVTOKO
RMG-I
JHOC-5
JHOC-7
RMG-II
TOV21G
CDCP1
GAPDH
b
Scramble
shCDCP1
Scramble
shCDCP1
Parental
CDCP1
– + Dox
CDCP1
pAkt-S473
tAkt
GAPDH
TOV21G
KOC7C
KK
c
Par
KK-WT
6 12 24 48 72
Dox – – + + + + +
Par
KK-Y734F
6 12 24 48 72 h
– – + + + + +
CDCP1
pAkt-S473
tAkt
GAPDH
pAkt-S473
CDCP1
tAkt
Relative level
Time after induction (hour)
pAkt-S473
tCDCP1
tAkt
Relative level
Time after induction (hour)
d
Control
KK
KOC7C
TOV21G
pCDCP1-Y734
CDCP1
GAPDH
e
Scramble
shCDCP1
Abs 490nm
TOV21G
Scramble
shCDCP1
Abs 490nm
KOC7C
Parental
CDCP1-WT
CDCP1-Y734F
Abs 490nm
KK
Hours
f
Relative migration
TOV21G
p=0.003
KOC7C
p=0.0002
KK
P=0.01
P=0.007
P=0.01
NS
Scr
shCDCP1
Scr
shCDCP1
Par
-Dox +Dox
WT
+Dox
Y734F
g
Number of spheres
TOV21G
P=0.002
KOC7C
P=0.002
KK
P=0.008
P=0.0004
Scr
shCDCP1
Scr
shCDCP1
Par
WT
Y734F
CDCP1


a
shScr
shCDCP1
Cell number
2,000
1,000
500
250
125
63
0
x10⁷
1.5
1.0
0.5
p/s/cm²/sr
b
Total flux
(10⁷ photons/sec)
p=0.028
shScr
shCDCP1
c
Tumors >8 mm³
p=0.0003
d
Ascites (ml)
p=0.002
e
CDCP1
GAPDH
Culture
Xenografts
f
CDCP1
pAkt-S473
g
Parental
KK-WT
KK parental +Dox
KK-WT -Dox
KK-WT +Dox
x10⁶
h
KK-WT +Dox
KK-WT -Dox
KK parental +Dox
p<0.001
Total flux
(10⁵ photons/sec)
Time after injection (week)

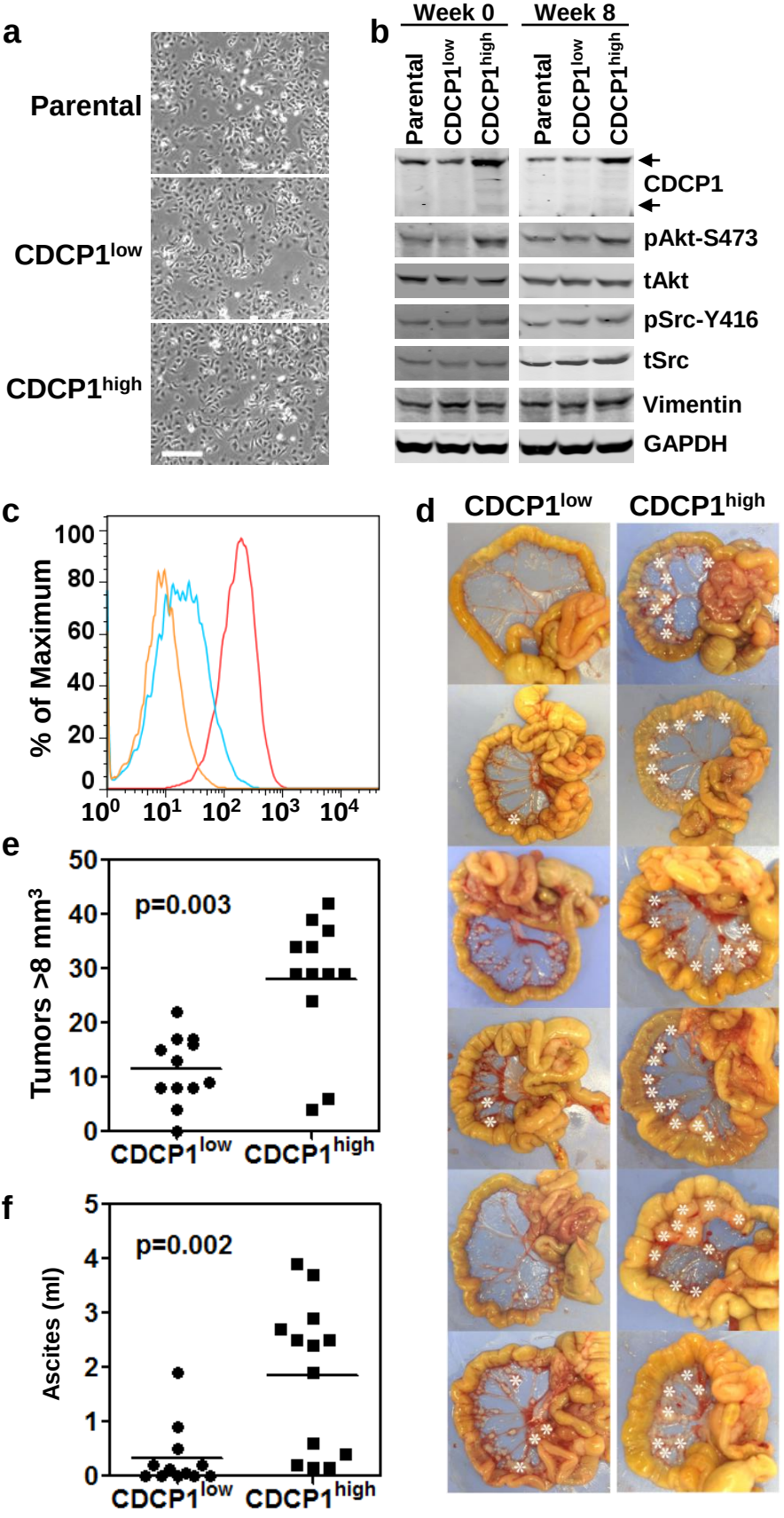

a
Parental
CDCP1low
CDCP1high
b
Week 0
Week 8
Parental
CDCP1low
CDCP1high
Parental
CDCP1low
CDCP1high
CDCP1
pAkt-S473
tAkt
pSrc-Y416
tSrc
Vimentin
GAPDH
c
% of Maximum
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
d
CDCP1low
CDCP1high
e
Tumors >8 mm3
50
40
30
20
10
0
p=0.003
CDCP1low
CDCP1high
f
Ascites (ml)
5
4
3
2
1
0
p=0.002
CDCP1low
CDCP1high

Figure 5

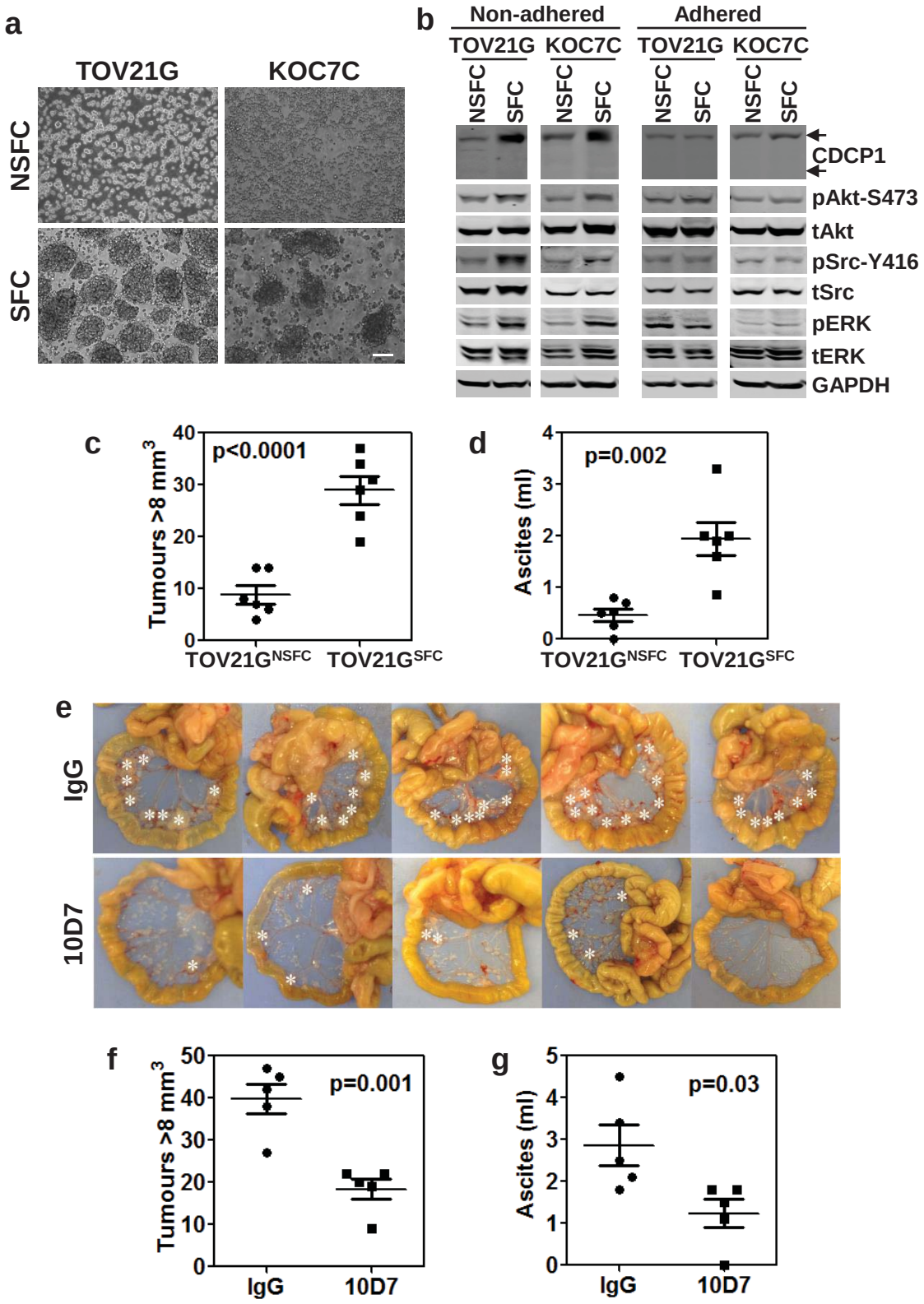
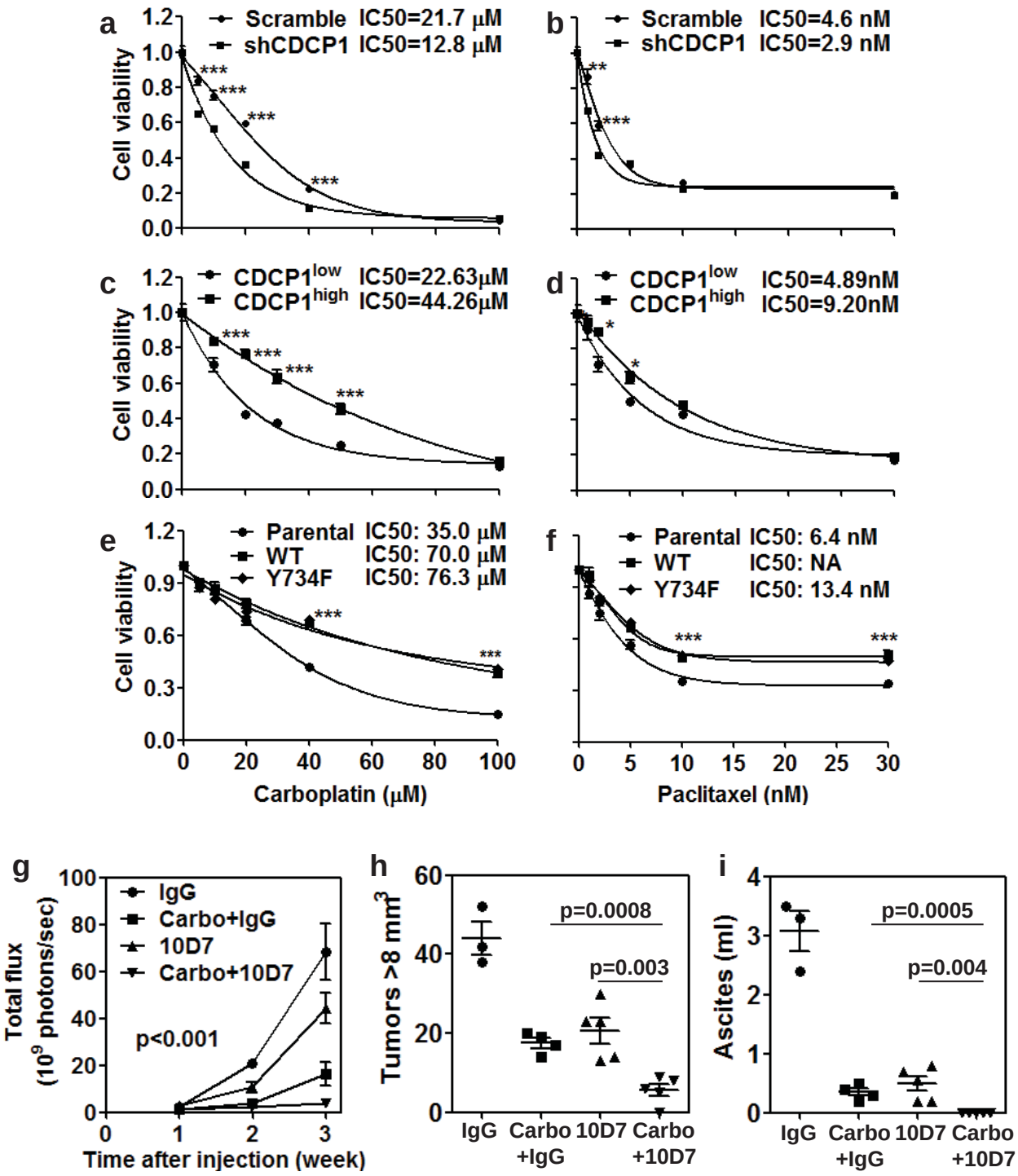
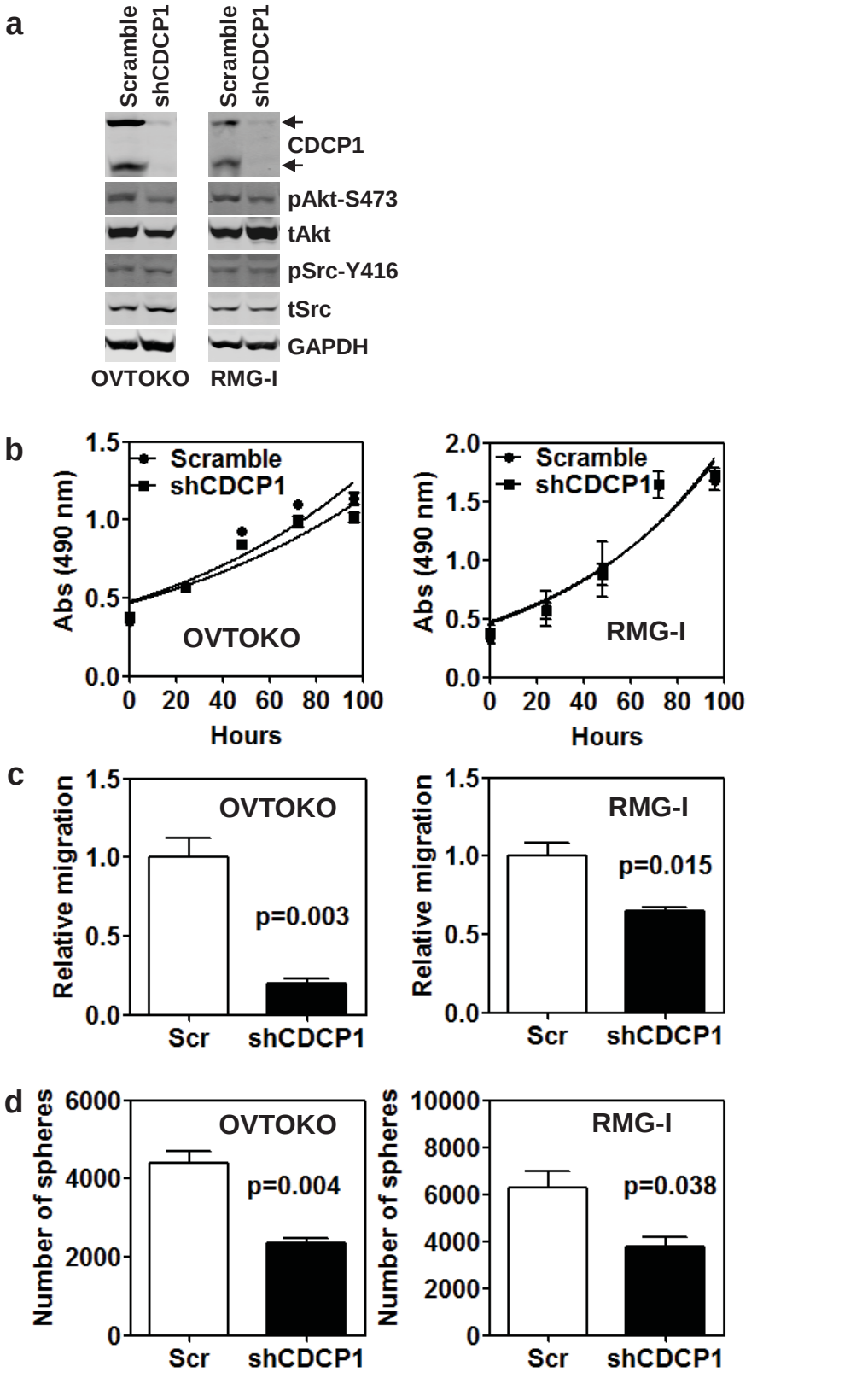

a
TOV21G
KOC7C
NSFC
SFC
b
Non-adhered
Adhered
TOV21G
KOC7C
TOV21G
KOC7C
NSFC
SFC
CDCP1
pAkt-S473
tAkt
pSrc-Y416
tSrc
pERK
tERK
GAPDH
c
Tumours >8 mm3
p<0.0001
TOV21GNSFC
TOV21GSFC
d
Ascites (ml)
p=0.002
TOV21GNSFC
TOV21GSFC
e
IgG
10D7
f
Tumours >8 mm3
p=0.001
IgG
10D7
g
Ascites (ml)
p=0.03
IgG
10D7

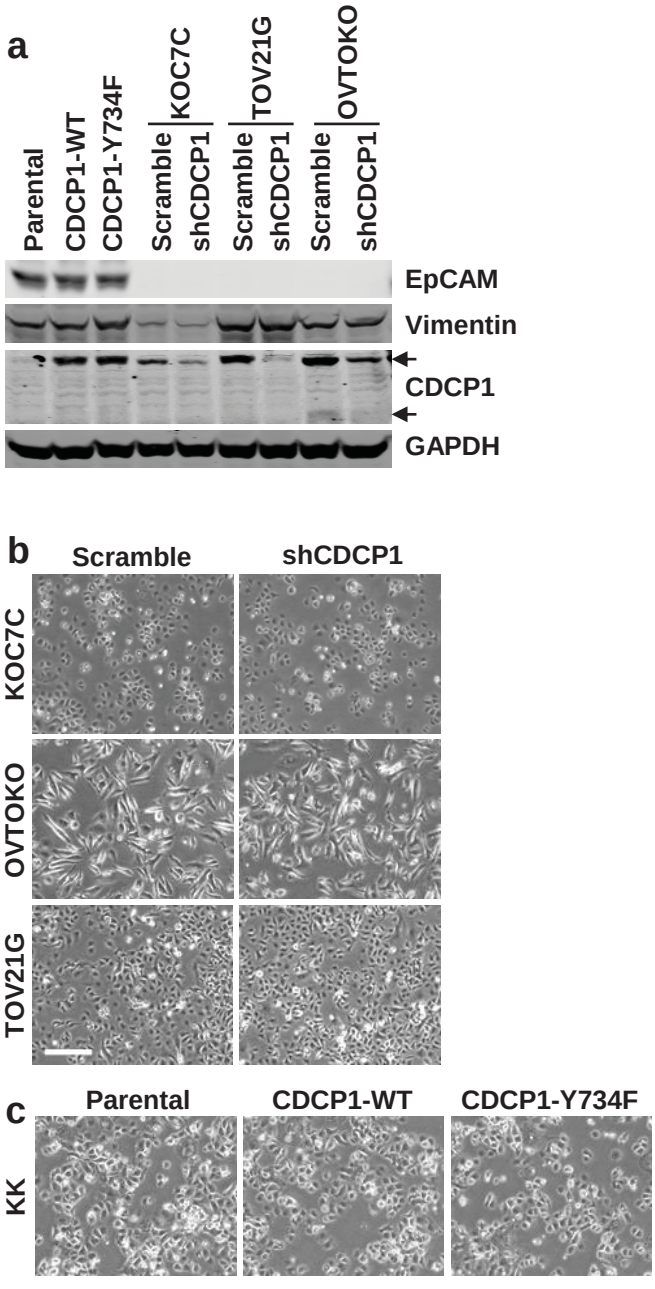
Figure 6


a
Scramble IC50=21.7 μM
shCDCP1 IC50=12.8 μM
Cell viability
b
Scramble IC50=4.6 nM
shCDCP1 IC50=2.9 nM
c
CDCP1low IC50=22.63μM
CDCP1high IC50=44.26μM
Cell viability
d
CDCP1low IC50=4.89nM
CDCP1high IC50=9.20nM
e
Parental IC50: 35.0 μM
WT IC50: 70.0 μM
Y734F IC50: 76.3 μM
Cell viability
Carboplatin (μM)
f
Parental IC50: 6.4 nM
WT IC50: NA
Y734F IC50: 13.4 nM
Paclitaxel (nM)
g
IgG
Carbo+IgG
10D7
Carbo+10D7
p<0.001
Total flux (10^9 photons/sec)
Time after injection (week)
h
p=0.0008
p=0.003
Tumors >8 mm3
IgG
Carbo +IgG
10D7
Carbo +10D7
i
p=0.0005
p=0.004
Ascites (ml)
IgG
Carbo +IgG
10D7
Carbo +10D7

Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3