**Abstract**

The genotoxic cisplatin is commonly used in chemotherapy to treat solid tumors, yet our understanding of the mechanism underlying the drug response is limited. In a focused siRNA screen, using an siRNA library targeting genes involved in ubiquitin and ubiquitin-like signaling, we identified the E3 ubiquitin ligase HOIP as a key regulator of cisplatin-induced genotoxicity. HOIP forms, with SHARPIN and HOIL-1L, the linear ubiquitin assembly complex (LUBAC). We show that cells deficient in the HOIP ligase complex exhibit hypersensitivity to cisplatin. This is due to a dramatic increase in caspase-8/caspase-3–mediated apoptosis that is strictly dependent on ATM–, but not ATR–mediated DNA damage checkpoint activation. Moreover, basal and cisplatin–induced activity of the stress response kinase JNK is enhanced in HOIP-depleted cells and, conversely, JNK inhibition can increase cellular resistance to cisplatin and reverse the apoptotic hyperactivation in HOIP-depleted cells. Furthermore, we show that HOIP depletion sensitizes cancer cells, derived from carcinomas of various origins, through an enhanced apoptotic cell death response. We also provide evidence that ovarian cancer cells classified as cisplatin-resistant can regain sensitivity following HOIP downregulation. Cumulatively, our study identifies a HOIP-regulated antiapoptotic signaling pathway, and we envisage HOIP as a potential target for the development of combinatorial chemotherapies to potentiate the efficacy of platinum-based anticancer drugs.

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**Introduction**

Platinum-based agents such as cisplatin and carboplatin are commonly used in chemotherapy. They display therapeutic activity against a wide variety of solid tumors such as testicular, ovarian, and non–small cell lung cancers (1). Cisplatin is a potent genotoxic that initially interferes with proliferation of the tumor cell (antiproliferative activity) but ultimately induces apoptosis in the tumor cell (cytotoxic activity). Despite the successful application of this drug over the last 30 years, side effects, in particular myelosuppression, nephrotoxicity, and neurotoxicity, remain the limiting factors for its therapeutic efficacy. Furthermore, efficient therapy is frequently challenging because tumor cells are either intrinsically resistant or acquire resistance to platinum drugs (2). In particular, resistance to platinum-based drugs remains a major problem in the management of advanced ovarian cancer. High-grade serous ovarian cancers frequently exhibit a complete clinical response to platinum-based drugs after initial chemotherapy. However, the majority of these patients will relapse with a progressive development of platinum drug resistance, resulting in overall poor prognosis. Hence, the underlying resistance mechanisms to these chemotherapeutic drugs are subject to intense studies (3).

Cisplatin reacts directly with DNA, inducing DNA inter- and intrastrand cross-link lesions (ICL) in proliferating tissues, and thereby triggering a DNA damage response (1). ICL repair is complex, involving multiple DNA-repair pathways, and their coordination and regulation relies heavily upon the interplay between phosphorylation and ubiquitin signaling (4–6). Initial recognition and removal of the ICL is coordinated by the Fanconi anemia tumor suppressor pathway (7), which targets the FANC/FANCD2 complex for mono-ubiquitylation (8). Subsequently, the mono-ubiquitylated FANC/FANCD2 complex promotes the removal of ICL lesions by translesion synthesis (TLS), as well as homologous recombination–dependent repair mechanisms (7). Moreover, DNA double-strand breaks (DSB) have been identified as intermediates in ICL repair during S phase, and their formation involves the activity of the Mus81–Eme1 endonuclease (9). DSB repair is initiated by the ATM (ataxia-telangiectasia mutated) checkpoint kinase-dependent phosphorylation of MDC1 (mediator of DNA damage checkpoint), and the subsequent assembly of an ubiquitin-signaling complex, consisting of the E3 ubiquitin ligases RNF8...
and RNF168 (4, 10). RNF168 and RNF8 cooperate with BRCA1 to promote repair of DNA lesions (4, 11). Cancers with defects in the ICL-repair pathway, which are frequently associated with BRCA1 and BRCA2/FANCDD1 deficiency, are hypersensitive to DNA cross-linking agents, including cisplatin. Hence, this provides a strong rationale to use cisplatin for the treatment of BRCA1/2 mutated tumors.

Less is known about the determinants and pathways that sense excessive DNA cross-link damage, and that activate the apoptotic cell death pathway that eliminates irreversibly damaged cells. The tumor suppressor p53, a transcription factor, is stabilized and activated by genotoxic stress and triggers multiple effector pathways, including the intrinsic (also known as mitochondrial) apoptotic pathway (reviewed in ref. 12). To induce apoptosis, activated homotetrameric p53 directly transcriptionally upregulates the expression of several proapoptotic BCL-2 family members such as PUMA, NOXA, and BAX. There is increasing evidence that excessive DNA damage, including cisplatin-induced lesions, can also activate the extrinsic death receptor apoptosis pathway (reviewed in refs. 13, 14). This pathway is triggered by the binding of death ligands of the TNF family to their cognate receptors, and the subsequent assembly of the receptor-associated death-inducing signaling complex (DISC). Initiator caspases 8 and 10 are activated within the DISC, and the death signal is amplified by the subsequent proteolytic activation of downstream effector caspases 3 and 7. Cisplatin treatment can also induce sustained activation of JNK (c-Jun N-terminal kinase), which triggers high levels of the transcription factor AP-1 (15). This leads to the expression of the FAS-L (FAS ligand) and consequently results in FAS receptor-mediated apoptosis.

The identification of novel determinants of the cellular response to cisplatin and other platinum-based cancer drugs might extend their clinical application, as well as provide potential insight into drug resistance mechanisms. The use of RNA interference (RNAi) as a tool for specifically silencing genes has opened up the possibility of performing high throughput loss-of-function and synthetic lethality screens. We have used a collection of siRNA pools targeting the expression of genes involved in ubiquitin and ubiquitin-like (UBL) signaling to address whether ablation of expression of these proteins can cause cisplatin hypersensitivity. The screen was carried out using human osteosarcoma U2OS cells, which are p53 proficient and exhibit a robust DNA damage response (16). We identified the RING-in-between-RING E3 ubiquitin ligase HOIP as a novel antiapoptotic regulator in response to cisplatin-induced genotoxicity.

Materials and Methods

U2OS cells used in the siRNA screen were grown in phenol red–free Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine. The cell lines A2780, ZR.75.1, MDA-MB-231, HEK293, HeLa, HCT116, PC3, and U2OS cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Mouse embryonic fibroblasts (MEF) were obtained from Prof. Philip Cohen (University of Dundee, Dundee, United Kingdom) and maintained as for HEK293 cells, with the addition of 1% sodium pyruvate and 1% nonessential amino acids. Stably transfected Flp-In T-Rex-293 cells were generated according to the manufacturer’s instructions (Invitrogen). A2780 and A2780 cisplatin-resistant cell lines were a kind gift from Dr. Gillian Smith (University of Dundee, Dundee, United Kingdom). PEA1, PEA2, PEO14, and PEO23 cell lines were a kind gift from Dr. Simon Langdon (University of Edinburgh, Edinburgh, United Kingdom). These cell lines were regularly examined for morphology and tested for cisplatin sensitivity status. All other cell lines were obtained from the American Type Culture Collection, where they were authenticated using short tandem repeat analysis, and passaged for a maximum of 2 months post resuscitation. All cell lines used were tested regularly for mycoplasma contamination.

Cell proliferation assay

HEK293 cells were seeded at 25% to 30% confluence in 10 cm² dishes and treated with the indicated siRNA for 48 hours before being seeded at 5,000 cells per well in a 96-well plate; five wells seeded for each dose of cisplatin. Cells were allowed to adhere for a minimum of 8 hours before addition of 0 to 5 μg/mL cisplatin. Cells were grown for 72 to 96 hours before MTS assays (Promega) were performed according to manufacturer’s instructions. Proliferation assays for all other cell lines were carried out as for HEK293 cells, except that siRNA treatments were performed on cells seeded in a 96-well plate.

Clonogenic survival assays

Clonogenic survival assays were performed in triplicate. Cells were seeded at 25% confluence and allowed to adhere before being transfected with the indicated siRNA for 48 hours. Cells were then seeded in 10 cm² dishes at 5,000 cells per dish and allowed to adhere before being treated with the indicated concentrations of genotoxic for 24 hours. Cells were then incubated in fresh medium for 10 days and colonies containing more than 50 cells were counted.

Cell lysis and immunoblot analysis

Whole-cell extracts were prepared from mammalian cells by lysis in 40 mmol/L HEPES pH 7.4, 120 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, and HALT protease and phosphatase inhibitor cocktail (Pierce Biotechnology). Clarified protein lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. ImageJ software was used to quantify immunoblots, and at least three independent experiments were used to obtain results.

Caspase activity in vitro assay

Cells were seeded at 25% to 30% confluence in 10 cm² dishes and treated with the indicated siRNA for 48 hours before plating 10,000 cells per well of a 96-well plate, in triplicate for each condition. Cells were grown in phenol red–free DMEM and were replica plated on a 96-well plate for MTS assays.
Caspase activity was measured 48 hours after treatment with the indicated dose of cisplatin, using the Caspase-Glo 3/7 or Caspase-Glo 8 assays (Promega), according to the manufacturer’s instructions. Caspase activity was normalized with respect to cell number per well, as calculated by an MTS assay (Promega).

**Immunofluorescence analysis**

Cells were treated with the indicated dose of genotoxic for the times indicated in the figure legends, and processed as described previously (17).

**JNK in vitro kinase assay**

JNK kinases were immunoprecipitated from 100 μg of HEK293 cell whole-cell lysate, treated as indicated in the figure legend, using 3 μg of anti-JNK1 antibody coupled to 10 μL protein G Sepharose. Immunoprecipitates were washed thoroughly in cell lysis buffer and then equilibrated in kinase assay buffer (50 mmol/L Tris/HCl pH 7.5, 0.1 mmol/L EGTA and 0.1% 2-mercaptoethanol). Assays were performed as described previously (18) using a peptide corresponding to GST-ATF2 amino acids 19 to 96, at a concentration of 0.2 mg/mL as a substrate.

**NF-κB luciferase reporter assay**

Cells to be analyzed for NF-κB activation were seeded at 25% to 30% confluence in 6-well plates and treated with the indicated siRNA for 24 hours, or induced with tetracycline for 24 hours, before transfection with 3 μg NF-κB ConA luciferase reporter plasmid. After 24 hours, the indicated concentrations of cisplatin were added to cells and luciferase activity was measured 24 or 48 hours later, as indicated. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Assays were performed in triplicate and luciferase signals were normalized with respect to the cell lysate protein concentration.

**Results**

**siRNA screen identifies HOIP as an enhancer of cisplatin-induced cytotoxicity**

We designed a robust, high-throughput RNAi platform to screen for enhancement of cisplatin-induced cell death in the human osteosarcoma cell line U2OS. We used an siRNA library targeting 1,067 human genes, which are either validated or computationally predicted components related to the ubiquitin- and UBL signaling machinery (Supplementary Data). These include ubiquitin, SUMO, NEDD8, E1s, E2s, E3s, UBL-specific proteases, and UBL-binding domain-containing proteins (siRNA ‘ubiquitome’ library). The design of our enhancer screen is outlined in Fig. 1A. Briefly, U2OS cells were reverse transfected in replicates with a library of siRNA pools (SMARTPools). Each plate contained nontransfected cells, negative control (siCON, nontarget), and cells transfected with siRNA against the positive control REV1L (siREV1L), a TLS polymerase required for ICL repair (19). Sixteen hours after transfection, one replica was treated with 3 μmol/L cisplatin and the other replica with the vehicle, dimethyl sulfoxide (DMSO). Cells were incubated for further 72 hours and viability of cells was assayed using an ATP-dependent cell viability assay. To quantify the robustness of this assay system, we calculated the Z’ factor. The average factor for our entire screen was Z’ = 0.58, indicating an excellent assay performance. The screen was completed in duplicate. First, data were filtered for lethal siRNA by calculating cell growth (CGE) and rejecting siRNAs causing CGE ≤ 50%. Second, we used a log2 surviving fraction (log2SF) threshold of −1.47 or less to identify 112 siRNAs that significantly sensitized cells to cisplatin (Supplementary Data).

siRNAs from the primary screen were subjected to a secondary screen in which cells were treated with 0 to 6 μmol/L of cisplatin (Supplementary Fig. S1A). This was performed in triplicate, and dose-dependent log2SF functions were plotted to determine the maximum log2SF for each siRNA (Fig. 1B). Setting the threshold of the maximum log2SF values to −1.07, we identified, with high confidence, 14 siRNAs that sensitized U2OS cells to cisplatin.

To test for off-target effects, the 14 siRNAs were assayed separately with each of the four different siRNA species from the SMARTPool that target the same gene. Such siRNAs were considered to function on-target when at least three of the four individual siRNAs caused enhanced cisplatin sensitivity. By applying these strict criteria, we identified the E3 ubiquitin ligase HOIP (RNF31) as being required for cellular resistance to cisplatin (Supplementary Fig. S1B). HOIP is the catalytic subunit of the E3 ubiquitin ligase linear ubiquitin assembly complex (LUBAC), which also consists of the components HOIL-1L and SHARPIN (20, 21). LUBAC is a critical regulator of the canonical NF-κB signaling pathway, and has been implicated in inflammatory diseases and immune regulation (22). No role, however, has been suggested for HOIP in response to cisplatin. We therefore chose to examine the biological significance of HOIP depletion, and the mechanisms impacting cisplatin hypersensitivity.

As further validation, we analyzed cisplatin sensitivity in mouse embryonic fibroblast (MEF) cell lines obtained from paired wild-type and HOIpc879s/c879s E3 ligase-dead knockin mice, in which there is no LUBAC activity and no detectable linear ubiquitin chain formation (21). The HOIpc879s/c879s MEFs exhibited cisplatin hypersensitivity, indicating that HOIP ligase activity is required for cellular resistance to cisplatin (Fig. 1C). In addition, we repeated cisplatin sensitivity assays using transformed human embryonic kidney 293 (HEK293) cells. HOIP depletion (siHOIP) was confirmed by immunoblot analysis and resulted in enhanced cisplatin sensitivity when compared with cells transfected with vehicle only (siCON; Fig. 1D and Supplementary Fig. S1C). Consistent with published data, we noted that depletion of HOIP in HEK293 and U2OS cells resulted in destabilization of the LUBAC complex members HOIL-1L and SHARPIN (Supplementary Fig. S1D and S1E; refs. 23–25). siRNA targeting FAN1, a structure-specific nuclease required for interstrand cross-link repair, was used as control for cisplatin hypersensitivity (26).

**HOIP is not required for DNA damage checkpoint activation**

To uncover the mechanisms underlying cisplatin hypersensitivity of HOIP-depleted cells, we first analyzed the integrity of
early DNA damage signaling mediated by the checkpoint kinases ATM and ATR. Cisplatin-induced phosphorylation of SMC1 on serine 966, a target of ATM, and phosphorylation of CHK1 kinase on serine 345, a substrate of ATR, were unaffected by HOIP depletion (siHOIP) in HEK293 cells (Fig. 2A). These results indicate a robust and timely DNA damage checkpoint activation. The presence of cisplatin-induced DNA lesions triggers the checkpoint kinase-dependent phosphorylation of the histone variant H2AX (γH2AX). Distinct nuclear foci of γH2AX can be visualized at sites of damage, allowing monitoring of DNA repair (27, 28). To test the possibility that accumulation of persistent DNA damage is the cause of cisplatin hypersensitivity, we monitored γH2AX foci formation in mock (siCON) and HOIP-depleted (siHOIP) HEK293 cells, following cisplatin exposure and recovery over 72 hours (Fig. 2B and C). HOIP-depleted cells show kinetics of accumulation and resolution of γH2AX foci similar to those of mock transfected cells, suggesting that cisplatin hypersensitivity after HOIP depletion is unlikely to be caused by an accumulation of persistent cisplatin-induced DNA lesions.
HOIP depletion sensitizes cells to genotoxin-induced apoptotic cell death

Cells trigger apoptotic cell death when genotoxic stress exceeds a certain threshold or upon the accumulation of persistent DNA lesions. It was recently shown that loss of the LUBAC component SHARPIN increased susceptibility to TNF-α–induced caspase-8/caspase-3–mediated apoptosis (24). Given this, we hypothesized that HOIP depletion might sensitize cells to apoptotic cell death following cisplatin-induced damage. We treated U2OS and HEK293 cells with 5 μmol/L cisplatin and observed, in both cell lines, that after 48 hours the proteolytic cleavage of the effector caspase, caspase-3, was almost undetectable in siCON control cells but greatly increased when HOIP was depleted (Fig. 3A). Similar results were obtained following treatment of cells with other genotoxins such as etoposide, hydroxyurea and ionizing radiation, or stress stimuli, including the broad-spectrum kinase inhibitor staurosporine and the mitotic inhibitor taxol (Supplementary Fig. S2A–S2E). We assayed caspase-3 activity in lysates of either siCON- or siHOIP-depleted cells treated with cisplatin and noted a modest elevation of caspase-3 activity in untreated siHOIP cells. However, 48 hours after cisplatin exposure, caspase-3 activity was significantly enhanced in siHOIP cells, compared with siCON cells (Fig. 3B and C). Importantly, we also observe increased cisplatin-induced caspase-3 cleavage in HOIP<sup>C879S/C879S</sup> MEFs when compared with control wild-type MEFs (Fig. 3D). We tested whether the phenotype of HOIP depletion could be mimicked by siRNA silencing of the LUBAC subunits SHARPIN and HOIL-1L. Indeed, SHARPIN, but not HOIL-1L, depletion causes a cisplatin-induced accumulation of cleaved caspase-3, suggesting that HOIP and SHARPIN cooperate in an antiapoptotic cell death response (Fig. 3E and F). Caspase-8 is one of the critical caspases upstream of caspase-3 and has been previously implicated in genotoxin-induced apoptosis (29–31). To test whether caspase-8 mediates the observed cisplatin-induced caspase-3 activation, cells were pretreated with the specific caspase-8 inhibitor Z-IETD-FMK before cisplatin treatment. Caspase-8 inhibition completely abolished siHOIP-enhanced caspase-3 activation (Fig. 3G and H) or stress stimuli, including the broad-spectrum kinase inhibitor staurosporine and the mitotic inhibitor taxol (Supplementary Fig. S2F). In agreement with this, we observed a significant elevation of caspase-8 activity in HOIP-depleted U2OS, as well as HEK293, cells after cisplatin treatment (Fig. 3H and I). These data suggest that HOIP protects cells from cisplatin-induced caspase-8–mediated apoptotic cell death.

Apoptotic sensitization of HOIP-depleted cells requires ATM but occurs in the absence of NF-κB activation

Recent evidence indicates that ATM drives LUBAC-dependent linear ubiquitin chain formation on NEMO, which promotes NF-κB activation in cells exposed to topoisomerase inhibitors, such as etoposide (32). We show that the highly specific ATM kinase inhibitor KU55933 (Fig. 4A, B, and E; ref. 33), but not the ATR kinase inhibitor ETP-4646 (Fig. 4C and D; ref. 34) blocks enhanced proteolytic caspase-3 cleavage in HOIP-depleted HEK293 and U2OS cells. Next, we analyzed whether NF-κB signaling is implicated in the hyperapoptotic cisplatin response observed in HOIP-depleted cells. We generated a HEK293 cell overexpressing a dominant mutant version of the NF-κB inhibitory protein IκBα (IκBα S32A/S36A), which cannot be phosphorylated by IκB kinase (IKK) and thus cannot be targeted for proteasomal degradation (35). Overexpression of IκBα S32A/S36A in HEK293 cells completely abolished cisplatin-induced NF-κB activation (Supplementary Fig. S3). We then transfected IκBα S32A/S36A expressing HEK293 cells with either siCON or siHOIP followed by cisplatin treatment for 48 hours. HOIP depletion in IκBα S32A/S36A cells resulted in an elevation of caspase-3 activation in untreated cells (<i>t</i> = 0 hours), and this was enhanced following cisplatin exposure (<i>t</i> = 48 hours; Fig. 4F). No significant caspase-3

Figure 2. HOIP is not required for DNA damage checkpoint activation. A, HEK293 cells were transfected with siCON or siHOIP and treated with 5 μmol/L cisplatin for the indicated times. Cell lysates were analyzed by immunoblotting with the antibodies indicated. B, siCON- or siHOIP-transfected HEK293 cells were treated with 8 μmol/L cisplatin for 2 hours before being allowed to recover for the times indicated. Cells were then fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize γ-H2AX antibody to visualize γ-H2AX foci. C, quantitation of γ-H2AX foci. Data in B: cells with more than six foci were classed as γ-H2AX positive. Data in C are presented as mean ± SEM from three independent experiments.
activation was detected in siCON-silenced IκBα S32A/S36A cells following cisplatin exposure. Cumulatively, this suggests that cisplatin-induced apoptotic cell death in HOIP-depleted cells occurs independently of NF-κB activation.

**JNK activity is elevated in HOIP-depleted cells**

In addition to NF-κB activation, extensive DNA damage triggers activation of the stress-responsive JNK and induces Fas-Associated Death Domain (FADD)-mediated pro-caspase-8 activation (36). We, therefore, assayed JNK activity by monitoring JNK-targeted phosphorylation of c-Jun (P-c-Jun) in response to cisplatin. We observed that HOIP-depleted cells respond with enhanced c-Jun phosphorylation, suggesting increased JNK activity (Fig. 5A). To assess JNK kinase activity directly, we immunoprecipitated JNK from cell lysates of mock (-) or cisplatin (+)-treated cells that were either siCON or siHOIP transfected, and assayed the kinase in vitro. In agreement with increased P-c-JUN, we observed a significantly enhanced basal, as well as cisplatin-induced, JNK activity in HOIP-depleted cells (Fig. 5B). To test whether ATM is required for cisplatin-induced JNK activation, we pretreated cells with ATM kinase inhibitor KU55933 (ATMi) following cisplatin treatment. ATM inhibition had no effect on JNK activation, suggesting that ATM checkpoint activation is not regulating JNK activity in response to cisplatin (Fig. 5B). Using the highly specific JNK inhibitor JNK-IN-8 (37), we next showed that caspase-8 processing in HOIP-depleted HEK293 cells was significantly suppressed (Fig. 5C), and cisplatin hypersensitivity reversed (Fig. 5D), following JNK inhibition. Consistent with HEK293 cells, HOIP depletion in U2OS cells triggered elevation of both basal and cisplatin-induced JNK activity (Fig. 5E). However, JNK inhibition did not alleviate caspase-3 hyperactivation in HOIP-depleted U2OS cells, suggesting a cell type-specific requirement of JNK in apoptotic cell death.
HOIP depletion induces cisplatin hypersensitivity in different types of cancer cells

We demonstrate here that HOIP depletion sensitizes U2OS osteosarcoma cells to cisplatin-induced apoptotic cell death. These findings suggest potential strategies to sensitize cancer cells or, even more relevantly, to resensitize cancer cells that have become resistant following prolonged cisplatin exposure. To test this concept, we studied the effect of HOIP depletion in a panel of cancer cell lines derived from different tissues. HCT116 (colon), HeLa (cervix), PC3 (prostate), ZR.75.1 (breast), MDA-MB-231 (breast), and PEA1 (ovary) were transfected with either siCON or siHOIP and analyzed for cisplatin-induced caspase-3 activation. In addition, cell viability was determined following exposure to a dose range of cisplatin (Fig. 6). Consistently, all siHOIP-transfected cancer cell lines exhibited, to varying degrees, elevated cleaved caspase-3 after cisplatin exposure, which coincided with increased cisplatin sensitivity.

The PEA1 ovarian cancer cell line exhibits particularly striking cisplatin hypersensitivity upon HOIP depletion. During platinum drug chemotherapy, ovarian cancers frequently develop drug resistance. Hence, we next addressed whether cisplatin-resistant ovarian cancer cells can be resensitized by HOIP-depletion. We analyzed paired platinum sensitive (A2780cisS) and resistant (A2780cisR) ovarian tumor cell lines. A2780cisR cells are in vitro derivatives of the sensitive A2780cisS cells that have acquired resistance through continuous treatment with cisplatin (38). A2780cisS and A2780cisR cells were transfected either with siCON or siHOIP and treated with 5 μmol/L cisplatin for the indicated times. Cell lysates were subjected to immunoblot analyses. Data in B and D are represented as mean ± SEM from three independent experiments and the Student t test was used to calculate significance.

Figure 4. Apoptotic sensitization of HOIP-depleted cells requires ATM but is independent of ATR activity. A, siRNA-transfected HEK293 cells were treated with vehicle only (−) or 10 μmol/L KU-55933 ATM inhibitor (+) 1 hour before treatment with 5 μmol/L cisplatin for the indicated times. Cell lysates were analyzed by immunoblotting. B, quantitation of the data shown in A. ImageJ software was used to quantify immunoblots; *, P = 0.0078. C, as in A, except that 5 μmol/L ETP-46464 ATR inhibitor was used. D, quantitation of the data shown in C. E, as in A, except that U2OS cells were used. F, human influenza haemagglutinin (HA)-IκBα (S32A, S36A)–expressing HEK293 cells were transfected either with siCON or siHOIP and treated with 5 μmol/L cisplatin for the indicated times. Cell lysates were subjected to immunoblot analyses. Data in B and D are represented as mean ± SEM from three independent experiments and the Student t test was used to calculate significance.
sensitivity in both cell lines (Fig. 7C and D). Similar results were obtained from the matching pair of PEO14CisS and PEO23CisR, suggesting that HOIP protects these cells from cisplatin genotoxicity (Fig. 7E and F).

Discussion
Managing resistance that occurs during platinum-based drug chemotherapy remains a major challenge in the treatment of ovarian and other cancers, and results in poor prognosis for successful eradication of the cancer. Cells respond to platinum drugs in several ways and the resistance mechanisms modulating these responses, to protect cells from platinum genotoxicity, are equally complex. Typically for platinum-based drugs, cancer cells frequently gain resistance by reduced cellular drug uptake, increased drug efflux, increased DNA repair and MHL1 hypermethylation (3). In this study, we identified HOIP, the catalytic E3 ubiquitin ligase component of LUBAC, as an antiapoptotic factor attenuating the genotoxic effect of genotoxic agents including cisplatin. We provide evidence that HOIP depletion in several cancer cell lines can potentiates cellular genotoxicity of cisplatin due to enhanced apoptotic cell death response.

We show that two subunits of LUBAC, HOIP and SHARPIN, are required to suppress cisplatin-induced apoptosis, whereas we have no evidence that the subunit HOIL-1L participates in the cisplatin response. Studies in vitro have demonstrated that HOIP on its own has only weak E3 ubiquitin ligase activity, but that its catalytic activity is strongly enhanced in the presence of either SHARPIN or HOIL-1L, assembling a HOIP/SHARPIN or HOIP/HOIL-1L complex respectively (40, 41). It is debatable whether these subcomplexes coexist in vivo; however, genetic evidence supports the idea that these LUBAC subcomplexes have separable functions (22). SHARPIN-deficient mice exhibit chronic proliferative dermatitis (cpdm), a pathology not observed in HOIL-1L–deficient mice. Moreover, these studies show that SHAPRIN-deficient MEFs have far higher levels of TNF-α–induced FADD and caspase-8–dependent apoptotic cell death than cells from HOIL-1L–deficient mice (24). TNF-α–induced apoptosis is triggered by the formation of a TRADD–TRAF2–RIP1–FADD–caspase-8 death-promoting

Figure 5. JNK activity is elevated and essential for apoptotic cell death in HOIP-depleted cells. A, siRNA-transfected HEK293 cells were treated with 25 μmol/L cisplatin for the indicated times. Cell lysates were analyzed by immunoblotting. B, siCON- or siHOIP-transfected HEK293 cells were pretreated with 3 μmol/L JNK inhibitor or 10 μmol/L ATM inhibitor before vehicle only (−) or 5 μmol/L cisplatin treatment (+) as indicated. JNK kinases were immunoprecipitated from cell lysates, and kinase activity was measured using an in vitro kinase assay. Activity is presented as mU/mg cell lysate; *, P = 0.0396 (Student t test). C, siCON- or siHOIP-transfected HEK293 cells were pretreated with vehicle only (−) or 3 μmol/L JNK inhibitor (+) before treatment with 5 μmol/L cisplatin for the indicated times. Cell lysates were analyzed by immunoblotting. D, cell viability of siCON- or siHOIP-transfected cells pretreated with JNK inhibitor in response to cisplatin was measured by MTS cell proliferation assay. E, as in C, except that U2OS cells were used. Data in B and D are represented as mean ± SEM from three independent experiments.
complex (TNFR1 complex II), whereas in response to genotoxic stress, an analogous ripoptosome complex, consisting of RIP1–FADD–caspase-8, is formed (42). Moreover, CUL3 RING ligase-mediated polyubiquitylation of caspase-8 stabilizes and promotes caspase-8 activity (43). It is tempting to speculate that a HOIP/SHARPIN ligase complex regulates cisplatin-induced apoptosis via ripoptosome assembly and activity. Indeed, this idea is supported by data indicating that the ripoptosome component RIP1 is modified with linear ubiquitin chains (23, 44). Whether the ripoptosome components are direct targets for HOIP/SHARPIN-mediated linear ubiquitylation following genotoxic stress will be the subject of future investigations.

Besides an essential function for ATM in promoting the apoptotic cell death response in response to cisplatin, we found that basal and cisplatin-induced JNK kinase activity is significantly upregulated in HOIP-depleted cells. It has been shown that sustained activation of JNK in ovarian cancer cells, in response to cisplatin, leads to activation of the AP-1 transcription factor and AP-1–induced FAS-L expression leading, in turn, to Fas receptor–triggered apoptosis (15). Moreover, JNK activation has previously been linked to the degradation of the antiapoptotic factor c-FLIPL, suggesting that JNK hyperactivation would lead to an enhanced ripoptosome/caspase-8 activity (45). The importance of the JNK/FAS apoptosis pathway is further demonstrated by the observation that several...
independently derived cisplatin-resistant cancer cell lines exhibit attenuated JNK activation (46).

The design of the RNAi approach applied in this study allowed us to screen directly for targets whose downregulation might either sensitize cancer cells for cisplatin or might reverse cisplatin resistance in tumors. In all cancer cells tested, siRNA depletion of HOIP reduced their viability and enhanced apoptotic cell death following cisplatin exposure. Enhanced cisplatin-induced apoptosis was also observed in HOIPC879S/C879S MEFs that express catalytically inactive HOIP. We, therefore, propose that HOIP E3 ligase activity plays a critical role in limiting apoptotic cell death upon cisplatin exposure. Indeed, our data show that HOIP-depleted cells exhibit enhanced apoptosis in response to treatment with a wide range of genotoxins, as well as nongenotoxic cellular stress inducers. This suggests that a HOIP-dependent antiapoptotic mechanism is likely to be conserved in the response to cellular stress in general. Hence, blocking or easing the antiapoptotic function of HOIP will render cancer cells more susceptible to apoptosis, and low levels of genotoxic stress will trigger apoptotic cell death.

Although it is clear that HOIP depletion renders cells hypersensitive to cisplatin treatment, based on our current knowledge it is not clear what, if any, contribution HOIP has to drug resistance. Some evidence that HOIP overexpression may contribute to cisplatin resistance in cancer comes from large-scale gene–drug association studies (https://www.oncomine.com; ref. 47). Expression profile data from a panel of several hundred cancer cell lines derived from tumors classified as cisplatin resistant or cisplatin sensitive have revealed that cisplatin-resistant cancer cell lines (n = 335) show a significantly higher expression of HOIP (P = 0.011) and SHARPIN (P = 0.016) when compared with cisplatin-sensitive cancer cell lines (n = 29; Supplementary Fig. S4). Oncomine analysis also revealed that both HOIP and SHARPIN are overexpressed in serous ovarian carcinomas patient samples compared with normal tissue (Supplementary Fig. S5). These data suggest that HOIP/SHARPIN expression is altered in cancer cell lines, and that an elevation of HOIP and HOIP’s antiapoptotic function may promote cisplatin resistance.

Enhancing or reestablishing the apoptotic cell death program in tumor cells is a promising strategy for cancer therapy. On the basis of our findings, we propose that small-molecule inhibitory drugs targeting HOIP E3 ligase activity, used in combination with cisplatin chemotherapy, might potentiate caspase-8–mediated death in cancer cells. This concept is supported by recent studies that describe Smac mimetics, i.e., small molecules that mimic the antagonistic activity of Smac toward inhibitor of apoptosis proteins (IAP), as efficient potentiators of cancer cell apoptosis. They stimulate IAP auto-ubiquitylation and proteasomal degradation, leading to TNF-α signaling, which, in turn, induces ripoptosome activation and caspase-8–dependent cell death in multiple cancer cell lines (48–50). We predict that HOIP inhibitors, although acting through mechanisms different from those of the Smac mimetics, would significantly upregulate cisplatin-induced caspase-8 activity, HOIP depletion or deficiency does not kill transformed cells and hence HOIP inhibitors are unlikely to be toxic when used alone. However, HOIP plays a critical role in the innate immune response and

Figure 7. HOIP depletion sensitizes cisplatin-resistant ovarian cancer cell lines. A and B (left), A2780CisS and A2780CisR cells were transfected with siCON or siHOIP as indicated and cell viability assessed by MTS cell proliferation assays. A and B (right), A2780CisS and A2780CisR cells were transfected with siCON or siHOIP and treated with 5 μmol/L cisplatin for the indicated time. Cell lysates were analyzed by immunoblotting. C–F, cell lines were transfected with siCON or siHOIP as indicated and cell viability assessed by MTS cell proliferation assays. Data shown are presented as mean ± SEM from three independent experiments.
the application of HOIP inhibitors bear the potential risk of stimulating inflammatory and/or auto-inflammatory side effects (22). An in-depth analysis of the impact of HOIP inhibition is needed to fully understand how HOIP can best be exploited as an anticancer drug target. This study, however, provides the rationale for the development of HOIP inhibitors to increase not only the efficacy of first-line cisplatin chemotherapy but also to achieve a better clinical outcome of cisplatin-based chemotherapy in drug-resistant cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. MacKay, E. Carroll, G.J. Inman, R.T. Hay, A.F. Alpi
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References

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