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ORIGINAL ARTICLE

Downregulation of the proapoptotic protein MOAP-1 by the UBR5 ubiquitin ligase and its role in ovarian cancer resistance to cisplatin

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Evasion of apoptosis allows many cancers to resist chemotherapy. Apoptosis is mediated by the serial activation of caspase family proteins. These proteases are often activated upon the release of cytochrome *c* from the mitochondria, which is promoted by the proapoptotic Bcl-2 family protein, Bax. This function of Bax is enhanced by the MOAP-1 (modulator of apoptosis protein 1) protein in response to DNA damage. Previously, we reported that MOAP-1 is targeted for ubiquitylation and degradation by the APC/C^{Cdh1} ubiquitin ligase. In this study, we identify the HECT (homologous to the E6-AP carboxyl terminus) family E3 ubiquitin ligase, UBR5, as a novel ubiquitin ligase for MOAP-1. We demonstrate that UBR5 interacts physically with MOAP-1, ubiquitylates MOAP-1 *in vitro* and inhibits MOAP-1 stability in cultured cells. In addition, we show that Dyrk2 kinase, a reported UBR5 interactor, cooperates with UBR5 in mediating MOAP-1 ubiquitylation. Importantly, we found that cisplatin-resistant ovarian cancer cell lines exhibit lower levels of MOAP-1 accumulation than their sensitive counterparts upon cisplatin treatment, consistent with the previously reported role of MOAP-1 in modulating cisplatin-induced apoptosis. Accordingly, UBR5 knockdown increased MOAP-1 expression, enhanced Bax activation and sensitized otherwise resistant cells to cisplatin-induced apoptosis. Furthermore, UBR5 expression was higher in ovarian cancers from cisplatin-resistant patients than from cisplatin-responsive patients. These results show that UBR5 downregulates proapoptotic MOAP-1 and suggest that UBR5 can confer cisplatin resistance in ovarian cancer. Thus UBR5 may be an attractive therapeutic target for ovarian cancer treatment.

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INTRODUCTION

The efficacy of conventional cancer chemotherapeutic agents, such as cisplatin and taxol, largely relies on activation of apoptosis. Importantly, cancer cells frequently alter and suppress their apoptotic pathways, thereby becoming resistant to the effects of chemotherapy.[1] Therefore, overcoming chemotherapeutic resistance depends critically on overcoming apoptotic suppression in tumors.

Different apoptotic pathways are engaged by different stimuli; cell damage induced by chemotherapeutic agents typically triggers the intrinsic pathway of apoptosis, resulting in mitochondrial outer membrane permeabilization (MOMP) and mitochondrial cytochrome *c* release.[2] Cytoplasmic cytochrome *c* then triggers caspase activation and, ultimately, cell death. MOMP can be positively and negatively regulated by the Bcl-2 family of proteins. It is well recognized that activation of the Bcl-2 family proteins Bax and Bak is critical for triggering MOMP. Most Bax/Bak-deficient mice die prenatally and exhibit multiple developmental defects.[3] Furthermore, fibroblasts derived from Bax/Bak-deficient mice are extremely resistant to apoptotic stimuli.

Modulator of apoptosis protein 1 (MOAP-1; also known as MAP-1) was identified as a factor that can bind and activate Bax, potentiating mitochondrial translocation of Bax and initiating MOMP in response to apoptotic stimuli.[4–6] MOAP-1 has a short half-life, and its destruction is mediated by the ubiquitin–proteasome protein degradation machinery.[7] Our laboratory previously showed that MOAP-1 can be targeted and degraded by the APC/C^{Cdh1} ubiquitin E3 ligase complex.[8] Our previous work also showed that another ubiquitin E3 ligase, TRIM39, negatively regulates APC/C^{Cdh1} to suppress its ability to target MOAP-1 for ubiquitylation-mediated degradation. Suppression of MOAP-1 degradation following knockdown of the APC/C activator Cdh1 enhanced apoptosis through Bax activation in cancer cells, demonstrating the importance of MOAP-1 in the intrinsic apoptotic pathway.

Here we identify the HECT (homologous to the E6-AP carboxyl terminus) family UBR5 ubiquitin ligase as an additional MOAP-1 regulator that targets MOAP-1 for ubiquitylation and degradation. MOAP-1 protein levels were regulated by UBR5-mediated ubiquitylation in cultured cells and UBR5 could directly ubiquitylate MOAP-1 *in vitro*. Interestingly, regulation of MOAP-1 protein levels by UBR5 was cell cycle dependent, acting specifically from late S phase to G2. In that, we previously identified the M-G1 phase E3 ligase, APC/C^{Cdh1}, as a MOAP-1-directed ligase; these data suggest that different E3 ligases (potentially in response to different stimuli) target MOAP-1 for degradation.

In addition to implicating UBR5 in MOAP-1 degradation, we identified Dyrk2, a component of a previously reported

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UBR5-containing E3 ligase complex, as a regulator of UBR5-mediated MOAP-1 ubiquitylation. We found that UBR5, Dyrk2 and the APC/C activator Cdh1 were all upregulated prior to cisplatin treatment in ovarian cancers from patients whose tumors were later shown to be cisplatin resistant. Conversely, MOAP-1 was downregulated in the resistant tumors, consistent with a role for MOAP-1 in cisplatin-induced ovarian cancer cell death. Accordingly, UBR5 depletion sensitized cultured ovarian cancer cells to cisplatin-induced Bax activation, and this Bax activation was dependent on MOAP-1. Moreover, depletion of UBR5 sensitized cisplatin-resistant cells to cisplatin-induced apoptosis. Taken together, these findings identify UBR5 as a new regulator of MOAP-1 and implicate this regulatory network in the sensitivity of ovarian cancers to cisplatin treatment.

RESULTS

Identification of UBR5 as a MOAP-1-interacting protein

We previously demonstrated that stability of the proapoptotic protein MOAP-1 was regulated by two ubiquitin E3 ligases, TRIM39 and APC/C^{Cdh1}.[8] In the previous work, we found that the APC/C^{Cdh1} targets and degrades MOAP-1 through ubiquitylation. Because DNA damage is known to increase MOAP-1 stability, in our current study, we sought to identify additional damage-controlled MOAP-1 interactors that might act upstream or downstream of MOAP-1.[7,9] To that end, Flag-tagged MOAP-1 was overexpressed in 293T cells that were cultured in the presence or absence of etoposide. As shown in Figure 1a, overexpressed MOAP-1 bound multiple proteins; among them a ~300-kD protein was found to be dissociated from MOAP-1 upon etoposide treatment (Figure 1a). We performed this experiment with cisplatin treatment and found a similar decrease in the silver stained ~300-kD band (Supplementary Figure S1A). Analysis by mass spectrometry identified this protein as the ubiquitin E3 ligase UBR5. The binding between overexpressed Flag-MOAP-1 and UBR5 was validated by co-immunoprecipitation (Co-IP) and immunoblotting using anti-UBR5 antibody (Figure 1b). We tested reciprocal Co-IP by overexpressing Flag-tagged UBR5 and confirmed endogenous MOAP-1 binding to Flag-UBR5 (Supplementary Figure S1B). In addition, we performed an *in vitro* binding assay using recombinant UBR5 and MOAP-1 proteins to demonstrate that these proteins interact directly (Figure 1c). These results suggest that UBR5 might, in addition to APC/C^{Cdh1}, be a regulator of MOAP-1 ubiquitylation.

UBR5 regulates MOAP-1 stability through ubiquitylation and degradation of MOAP-1

UBR5, also known as EDD or HYD, is an ubiquitin E3 ligase containing a HECT domain and known to mediate degradative ubiquitylation.[10–14] Knockdown of UBR5 induced accumulation of MOAP-1, consistent with UBR5 functioning in degradative ubiquitylation of MOAP-1 (Figure 2a). We validated the specificity of the UBR5 E3 ligase activity by monitoring the accumulation of PEPCK and DUBA, which are the previously reported substrates of UBR5.[11,15] The half-life of MOAP-1 has been reported to be ⩽~30 min.[7] In fact, MOAP-1 protein levels were rapidly decreased after addition of the protein translation inhibitor cycloheximide, while MOAP-1 stability was dramatically enhanced by UBR5 knockdown (Figure 2b).

We next examined whether the altered half-life of MOAP-1 following UBR5 knockdown resulted from UBR5-mediated MOAP--1 ubiquitylation. Flag-MOAP-1 was immunoprecipitated from 293T cells transfected with plasmid encoding Flag-tagged MOAP-1 to detect ubiquitylation. A typical laddering pattern demonstrating polyubiquitylation of MOAP-1 was observed (Figure 2c). This polyubiquitylation was dramatically decreased when UBR5 was depleted by small interfering RNA (siRNA)-mediated knockdown (Figure 2c). We also tested MOAP-1 ubiquitylation in human lung cancer H1299 cells and obtained a similar result (Supplementary Figure S2A). In order to determine whether UBR5 directly ubiquitylates MOAP-1, we assayed ubiquitylation *in vitro*. Recombinant MOAP-1 protein was incubated with E1, E2 (Ubc5Hb), recombinant UBR5 protein, ubiquitin and ATP. Polyubiquitylation of MOAP-1 was observed in the presence, but not the absence, of UBR5 (Figure 2d). In addition, a catalytically inactive mutant of UBR5 (C2768A) failed to ubiquitylate MOAP-1

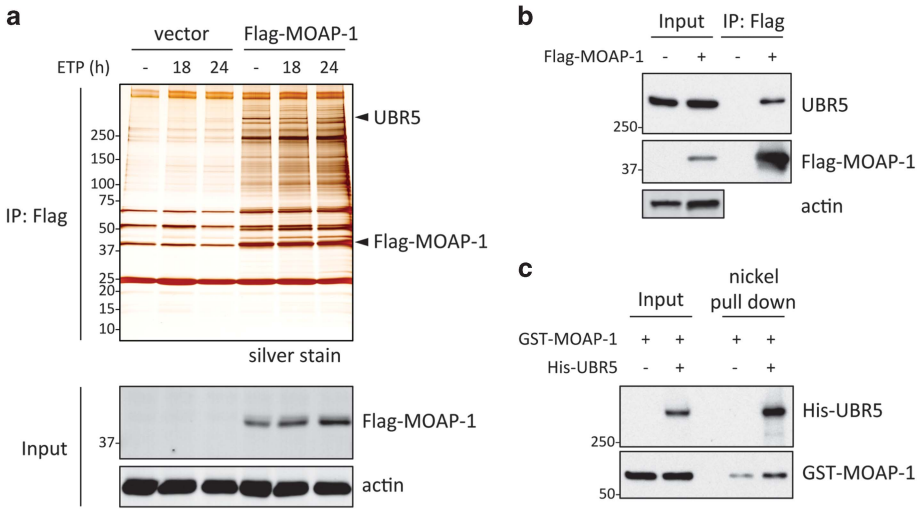

Figure 1. UBR5 is identified as a novel interacting factor of MOAP-1. (**a**) Flag-MOAP-1 was transfected into 293T cells, treated with or without 100 μM of etoposide (ETP) for 18 or 24 h and lysates were prepared for co-immunoprecipitation (Co-IP) with Flag M2 agarose beads. Co-IP samples were applied for SDS–PAGE and proteins were visualized by silver staining (top). Whole-cell lysates were immunoblotted with Flag antibody for Flag-MOAP-1 (bottom). $n=2$ independent experiments. (**b**) Transfection was performed as in panel (**a**), and Co-IP samples with Flag beads were immunoblotted as indicated. $n=3$ independent experiments. (**c**) GST-MOAP-1 recombinant protein was incubated with or without His-UBR5 recombinant protein on ice for 4 h, and nickel beads were added and incubated for 45 min. Beads were washed with 0.5% TritonX-100 wash buffer for five times. The proteins were immunoblotted with UBR5 or MOAP-1 antibody. $n=3$ independent experiments. Molecular weight markers are in kDa.

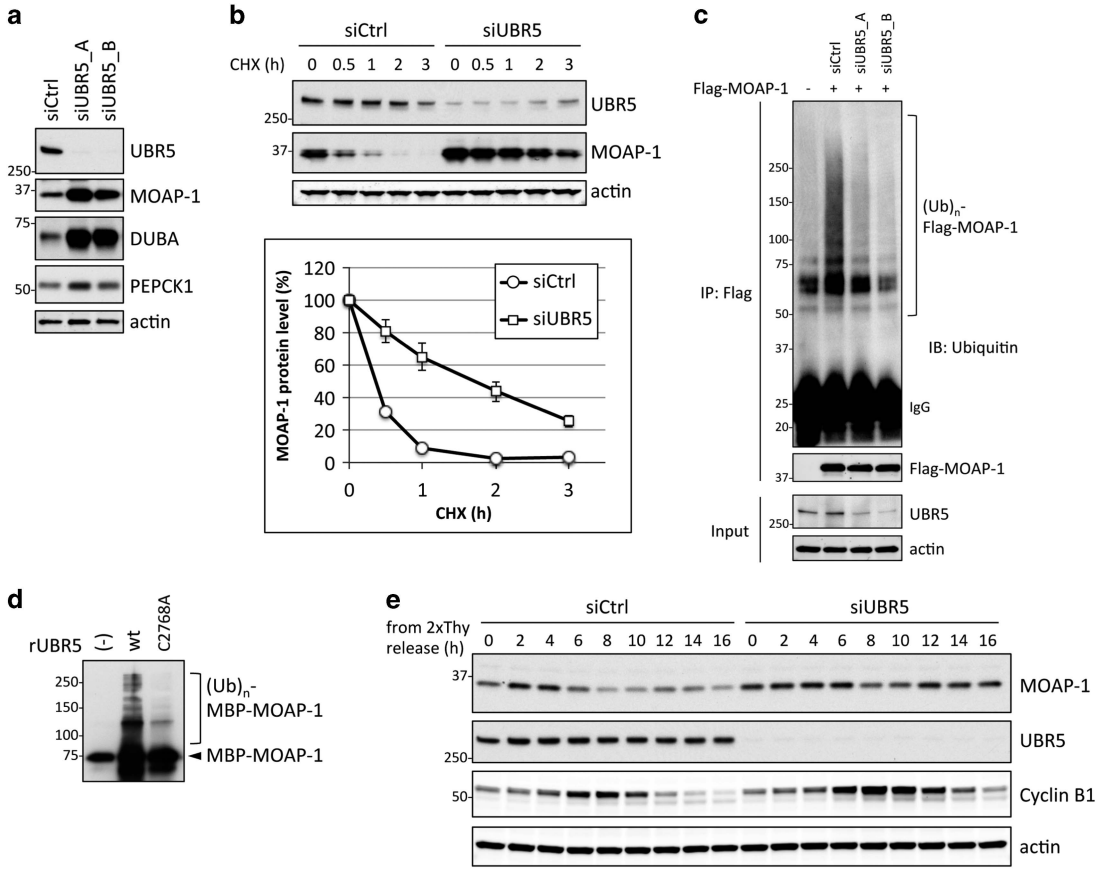

Figure 2. UBR5 regulates MOAP-1 protein stability by ubiquitylation. (**a**) 293T cells were transfected with control or UBR5-targeting siRNA (siCtrl, siUBR5_A or siUBR5_B). Forty-eight hours posttransfection, lysates were prepared and immunoblotted as indicated. $n=3$ independent experiments. (**b**) 293T cells transfected with siCtrl or siUBR5_A were treated with cycloheximide (CHX) 48 h posttransfection. Cells were collected at the indicated times after CHX treatment, and lysates were prepared and immunoblotted as indicated (top). MOAP-1 protein level was quantified and plotted (bottom). The MOAP-1 abundance at 0 time point was set at 100%. $n=3$ independent experiments (means ± s.e.m.). (**c**) siCtrl, siUBR5_A or siUBR5_B was transfected into 293T cells, and Flag-MOAP-1 was transfected into each siRNA transfectant 24 h posttransfection. Cell lysates were prepared under denaturing condition; Flag-MOAP-1 was immunoprecipitated and immunoblotted with ubiquitin antibody. Same membrane was re-blotted with Flag antibody for Flag-MOAP-1. $n=3$ independent experiments. (**d**) MBP-MOAP-1 recombinant protein was incubated with or without UBR5 recombinant protein in the presence of E1, E2 (UbcH5b), ubiquitin and ATP at 30 °C for 1 h. Active wild-type (wt) or catalytically inactive mutant (C2768A) of UBR5 recombinant protein was used. Reaction was immunoblotted with MOAP-1 antibody. $n=3$ independent experiments. Original uncropped image is shown in Supplementary Figure S6. (**e**) HeLa cells transfected with siCtrl or siUBR5_A were synchronized with double thymidine and released from G1/S phase. Cells were collected at the indicated time points after the release and lysates were immunoblotted with antibodies as indicated. $n=2$ independent experiments. Cell cycle data from the same experiment is shown in Supplementary Figure S2B. Molecular weight markers are in kDa.

robustly. These results indicate that UBR5 directly catalyzes ubiquitylation of MOAP-1 both in intact cells and *in vitro*.

UBR5 has been reported to regulate the S–G2 cell cycle checkpoint in response to irradiation or phleomycin, which induces DNA double-stranded breaks.[16] In addition, UBR5 regulates cell cycle progression by ubiquitylating the katanin p60 ATPase.[10] These observations led us to examine whether MOAP-1 regulation by UBR5 was dependent on the phase of the cell cycle. We synchronized cells at the G1/S boundary using a double thymidine block (Supplementary Figure S2B) and monitored MOAP-1 protein levels after cells were released from thymidine. MOAP-1 protein levels were relatively high at the 2- and 4-h time points after release (at early S phase); protein levels were then decreased at the 6–16-h time points (at late S to early G1 phase, as indicated by cyclin B1 protein levels). In contrast, when UBR5 was depleted by siRNA, MOAP-1 levels were maintained throughout G2 (or as long as time points were taken; Figure 2e), consistent with previous reports showing that UBR5 functions from S to G2 phases.[16] Although MOAP-1 degradation in G2 phase is abrogated by UBR5 knockdown, our previous study showed that ubiquitylation of MOAP-1 in G1 phase is mediated by the APC/C^{Cdh1} E3 ligase. This finding is consistent with the fact that the APC/C^{Cdh1} E3 ligase is most active during G1.[8,17] These data indicate that UBR5 is active and targets MOAP-1 for ubiquitylation and degradation during S–G2 phase, whereas the APC/C^{Cdh1} mediates degradation during G1 phase.

UBR5-containing EDVP E3 ligase complex interacts with and regulates MOAP-1 ubiquitylation and stability

It has been reported previously that UBR5 is a component of the E3 ligase EDVP complex, which includes UBR5, DDB1, VprBP and Dyrk2.[10] In fact, we found that Flag-MOAP-1 co-immunoprecipitated with endogenous DDB1, Dyrk2 and VprBP proteins (Figure 3a). Accordingly, overexpressed Flag-Dyrk2 also interacted with endogenous UBR5, VprBP and MOAP-1 (Supplementary Figure S3A). This raised the possibility that UBR5 functions within the EDVP E3 ligase complex to regulate MOAP-1. We examined the contribution of EDVP ligase components to MOAP-1 ubiquitylation *in vitro*. Indeed, addition of other EDVP components did enhance *in vitro* ubiquitylation of MOAP-1 (Figure 3b).

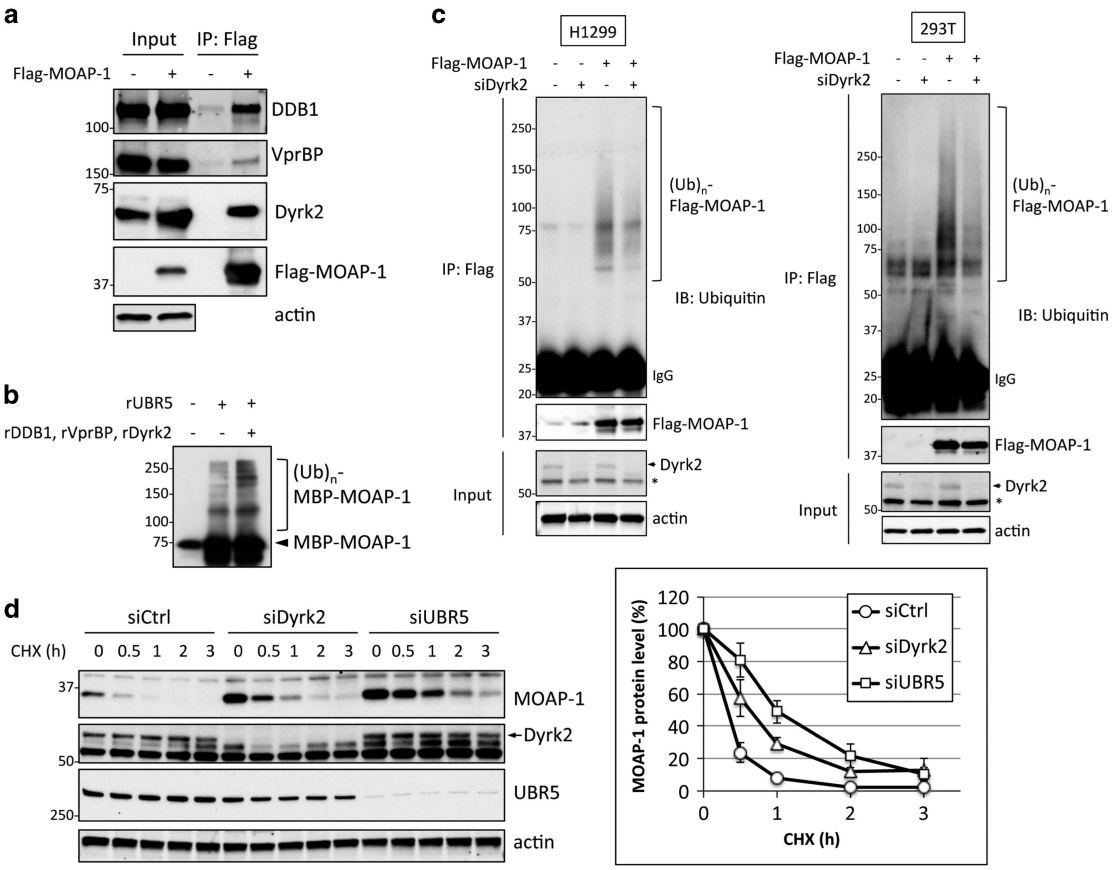

Figure 3. UBR5-containing EDVP E3 ligase complex interacts and regulates MOAP-1 ubiquitylation and stability. (**a**) Flag-MOAP-1 was transfected into 293T cells, and lysates were prepared 48 h posttransfection. Co-IP with Flag M2 agarose beads were performed and immunoblotted with antibodies as indicated. $n=3$ independent experiments. (**b**) *In vitro* ubiquitylation assay was performed as Figure 2d in the presence or absence of recombinant DDB1, VprBP and Dyrk2. $n=3$ independent experiments. Original uncropped image is shown in Supplementary Figure S6. (**c**) siCtrl or siDyrk2 was transfected into H1299 or 293T cells, and Flag-MOAP-1 was transfected into each siRNA transfectant 24 h post-siRNA transfection. Cell lysates were prepared under denaturing condition; Flag-MOAP-1 was immunoprecipitated and immunoblotted with ubiquitin antibody. Same membrane was re-blotted with Flag antibody for Flag-MOAP-1. $n=3$ independent experiments. Asterisk in Dyrk2 blot indicates non-specific band. Quantification of MOAP-1 ubiquitylation is shown in Supplementary Figure S3B. (**d**) 293T cells transfected with siCtrl, siDyrk2 or siUBR5 were treated with CHX 48 h posttransfection. Cells were collected at the indicated times after CHX treatment, and lysates were prepared and immunoblotted as indicated (left). MOAP-1 protein level was quantified and plotted (right). The MOAP-1 abundance at 0 time point was set at 100%. $n=4$ independent experiments (means ± s.e.m.). Molecular weight markers are in kDa.

Dyrk2 functions as a scaffold in the EDVP E3 ligase complex.[10,14] Therefore, we tested the effect of Dyrk2 knockdown on MOAP-1 ubiquitylation and found that depletion of Dyrk2 decreased MOAP-1 ubiquitylation in both H1299 and 293T cells (Figure 3c, Supplementary Figure S3B). Knockdown of Dyrk2 also induced MOAP-1 accumulation and enhanced MOAP-1 stability after cycloheximide treatment, although the effect was less dramatic than UBR5 knockdown (Figure 3d). These results strongly suggest that EDVP components function cooperatively with UBR5 to control the ubiquitylation and stability of MOAP-1.

UBR5 downregulation leads to MOAP-1 accumulation and potentiates cell death through the intrinsic apoptosis pathway

Knowing that UBR5 mediates MOAP-1 ubiquitylation, we then asked whether MOAP-1 protein stability controlled by UBR5 affects apoptosis. MOAP-1 protein levels were increased when UBR5 was knocked down in ovarian cancer A2780, prostate cancer PC-3 and lung cancer H1299 cells (Figure 4a, Supplementary Figure S4A). We then measured apoptosis in the A2780 cells. Consistent with our hypothesis, UBR5 knockdown increased apoptosis induced by cisplatin treatment in A2780 cells (Figure 4b). Importantly, additional knockdown of MOAP-1 diminished the apoptotic sensitization induced by UBR5 knockdown, indicating that the sensitization was mediated, at least in part, by MOAP-1 (Figure 4b). We performed a similar experiment examining cell death following knockdown of UBR5 and/or MOAP-1 in PC-3 and H1299 cells and observed similar results to those obtained using A2780 cells (Supplementary Figure S4B). Because MOAP-1 enhances Bax activation and the release of cytochrome *c* from mitochondria,[5,7] we examined the status of Bax activation using the anti-Bax 6A7 antibody, which specifically recognizes the active conformation of Bax. Consistent with the results of the apoptosis assay in Figure 4b, UBR5 knockdown increased cisplatin-induced Bax activation, whereas co-knockdown of both MOAP-1 and UBR5 decreased Bax activation to levels similar to those seen in the control knockdown cells (Figure 4c). These results indicate that UBR5 regulates the intrinsic apoptosis pathway through MOAP-1-mediated Bax activation.

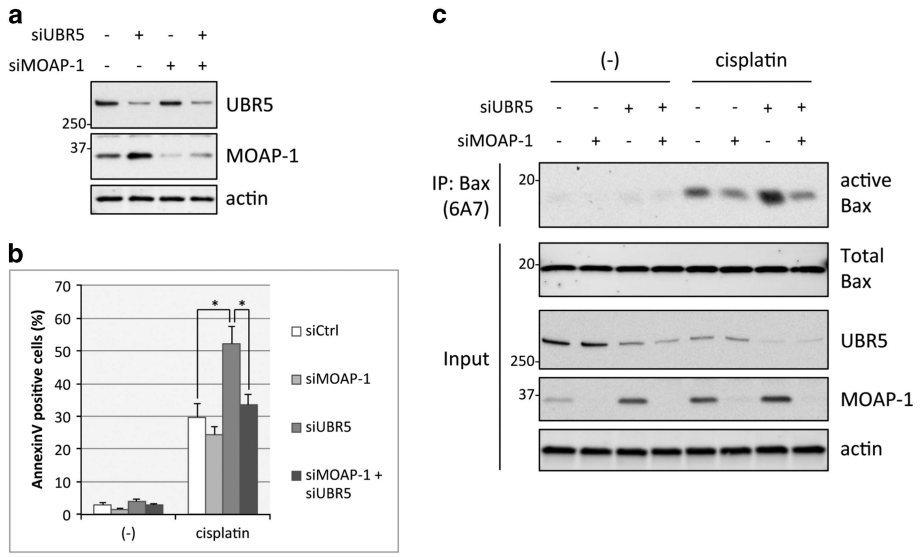

Figure 4. UBR5 knockdown enhances apoptosis dependent on MOAP-1. (**a**) A2780 cells were transfected with siCtrl, siUBR5, siMOAP-1 or siUBR5 and siMOAP-1. Cell lysates were prepared 48 h posttransfection and immunoblotted as indicated. $n = 3$ independent experiments. (**b**) A2780 cells were prepared as described in panel (**a**) and treated with or without 10 μM of cisplatin for 48 h and stained with Alexa Fluor 488-conjugated Annexin V for flow cytometric analysis. Percentage of apoptotic cells is indicated as Annexin V-positive cells. $n = 4$ independent experiments (means+s.e.m.). $^*P < 0.05$ by unpaired two-tailed *t*-test. (**c**) A2780 cells were transfected as in panel (**a**) and treated with or without 10 μM of cisplatin for 12 h. Cell lysates were used for IP with active Bax-specific antibody (6A7). IP samples were immunoblotted with total Bax antibody. $n = 3$ independent experiments.

UBR5–MOAP-1 pathway contributes to resistance of ovarian cancer

Platinum-based antineoplastic agents, such as cisplatin, are widely used to treat ovarian cancer; unfortunately, resistance to platinum-based therapy remains a significant clinical barrier for the treatment of patients with this disease.[18] To further understand whether the UBR5–MOAP-1 axis is involved in cisplatin resistance, we utilized pairs of cisplatin-sensitive and -resistant ovarian cancer cell lines derived from two ovarian cancer cell lines: A2780 and Tyknu. First, we measured MOAP-1 expression levels in these cells: although MOAP-1 accumulated in the sensitive cells following cisplatin treatment, cisplatin failed to robustly induce MOAP-1 in resistant cells (A2780CIS and Tyknu cisR) (Figure 5a). Of note, cisplatin treatment markedly decreased UBR5 protein in A2780 cells; however, this decrease was not observed in the cisplatin-resistant A2780CIS cells (Figure 5a, left). These results (as shown in Figure 5a) raised the possibility that the UBR5–MOAP-1 axis becomes lost in cisplatin-resistant cells. Of note, UBR5 knockdown induced MOAP-1 accumulation in both sensitive and resistant cells (Figure 5b), indicating that the UBR5–MOAP-1 axis remains present in these cells. Nonetheless, the induction of MOAP-1 was weaker in resistant cells. Based on these findings, we tested whether MOAP-1 induction by UBR5 knockdown could potentiate apoptosis in cisplatin-resistant cells. Interestingly, UBR5 knockdown significantly enhanced apoptosis in A2780CIS and Tyknu cisR cells in response to cisplatin treatment (Figure 5c). These data raise the possibility that UBR5 enzymatic activity (and not simply abundance) may be controlled in response to DNA damage in some cell types.

We then assessed the relationship between UBR5 reduction and MOAP-1 accumulation following cisplatin treatment in a broader range of ovarian cancer cells (OVCA432, OVCAR8, DOV13, ES-2 and SK-OV-3). Importantly, the reduction of UBR5 that was observed in the cisplatin-sensitive A2780 line in response to DNA damage was also observed in multiple other ovarian cancer cells (Supplementary Figure S5A). Although this trend is very clear, we observed some heterogeneity with regards to the linkage between UBR5 reduction and MOAP-1 elevation (Supplementary Figure S5A). Because MOAP-1 can be ubiquitylated by E3 ligases in addition to UBR5 (for example, APC/C^{Cdh1}), we suspect that, even following loss of UBR5 in some cell lines, it is possible that the presence of other active MOAP-1-directed ligases diminish the accumulation of MOAP-1.

Notwithstanding heterogeneity among cell lines, the promising pattern observed in paired sensitive and resistant cell lines prompted us to ask more directly whether the UBR5–MOAP-1 axis might be contributing to cisplatin resistance in a clinical setting. To this end, we obtained samples of ovarian tumors removed from patients prior to treatment with cisplatin. The tumors were later categorized as resistant or sensitive based on the eventual clinical responsiveness of the patient to treatment with platinum-based chemotherapy. We first stained patient samples with hematoxylin and eosin to demarcate areas for tumor microdissection, which enriched the tumor regions in the tissue extracts. We then assessed the expression level of UBR5, MOAP-1, Dyrk2 and Cdh1 by immunoblotting in these enriched samples. Interestingly, UBR5 was significantly elevated in patient tumor samples classified as 'cisplatin resistant' compared with those from patient tumor samples classified as 'cisplatin responsive' (Figures 5d and e). Dyrk2 and Cdh1 showed higher expression in cisplatin-resistant patient tissues as well. In line with this finding, MOAP-1 was slightly downregulated in cisplatin-resistant patient tissues, although we did not have sufficient samples to prove statistical significance. Immunohistochemical staining results were consistent with immunoblotting data, demonstrating increased UBR5 protein in cisplatin-resistant tumors and a trend towards increased MOAP-1 protein in cisplatin-responsive tumors (Figure 5f). These data support our hypothesis that UBR5 and Dyrk2 target MOAP-1 for degradation and suggest that this regulation may contribute to resistance to platinum therapy in ovarian cancer patients.

DISCUSSION

Regulation of MOAP-1 by both UBR5 and APC/C^{Cdh1}

It has been reported that UBR5 regulates several cell cycle-related factors. UBR5 is required for full CHK2 activation, which induces

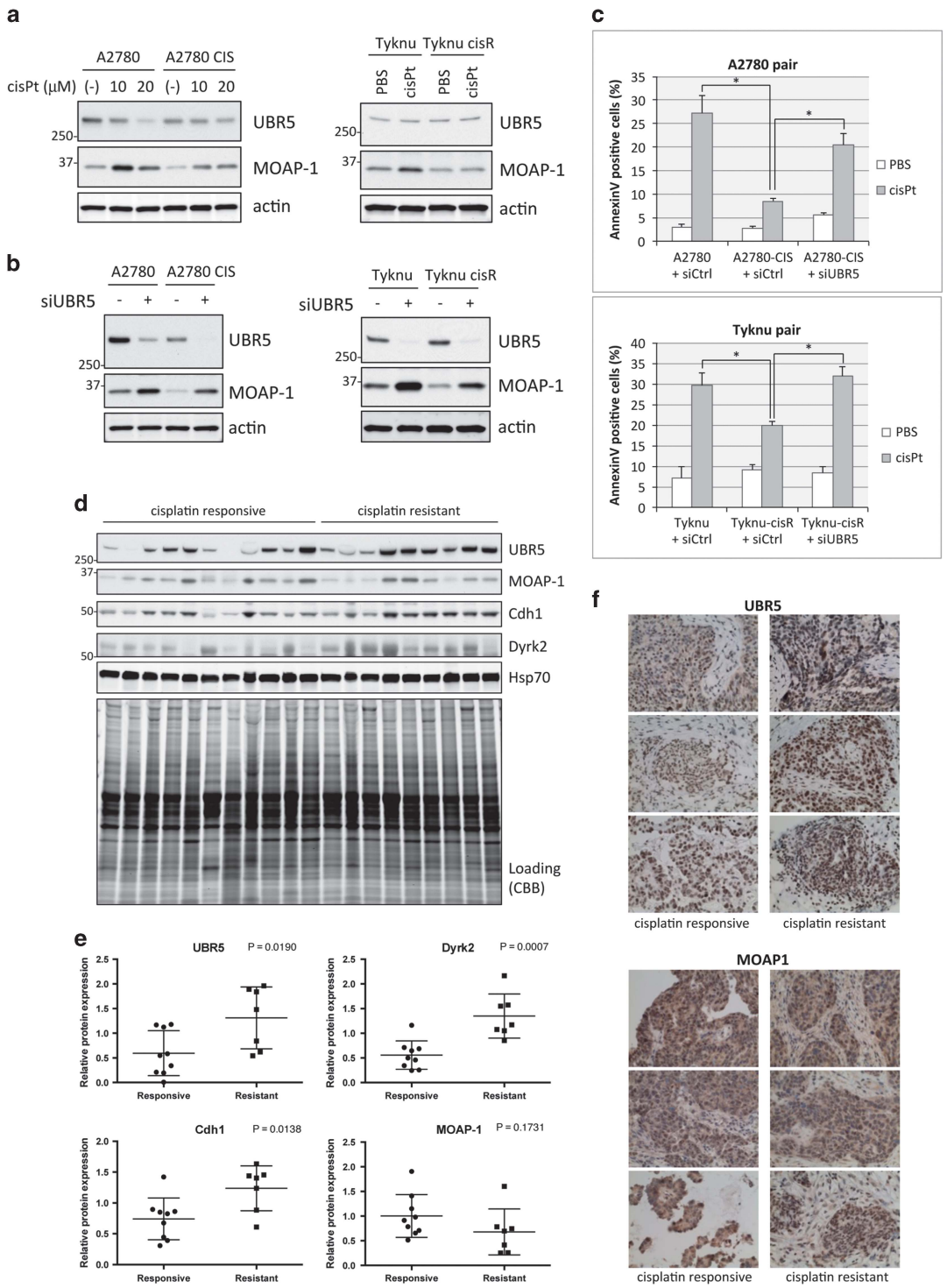

Figure 5. UBR5–MOAP-1 axis contributes to cisplatin resistance in ovarian cancer. (**a**) A2780 or A2780CIS cells were treated for 24 h with the indicated concentration of cisplatin (cisPt). Tyknu or Tyknu cisR cells were treated for 6 h with 10 μM of cisplatin. Cell lysates were prepared and immunoblotted as indicated. $n = 3$ independent experiments. (**b**) Pairs of sensitive and resistant of A2780 and Tyknu cells were transfected with siCtrl or siUBR5; lysates were prepared 48 h posttransfection and immunoblotted as indicated. $n = 2$ independent experiments. (**c**) Cells prepared as described in panel (**b**) were treated with 10 μM of cisplatin for 48 h. Cells were stained with Alexa Fluor 488-conjugated Annexin V and analyzed by flow cytometry. $n = 4$ independent experiments (means+s.e.m.). $^*P < 0.005$ (A2780 pair) and $P < 0.05$ (Tyknu pair) by unpaired two-tailed *t*-test. (**d**) Ovarian cancer tissue extracts from treatment-naive patients with high-grade serious ovarian cancer (from 11 or 9 patients, cisplatin-responsive or cisplatin-resistant group, respectively) were prepared as described in Materials and Methods section and immunoblotted as indicated. Additional information of ovarian cancer patients is shown in Supplementary Figure S5B. (**e**) Quantification results of panel (**d**). Band intensities were calculated by the ImageJ software and normalized based on Coomassie Brilliant Blue (CBB). The band intensities were plotted as means ± s.d. $^*P$-values by unpaired two-tailed *t*-test. (**f**) Representative images of immunohistochemical staining of UBR5 and MOAP-1 in tumor samples. Formalin-fixed, paraffin-embedded tissues were stained as described in Materials and Methods section.

the S and G2/M checkpoint in response to DNA stress.[16] UBR5 also contributes to p53 activation by regulating ATM.[19] In our study, under unstressed conditions, both UBR5 and APC/C^{Cdh1} appear to regulate MOAP-1 protein levels, albeit at different stages of the cell cycle. Previously, we reported that APC/C^{Cdh1} ubiquitylates and degrades MOAP-1 during G1 phase.[8] In contrast, UBR5-mediated MOAP-1 degradation occurs during the late S and G2 phases (Figure 2e). MOAP-1 ubiquitylation by APC/C^{Cdh1} requires the D-box domain, a Cdh1 recognition motif, which is located in the middle of the MOAP-1 protein.[8] Mutation of the D-box abolished the interaction between Cdh1 and MOAP-1, attenuating ubiquitylation of MOAP-1. However, the D-box mutant of MOAP-1 could still be ubiquitylated (data not shown). Tan *et al.*[6] could not identify any specific lysine residues of MOAP-1 on which ubiquitylation occur. Therefore, they concluded that MOAP-1 potentially undergoes ubiquitylation through an unconventional mechanism. UBR5 is a member of the family of UBR E3 ligases; these ligases contain a UBR motif for N-end rule-mediated ubiquitylation of substrates.[20] These facts raise the possibility that UBR5 may ubiquitylate MOAP-1 via N-end rule.

It seems that several ubiquitin E3 ligase complexes regulate MOAP-1 by targeting different recognition sites. This phenomenon of multiple E3 ligase regulation of a substrate is similar to what has been described in other proapoptotic proteins; for example, p53 is regulated by MDM2, Huwe1, TRIM39 and others.[21,22] Being regulated by two distinct ubiquitin E3 ligases allows fine control of MOAP-1 protein levels by different stimuli at different cell cycle stages, potentially allowing diverse apoptotic triggers to be enhanced through MOAP-1 and also helping to maintain low basal levels of MOAP-1 in order to avoid an accidental initiation of apoptosis.

Mechanism of MOAP-1 ubiquitylation by UBR5

As described above, UBR5 is a HECT family ligase that can function in cooperation with EDVP complex proteins.[10,23,24] In previous studies of the EDVP complex, its substrates, including katanin p60 and TERT, required prior phosphorylation by Dyrk2 for recognition by VprBP.[10,13] In addition, UBR5 has been reported to target several other proteins for ubiquitylation, including CDK9,[25] PEPCK1,[11] RNF168[12] and PXR;[14] although, it is not clear whether the EDVP E3 ligase complex is required for targeting these substrates. In this study, we showed that UBR5 knockdown reduced MOAP-1 ubiquitylation dramatically and recombinant UBR5 protein possessed direct ubiquitylation activity toward MOAP-1 *in vitro* (Figures 2c and d). In addition, MOAP-1 interacted with the EDVP components, DDB1, VprBP and Dyrk2, and *in vitro* ubiquitylation assay showed that the addition of the components enhanced ubiquitylation of MOAP-1 (Figure 3a and b). Our data suggest UBR5 is sufficient to bind and ubiquitylate MOAP-1 (Figures 1c and 2d). This is in contrast to other studies suggesting that all other components of the EDVP complex are necessary for ubiquitylation of UBR5's substrates.[10,13] We did find that knockdown of Dyrk2, the scaffold of the EDVP complex, significantly altered ubiquitylation of MOAP-1 and enhanced MOAP-1 stability (Figure 3c and d, and Supplementary Figure S3B), suggesting at least some participation of the EDVP E3 ligase complex *in vivo*.

Decrease of UBR5 activity and MOAP-1 accumulation after DNA damage

By using pairs of cisplatin-sensitive and -resistant cell lines as culture models, we found that MOAP-1 protein levels were highly induced by cisplatin treatment in sensitive cells but only slightly induced in resistant cells (Figure 5a). Downregulation of UBR5 occurred after cisplatin treatment and resulted in MOAP-1 accumulation in cisplatin-sensitive A2780 cells but much less in cisplatin-resistant A2780CIS cells (Figure 5a, left), indicating that UBR5 protein levels might contribute to the sensitivity to apoptotic stimuli. On the other hand, in Tyknu cells, UBR5 protein levels were not detectably altered by cisplatin treatment (Figure 5a, right). Nevertheless, UBR5 knockdown induced MOAP-1 accumulation in both pairs of A2780 and Tyknu cells. These data suggest that UBR5 activity, rather than just abundance, may be targeted by DNA-damaging agents. Decreases in either abundance or activity could culminate in the same outcome—MOAP-1 accumulation and apoptotic initiation. Interestingly, we found that multiple ovarian cancer cell lines showed decreased UBR5 protein after DNA damage (Supplementary Figure S5A). This decrease could be regulated by either transcriptional or post-translational mechanisms as some, but not all, of the cell lines showed a reduction of UBR5 mRNA levels following DNA damage (data not shown). Recently, it was reported that the deubiquitinase DUBA regulates ubiquitylation of UBR5.[15] Therefore, it would be interesting to determine whether DUBA regulates UBR5 protein levels or activity after DNA damage in ovarian cancer cells.

Involvement of UBR5 and MOAP-1 in cell death and cisplatin resistance in ovarian cancer

The *UBR5* gene is located on chromosome 8q, a region that is often mutated and/or overexpressed in several types of cancers, including breast cancer, hepatocellular carcinoma, squamous cell carcinoma of the tongue, metastatic melanoma[26] and mantle cell lymphoma.[27] Based on our observations, we postulate that *UBR5* gene alteration may have an effect on responsiveness of these tumors to chemotherapy. UBR5 was previously shown to be highly expressed in ovarian carcinoma and its expression level was associated with increased risk of disease recurrence and death in patients.[28] In addition to UBR5, we found that Dyrk2 and Cdh1 were also upregulated in cisplatin-resistant tumors. Despite the fact that a smaller difference of MOAP-1 protein level between cisplatin-sensitive and -resistant cancer tissues was observed (Figures 5d–f), the basal expression of MOAP-1 was found to be very low by immunohistochemical analysis across a large panel of cancer tissues.[29] This is consistent with recent data showing that MOAP-1 expression is downregulated in several types of cancer.[30] From our data, the increased expression of UBR5, Dyrk2 and Cdh1 might be useful as indicators to predict chemoresponsiveness in ovarian cancer. Most importantly, targeting UBR5, Dyrk2 and Cdh1 might be beneficial in sensitizing cancer cells to chemotherapy. In this study, we have shown that the proapoptotic activity of MOAP-1 is regulated by the E3 ligase activity of UBR5. However, it has also been reported that UBR5 upregulates the antiapoptotic protein Mcl-1 independently of its E3 ligase activity.[31] Therefore, UBR5 may regulate cell death in several intriguing ways that can affect responsiveness to chemotherapy.

MATERIALS AND METHODS

Reagents and cell culture

Etoposide, cisplatin, cycloheximide, thymidine, Anti-Flag M2 Affinity Gel (Cat. no. A2220), *N*-ethylmaleimide were purchased from Sigma (St Louis, MO, USA). MG-132 was from Calbiochem (Billerica, MA, USA). AlexaFluor488-conjugated Annexin V (Cat. no. A13201) and Lipofectamine RNAiMAX Transfection Reagent (Cat. no. 13778150) were from Invitrogen (Grand Island, NY, USA). X-tremeGENE 9 DNA Transfection Reagent (Cat. no. 06365809001) was from Roche (Indianapolis, IN, USA). Nickel-NTA Agarose (Cat. no. 30210) was from Qiagen (Germantown, MD, USA). A2780 and A2780CIS cells were purchased from Sigma. PC-3, H1299, ES-2, SK-OV-3, HeLa and 293T cells were obtained from Duke Cell Culture Facility (Durham, NC, USA). Tyknu, Tyknu-cisR, OVCA432, OVCAR8 and DOV13 cells were kindly provided by Dr Susan Murphy (Duke University).

Antibodies

Antibodies used in this study are as follows: MOAP-1 rabbit polyclonal (Cat. no. HPA000939, Sigma/Atlas antibodies, Stockholm, Sweden), UBR5 rabbit polyclonal (Cat. no. 8755, Cell Signaling Technology, Danvers, MA, USA), VprBP

rabbit polyclonal (Cat. no. A301-887A, Bethyl Laboratories, Montgomery, TX, USA), DDB1 rabbit polyclonal (Cat. no. A300-462A, Bethyl Laboratories), DUBA rabbit polyclonal (Cat. no. A302-919A, Bethyl Laboratories), Flag M2 mouse monoclonal (Cat. no. F1804, Sigma), Flag rabbit polyclonal (Cat. no. PA1-984B, Thermo Scientific, Waltham, MA, USA), Cdh1 mouse monoclonal (DH01, Cat. no. MS-1116-P, Thermo Scientific), Cyclin B1 mouse monoclonal (GNS1, Cat. no. sc-245, Santa Cruz Biotechnology, Dallas, TX, USA), β-actin mouse monoclonal (Cat. no. ab8224, Abcam, Cambridge, UK), actin rabbit polyclonal (I-19-R, Cat. no. sc-1616-R, Santa Cruz Biotechnology), Dyrk2 rabbit polyclonal (Cat. no. ab37912, Abcam or Cat. no. 8143, Cell Signaling Technology), PEPCK1 rabbit monoclonal (D12F5, Cat. no. 12940, Cell Signaling Technology), ubiquitin mouse monoclonal (P4D1, Cat. no. sc-8017, Santa Cruz Biotechnology), active Bax 6A7 mouse monoclonal (Cat. no. 556467, BD Biosciences, Franklin Lakes, NJ, USA), Bax rabbit polyclonal (N-20, Cat. no. sc-493, Santa Cruz Biotechnology), Hsp70 mouse monoclonal (Cat. no. 610607, BD Biosciences).

Plasmids

MOAP-1 expression constructs were described previously.[8] pCMV-Tag2B EDD, which encodes Flag-UBR5, was a gift from Darren Saunders and Charles Watts (Addgene, Cambridge, MA, USA, plasmid no. 37188).[32] pDEST12.2 DYRK2 WT, which encodes Flag-Dyrk2, was a gift from Anjana Rao (Addgene plasmid no. 20005).[33]

siRNA

siRNA sequences used in this study are as follows: siCtrl directed against firefly luciferase (5'-CGUACGCGGAAUACUUCGA-3'): siUBR5_A (5′-CAA CUUAGAUCUCCUGAAA-3′): previously described,[10] siUBR5_B (5′-GAUUGU AGGUUACUUAGAA-3′), siDyrk2_A (5′-UCACGUGGCUUACAGGUAU-3′), and siDyrk2_B (5′-GGCCUACGAUCACAAAGUC-3′): previously described.[13] siRNA for MOAP-1 was purchased from Dharmacon (Lafayette, CO, USA; Cat. no. L-004430-00). siRNA transfection was performed following the manufacturer's protocol of Lipofectamine RNAiMAX Transfection Reagent. In all, 20 nM (final concentration) of siRNA was transfected twice in a 24-h interval.

Co-IP and mass spectrometric analysis

Plasmid encoding Flag-tagged MOAP-1 was transfected into 293T. Twenty-four hours posttransfection, cells were treated with or without 100 μM of etoposide for another 18 or 24 h. Cells were collected and lysed in 0.5% TritonX-100 lysis buffer (0.5% TritonX-100, 20 mM Hepes (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT) supplemented with aprotinin, leupepcin, phenylmethanesulfonylfluoride and phosphatase inhibitor cocktails (Sigma) and then Flag-MOAP-1 and its interacting protein was co-immunoprecipitated by using Flag M2 agarose beads. Co-IP samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein was visualized with Pierce Silver Stain Kit for Mass Spectrometry (Thermo Scientific). The bands of interest were analyzed by mass spectrometry at Duke Proteomics and Metabolomics Core Facility (Durham, NC, USA).

Cell cycle synchronization by double thymidine block

Double thymidine block in HeLa cells was performed as described previously.[8] Cells were plated at about 40% confluence. In all, 2 mM of thymidine was added, and cells were cultured for 19 h. Thymidine was washed out with phosphate-buffered saline twice, and cells were cultured 9 h. Then thymidine was added back, and cells were cultured for 16 h. Cells synchronized at the G1/S boundary were released by washing with phosphate-buffered saline twice and subsequently collected every 2 h. Cell cycle synchronization was confirmed by propidium iodide staining and flow cytometric analysis by FACScan Analyzer (BD Biosciences).

Detection of ubiquitylation *in vivo*

Immunoprecipitation under denaturing conditions was performed as described previously with some modifications.[34] Briefly, Flag-MOAP-1-expressing cells were suspended in denaturing cell lysis buffer (1% SDS, 1% TritonX-100, 150 mM NaCl, 10 mM Tris (pH 8.0), 2 mM EDTA) containing 10 mM of *N*-ethylmaleimide and proteinase inhibitors. Cell lysates were boiled at 100 °C, and DNA was sheared by sonication, following dilution with lysis buffer without SDS. Lysates were immunoprecipitated with anti-Flag M2 agarose. The samples were analyzed by SDS–PAGE and immunoblotted with anti-ubiquitin antibody.

Recombinant protein

Gateway system plasmids of UBR5, VprBP, DDB1 and Dyrk2 are kindly provided from Dr Maddika (The Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India).[10] Coding regions of UBR5, VprBP or DDB1 were transferred to pDONR221 using BP Clonase (Invitrogen) and obtained entry plasmids. Then genes were transferred to pDEST10 using LR Clonase (Invitrogen). The destination plasmids were transformed into DH10Bac competent cells to obtain bacmids coding each gene with an N-terminal His_6-tag. Each bacmid was transfected to SF9 cells with FuGENE HD transfection reagent (Promega, Madison, WI, USA), and the resultant baculovirus was repeatedly amplified for high titer. The virus was infected into SF9 cells, and His_6-tagged recombinant protein was purified using Nickel-NTA Agarose. GST-Dyrk2 was produced in Rosetta2(DE3)pLysS competent cells (EMD Millipore, Billerica, MA, USA) and purified by using glutathione sepharose beads (GE Healthcare, Little Chalfont, UK). MBP- or GST-tagged MOAP-1 recombinant protein was described previously.[8]

In vitro ubiquitylation assay

Recombinant MBP-MOAP-1 protein was incubated in ubiquitylation assay buffer (25 mM Hepes (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.05% TritonX-100, 1 mM DTT, 5 mM ATP) with or without recombinant UBR5 (wild-type or C2768A mutant), E1, E2-UbcH5b and ubiquitin (Boston Biochem, Cambridge, MA, USA) at 37 °C for 2 h. The reaction was stopped by adding SDS sample buffer and boiling at 100 °C for 5 min. Samples were run on SDS–PAGE and ubiquitylation of MOAP-1 was detected by immunoblotting with anti-MOAP-1 antibody.

Protein extraction from human tumor tissues of ovarian cancer patients and patient information

All tissue specimens were obtained from the Duke Gynecology Oncology Tissue Bank (Durham, NC, USA), with patient consent and were used under a protocol approved by the Duke Institutional Review Board. Frozen specimens from $N = 20$ women with serous epithelial ovarian cancer were used in this study and were acquired between 1999 and 2002. Specimens were received at initial surgery prior to initiation of chemotherapy. All patients received platinum-based combination chemotherapy postoperatively. Patients were classified as cisplatin-responsive or -resistant based on response to cisplatin treatment following tumor debulking. Patients with progression-free interval >6 months from stopping treatment were defined as 'cisplatin-responsive' whereas patients with progression-free interval <6 months were defined as 'cisplatin-resistant'. Additional information about the patients (age, FIGO (International Federation of Gynecology and Obstetrics) stage, histological types and cancer antigen 125) is shown in Supplementary Figure S5B. Tissues were stored in approximately 1 mm^3 segments in cryo-OCT block. Prior to extraction, tissues were graded for percentage of tumor relative to stroma. To prepare tissue homogenates, samples were lysed in RIPA Buffer (Cat. no. R0278, Sigma), supplemented with Halt protease and phosphatase inhibitor (Cat. no. 78440, Thermo Scientific), using Fast Prep Beadbeater tubes. Samples were processed using a Mini Beadbeater (BioSpec Products, Bartlesville, OK, USA) for 20 s at 25 r.p.m. × 100 s. Samples were then centrifuged at 10 000 r.p.m. for 3 min. Tissue supernatant was transferred to precooled microfuge tube and maintained under constant agitation for 30 min at 4 °C. Samples were centrifuged at 12 000 r.p.m. for 20 min. Supernatant was collected and analyzed by immunoblotting.

Immunohistochemistry

Three representative cisplatin-responsive and three representative cisplatin-resistant tumor tissues were used for staining. Formalin-fixed and paraffin-embedded tissues were prepared, sectioned and stained as previously described.[35] UBR5 and MOAP-1 were detected using rabbit polyclonal antibodies against UBR5 and MOAP-1, respectively.

Statistical methods

Statistical significance for all comparisons was determined using unpaired, two-tailed Student's *t*-test. Exact number of sample size (*n*) is indicated in the figure legend. Values are shown as mean ± s.e.m. For human tumor tissues, immunoblotting results were quantified by the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Signal intensity of each protein band was normalized using the signal intensity of Coomassie brilliant blue staining of each gel lane. Statistical analysis was performed using the Prism software (GraphPad Software, La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

KM, N-JH, LZ and SK designed the experiments and analyzed the data. KM, N-JH, KC and SK wrote the manuscript. KM and N-JH performed most of the experiments with help from KC for the experiments with ovarian cancer patient samples.

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