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ORIGINAL ARTICLE

P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism

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P73 is important in drug-induced apoptosis in some cancer cells, yet its role in the regulation of chemosensitivity in ovarian cancer (OVCA) is poorly understood. Furthermore, if and how the deregulation of p73-mediated apoptosis confers resistance to cisplatin (CDDP) treatment is unclear. Here we demonstrate that TAp73α over-expression enhanced CDDP-induced PARP cleavage and apoptosis in both chemosensitive (OV2008 and A2780s) and their resistant counterparts (C13* and A2780cp) and another chemoresistant OVCA cells (Hey); in contrast, the effect of ΔNp73α over-expression was variable. P73α downregulation attenuated CDDP-induced PUMA and NOXA upregulation and apoptosis in OV2008 cells. CDDP decreased p73α steady-state protein levels in OV2008, but not in C13*, although the mRNA expression was identical. CDDP-induced p73α downregulation was mediated by a calpain-dependent pathway. CDDP induced calpain activation and enhanced its cytoplasmic interaction and co-localization with p73α in OV2008, but not C13* cells. CDDP increased the intracellular calcium concentration ($[Ca^{2+}]_i$) in OV2008 but not C13* whereas cyclopiazonic acid (CPA), a Ca^{2+}-ATPase inhibitor, caused this response and calpain activation, p73α processing and apoptosis in both cell types. CDDP-induced $[Ca^{2+}]_i$ increase in OV2008 cells was not effected by the elimination of extracellular Ca^{2+}, but this was attenuated by the depletion of internal Ca^{2+} store, indicating that mobilization of intracellular Ca^{2+1} stores was potentially involved. These findings demonstrate that p73α and its regulation by the Ca^{2+}-mediated calpain pathway are involved in CDDP-induced apoptosis in OVCA cells and that dysregulation of Ca^{2+}/calpain/p73 signaling may in part be the pathophysiology of CDDP resistance. Understanding the cellular and molecular mechanisms of chemoresistance will direct the development of effective strategies for the treatment of chemoresistant OVCA.
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Introduction

Chemoresistance is a major concern in cancer chemotherapy and remains an obstacle for the successful treatment of ovarian cancer (OVCA). Although cisplatin (CDDP) and its derivatives are effective anticancer agents for OVCA, its efficacy is limited by the development of resistance. Whereas chemoresistance could be attributed to altered DNA repair, drug transport and metabolism, deregulation of cell death seems a key determinant in CDDP resistance in OVCA, resulting from the upregulation and reduction of either the pro- or anti-apoptotic factors (Eltabbakh and Awtrey, 2001; Fraser *et al*., 2003a).

The expression of the *TP73* gene is frequently altered in cancer and its modulation enhances cancer cell sensitivity to drug-induced apoptosis (Melino *et al*., 2002; Irwin *et al*., 2003; Vayssade *et al*., 2005). The gene products include at least seven spliced isoforms with different carboxyl termini, termed TA variants (TAp73α-η). In addition, the gene product gives rise to at least another seven isoforms transcribed from a cryptic promoter in intron 3, these isoforms lack the TA domain, thus are termed ΔN variants (ΔNp73α-η) (Pietsch *et al*., 2008). TAp73 is a transcription factor that causes cell cycle arrest and apoptosis through the activation of p53-like target genes such as PUMA and NOXA (Melino *et al*., 2003; Muller *et al*., 2005). It also

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activates unique downstream targets, suggesting a role that is distinct from that of p53 (Fontemaggi *et al.*, 2002). In contrast, the ΔNp73 isoforms are transcriptionally inactive and act as endogenous dominant negative proteins that inhibit both TAp73- and p53-mediated apoptosis by either competing for the same responsive elements or by sequestration of the active isoforms into non-active hetero-tetramers (Muller *et al.*, 2006).

Calpains are a family of widely expressed calcium (Ca^{2+})-dependent proteases. The most ubiquitously expressed isoforms, known as μ- and m-calpain, are heterodimers consisting of a distinct large 80-kDa catalytic subunit and a common small 28-kDa regulatory subunit (Perrin and Huttenlocher, 2002). Calpains are important regulators of apoptosis by its proteolytic function in cleaving both pro- (Gao and Dou, 2000) and anti- (Kobayashi *et al.*, 2002) apoptotic proteins. Ca^{2+} homeostasis is known to have a vital role in apoptosis, and its modulation influences the activation of calpains (Monteith *et al.*, 2007).

P73 degradation is regulated, in part, by the ubiquitin proteasome pathway (Bernassola *et al.*, 2004; Rossi *et al.*, 2005; Bernassola *et al.*, 2008). A recent finding demonstrated that p73, in addition to the degradation by the ubiquitin E3 ligase ITCH (Rossi *et al.*, 2005) is a substrate of calpain *in vitro*, and that calpain-mediated cleavage sites are found at both the N- and the C-termini (Munarriz *et al.*, 2005). However, whether calpain-mediated p73 cleavage has a role in the physiological function of p73 has not been established. The pathophysiological relevance of Ca^{2+} homeostasis and calpain regulation of p73α and the potential contribution of such pathway to the regulation of CDDP sensitivity in OVCA cells have not been studied, prompting the direction of our investigations.

Here we demonstrate that p73α content is regulated by calpain in CDDP-induced apoptosis in OVCA cells. CDDP induced TAp73α and ΔNp73α downregulation/cleavage in chemosensitive cells, but not in its resistant counterpart and it is mediated by the calpain pathway. CDDP induced $[Ca^{2+}]_i$ increase and calpain activation, enhancing its cytoplasmic interaction and co-localization with p73α in sensitive, but not resistant, cells, potentially via mobilization of intracellular Ca^{2+} stores. These findings illustrate a vital role of the Ca^{2+}/calpain/p73α pathway in regulating OVCA cell sensitivity to CDDP.

Results

P73α contributes to CDDP-induced apoptosis in human ovarian cancer cells

Although p73 is important in drug-induced apoptosis in some cancer cells (Irwin, 2004; Ozaki and Nakagawara, 2005; Ramadan *et al.*, 2005), its role in the regulation of chemosensitivity in OVCA is poorly understood; this is of particular importance because the apoptotic capacity of ovarian cancer cells appears to be tightly linked to their sensitivity to chemotherapeutic agents, such as CDDP (Fraser *et al.*, 2003a). To determine the role of p73α, two pairs of chemosensitive OVCA cell lines (OV2008 and A2780s), their resistant isogenic counterparts (C13* and A2780cp, respectively) and an additional CDDP-resistant OVCA line (Hey) were transfected with TAp73α or ΔNp73α cDNA (2 μg; 24 h) or with empty pcDNA3.1 vectors and then treated with CDDP (5 μM, OV2008; 10 μM, C13*, A2780s, A2780cp and Hey; 24 h). Over-expression of TAp73α and ΔNp73α was confirmed by immunoblot (Figures 1a-c). Whereas TAp73α over-expression consistently enhanced CDDP-induced apoptosis in these cells (OV2008, A2780s, A2780cp and Hey; $P<0.01$ and C13*; $P<0.001$), the effect of ΔNp73α over-expression was variable. Over-expression of ΔNp73α promoted basal and CDDP-induced apoptosis in OV2008 and C13* compared with controls (OV2008, $P<0.05$); C13*, $P<0.01$). In contrast, ΔNp73α failed to sensitize A2780s and Hey cells to CDDP-induced apoptosis, but it did increase the basal apoptosis in A2780cp cells. The enhancement of CDDP-induced apoptosis by either isoform was associated with increased cleavage of PARP, a substrate of caspase-3.

To further examine the role of TAp73α and ΔNp73α in the regulation of CDDP sensitivity, OV2008 cells were transfected with p73α siRNA, targeting both isoforms (0–100 nM; 48 h), prior to CDDP treatment (10 μM; 24 h). CDDP alone decreased TAp73α and ΔNp73α levels, upregulated the content of the p53-responsive gene products PUMA and NOXA and induced apoptosis (Figure 1d). P73α siRNA markedly downregulated both TAp73α and ΔNp73α contents and significantly attenuated these responses induced by CDDP ($P<0.001$), thus leaving open the question on the isoform responsible and their relative balance. P73α siRNA had no effect on p53 content, suggesting that the changes in PUMA and NOXA level were specific to p73α, and not secondary to a decrease in p53.

CDDP-induced p73α downregulation is mediated by calpain-dependent pathway

We next determined whether CDDP has a differential effect on TAp73α and ΔNp73α expression in chemosensitive and chemoresistant cells. TAp73α was highly expressed in both cell lines, whereas ΔNp73α content was high in OV2008 compared with C13* (Figure 2a). CDDP significantly decreased both TAp73α and ΔNp73α contents and induced apoptosis in a concentration-dependent manner in OV2008, but not C13* after 24 h. In contrast, CDDP had no effect on their mRNA levels in either cell line (Figure 2b). The CDDP-induced TAp73α and ΔNp73α downregulation appeared not to be a consequence of protein degradation secondary to cell death or of proteasome degradation. Inhibition of apoptosis by Xiap over-expression or inhibition of the proteasome pathway failed to significantly attenuate these responses (Supplementary Figure 1A and B).

Calpain regulates the steady-state level of p73 isoforms (Munarriz *et al.*, 2005). To test the involvement of calpain in CDDP-induced TAp73α and ΔNp73α processing, OV2008 cells were pre-treated with calpeptin

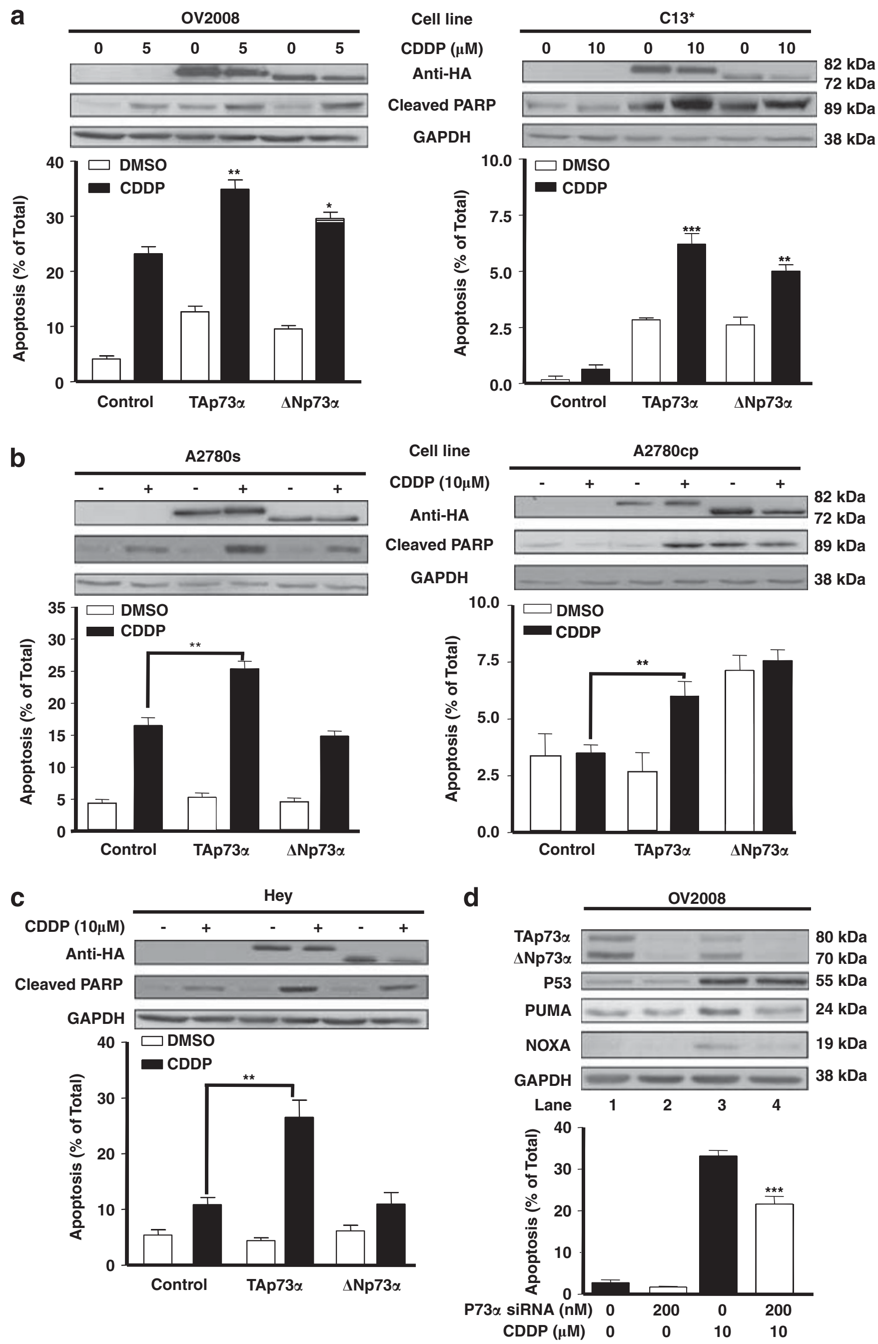

Figure 1 P73α is required for CDDP-induced apoptosis in OVCA cells. (**a**) TAp73α over-expression enhanced CDDP-induced PARP cleavage and apoptosis in OVCA cell lines. The effect of ΔNp73α over-expression was cell-type specific. (**a**) OV2008 and C13*, (**b**) A2780s and A2780cp and (**c**) Hey cells were transfected with either TAp73α cDNA, ΔNp73α cDNA (2 μg; 24 h) or an empty vector (CTL) followed by CDDP (10 μM; 24 h). TAp73α and ΔNp73α over-expression was assessed by western blot (top), and apoptosis by Hoechst staining (bottom). Effective over-expression of TAp73α and ΔNp73α was confirmed by western blot using anti-HA antibody. The expression of HA-TAp73α sensitized all the tested cell lines to CDDP-induced apoptosis when compared with the control groups (OV2008, A2780s, A2780cp and Hey; $P<0.01$ and C13*; $P<0.001$). The effect of HA-ΔNp73α over-expression was variable. As observed with TAp73α, the over-expression of ΔNp73α promoted basal and CDDP-induced apoptosis in OV2008 and C13* compared with controls (OV2008 ($P<0.05$); C13* ($P<0.01$)). In contrast, ΔNp73α failed to sensitize A2780s and Hey cells to CDDP-induced apoptosis but it did increase the basal level of apoptosis in A2780cp cells. The enhancement of CDDP-induced apoptosis by either isoform was associated with increased cleavage of PARP, a substrate of caspase-3. (**d**) P73α downregulation by p73α siRNA attenuated CDDP-induced PUMA and NOXA upregulation and apoptosis in OV2008. OV2008 cells were transfected with p73α siRNA (0–100 nM; 48 h) and treated with CDDP (10 μM; 24 h). TAp73α, ΔNp73α, PUMA and NOXA contents (Top) and apoptosis (Bottom) were determined by western blot and Hoechst stain, respectively. P73α siRNA markedly downregulated TAp73α and ΔNp73α contents (lane 2 vs 1 and lane 4 vs 3) and had no effect on p53 content. TAp73α and ΔNp73α downregulation significantly attenuated CDDP-induced apoptosis compared with siRNA controlled-group treated with CDDP ($P<0.001$). Figures indicate the mean ± s.e.m. of three independent experiments assessed by two way-ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

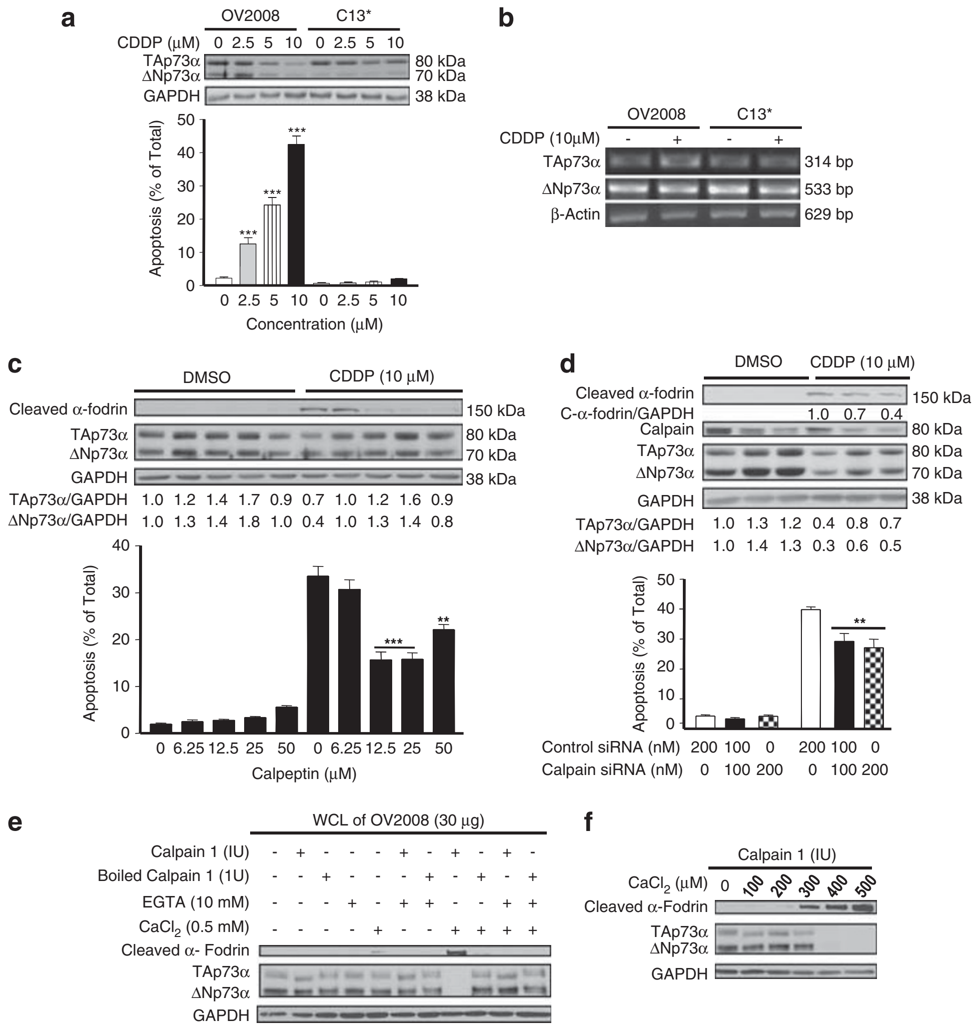

Figure 2 CDDP-induced p73α downregulation is calpain-dependent. (**a**) CDDP decreased both TAp73α and ΔNp73α contents in OV2008, but not C13*, in a concentration-dependent manner. OV2008 and C13* cells were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM; 24 h). TAp73α and ΔNp73α content (Top) and apoptosis (Bottom) were assessed as with the previous methodology (**b**) CDDP had no effect on TAp73α and ΔNp73α mRNA abundance in both OV2008 and C13* cells. OV2008 and C13* were treated with CDDP (10 μM; 24 h). TAp73α and ΔNp73α mRNA levels were assessed by RT-PCR. Calpain was inhibited by either calpeptin (**c**) or specific calpain siRNA (**d**) decreased cleaved α-fodrin restored TAp73α and ΔNp73α content and attenuated CDDP-induced apoptosis. OV2008 cells were pre-treated for 4 h with different concentrations of calpeptin (0, 6.25, 12.5, 25 and 50 μM) or transfected with calpain siRNA (0–200 nM; 48 h). (**e**) OV2008 cell lysates were incubated with recombinant calpain 1 for 1 h at 30 °C, where calpain activity was inhibited by boiling, EGTA and absences of Ca^{2+}. (**f**) OV2008 cells lysate was incubated as above and calpain was activated by increasing concentration of Ca^{2+}. Calpain downregulation, p73α isoforms contents and cleaved α-fodrin and apoptosis were assessed as above. Results were obtained from the mean ± s.e.m. of three independent experiments using two-way ANOVA (**$P<0.01$, ***$P<0.001$).

(exogenous inhibitor; 0–50 μM) and a specific siRNA before CDDP treatment (10 μM; 24 h). Both calpeptin (Figure 2c) and calpain siRNA (Figure 2d) inhibited calpain activity (as indicated by cleaved α-fodrin), restored TAp73α and ΔNp73α contents and attenuated CDDP-induced apoptosis.

To validate the processing of TAp73α and ΔNp73α by calpain in OV2008 cells, their cell lysates were incubated with recombinant calpain 1 followed by immunoblotting. Ca^{2+}-activated calpain 1 induced α-fodrin cleavage and TAp73α and ΔNp73α processing; however, these events were prevented by EGTA or when calpain was inactivated by boiling (Figure 2e). The Calpain-mediated α-fodrin cleavage and TAp73α and ΔNp73α processing were consistent with the different concentration of Ca^{2+}, suggesting that calpain activation is controlled by Ca^{2+}. Unfortunately, the antibody used to detect TAp73α and ΔNp73α content failed to recognize their cleaved products induced by calpain 1. Collectively, our studies provided strong evidence for an involvement of calpain in the regulation of TAp73α and ΔNp73α protein level both alone and in the presence of CDDP.

CDDP induced calpain activation and influenced its interaction and co-localization with p73α in chemosensitive, but not chemoresistant cells

As CDDP induced p73α downregulation only in chemosensitive cells, we examined the effect of CDDP on calpain activation. Time course and concentration-response studies on the effects of CDDP indicate that CDDP caused calpain activation, as evident by α-fodrin cleavage, in a time- and a concentration-dependent manner in OV2008, but not C13* cells (Figure 3a). This supports the notion that the absence of CDDP-induced p73α processing in C13* could be attributed to the lack of calpain activation.

We then speculated that calpain and p73α isoforms may interact in the chemosensitive OV2008 cells. To explore this possibility, OV2008 cells were over-expressed with exogenous HA-TAp73α and HA-ΔNp73α and then treated with CDDP (10 μM). Both proteins were immunoprecipitated with anti-HA antibody, and calpain interaction was then detected by western blot. Figure 3b showed that both TAp73α and ΔNp73α bind to calpain in control and CDDP-treated groups, suggesting that both isoforms interact with calpain. To validate that the endogenous proteins also interact, calpain and p73α interaction was examined by co-immunoprecipitation. Both p73α isoforms were successfully co-precipitated with a common anti-p73α antibody, due to the lack of discriminating antibodies for each isoforms. In keeping, Figure 3c revealed that p73α and calpain interact at the endogenous level in OV2008 cells.

Immunolocalization studies on p73α in OV2008 treated with DMSO (control) showed that p73α was localized in the nucleus and in the perinuclear region (Figure 3d). CDDP (10 μM; 24 h) decreased nuclear p73α immunoreactivity and increased localization in the perinuclear region. Conversely, p73α immunoactivity was low or not detectable in the nucleus of C13* and was not influenced by CDDP (Figure 3d). Calpain immunoreactivity in OV2008 cells was found only at the cytoplasm and was not mediated by CDDP (Figure 3d). The overlay of p73α and calpain immunosignals demonstrated that CDDP treatment of OV2008 cells

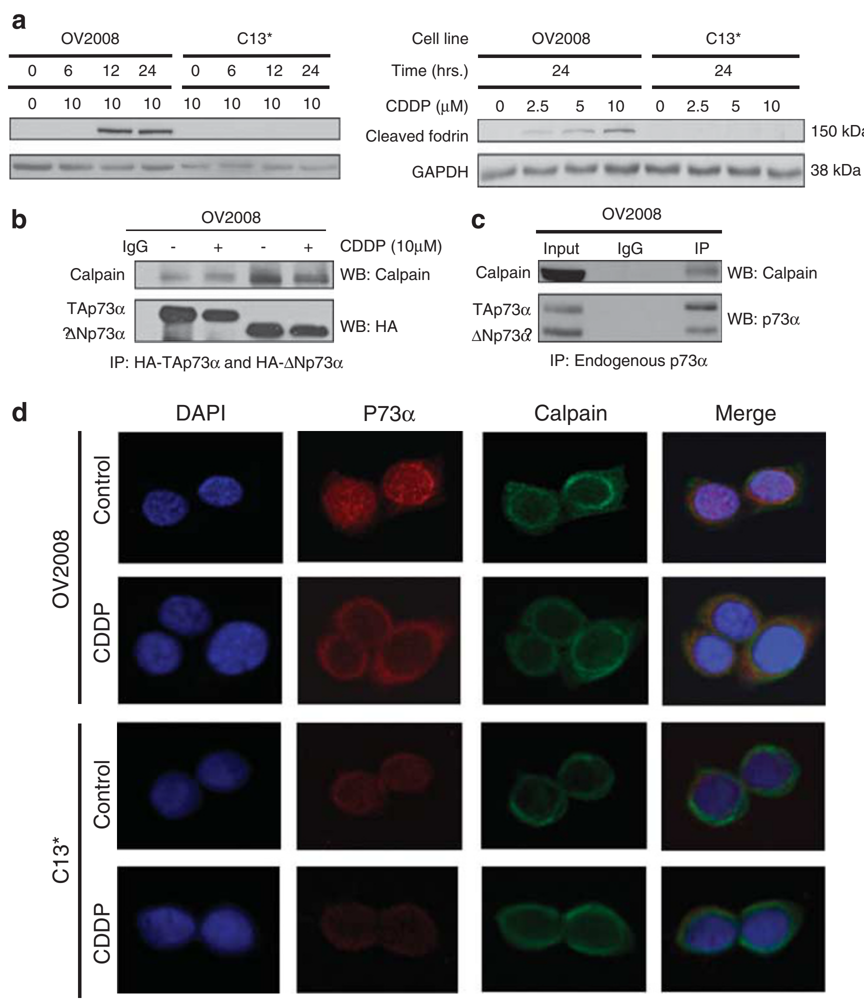

Figure 3 CDDP induced calpain activation and enhanced its interaction and co-localization with p73α in OV2008, but not C13* cells. (**a**) CDDP induced calpain activation in OV2008, but not C13*. OV2008 and C13* cells were treated with different concentration of CDDP (0, 2.5, 5 and 10 μM; 24 h) and harvested at different times (0, 6, 12 and 24 h; 10 μM). Calpain activation was illustrated by cleaved α-fodrin (determined by western blot) (**b**) Exogenous TAp73α and ΔNp73α bind to calpain in control and CDDP-treated groups (**c**) Endogenous p73α and calpain interact in OV2008 cells. HA- TAp73α, HA-ΔNp73α and endogenous p73α were immunoprecipitated and then p73α isoforms and calpain binding was detected by western blot. (**d**) CDDP enhanced cytoplasmic p73α/calpain co-localization in OV2008 but not C13* cells. OV2008 and C13* cells were treated with CDDP (10 μM; 12 h) and p73α/calpain co-localization was detected by immunocytochemistry. Images represent three independent experiments.

resulted in the co-localization of immunosignals in clusters and with increased intensity in the cytoplasm. C13* cells exhibited a similar immunoreactive pattern irrespective of the presence of CDDP, in which the co-localization of p73 and calpain was evident only in the cytoplasm. Differential co-localization of both proteins in the two cell lines could be the precursor for the regulation of p73α by calpain. Changes in p73 localization have already been reported, because interactors of p73 such as Wwox are able to affect the subcellular localization of p73, resulting in a change of its apoptotic effect (Aqeilan *et al*., 2004). Our results involve calpain in the subcellular localization of p73.

CDDP differentially effects the intracellular calcium concentration $[Ca^{2+}]_i$ in OVCA cells

As calpain is a calcium-dependent protease, we tested if CDDP increased $[Ca^{2+}]_i$ in both OV2008 and C13* cells by confocal microscopy. Representative images of OV2008 cells showed a marked increase in the intensity of green fluorescence following CDDP treatment; there was no difference in C13* (Figure 4a). Increases in the $[Ca^{2+}]_i$ were evident around 10 min following the addition of CDDP to the cultures, plateauing after 50 min, when the experiment was terminated. The differential influence of CDDP on $[Ca^{2+}]_i$, calpain activation and p73α processing in the chemosensitive and chemoresistant cells suggest that these pathways regulate OVCA cell sensitivity to CDDP.

The inability of the C13* cells to undergo apoptosis in response to CDDP was related to the observed dysregulation of CDDP-induced $[Ca^{2+}]_i$, we examined the influence of cyclopiazonic acid (CPA; 100 μM) on $[Ca^{2+}]_i$, calpain activation (illustrated by cleaved α-fodrin), TAp73α and ΔNp73α content and apoptosis in both OV2008 and C13* cells. CPA is a selective Ca^{2+}-ATPase inhibitor, which depletes the endoplasmic reticulum (ER) of Ca^{2+}(Moncoq *et al*., 2007). In both cell lines, CPA significantly increased $[Ca^{2+}]_i$ (Figure 4b) and induced calpain activation (evident by cleaved α-fodrin), as well as TAp73α and ΔNp73α downregulation and apoptosis (Figure 4c). Glucose-regulated protein 78 (GRP78) was used as an indicator of ER stress (Di Sano *et al*., 2006). Dysregulation is neither at the level of calpain nor p73, but rather in the $[Ca^{2+}]$ signaling in response to CDDP treatment as interpreted from our data. These findings also suggest that Ca^{2+} is required for calpain activation and subsequent TAp73α and ΔNp73α cleavage and apoptosis. As the machinery of Ca^{2+} mobilization was functional in both OVCA cell lines and that the inability of C13* cells to respond to CDDP with increased $[Ca^{2+}]_i$ might be due to a defect in the CDDP-induced Ca^{2+}signaling, then a compromised apoptotic response and CDDP resistance would justify the observations.

CDDP-induced $[Ca^{2+}]_i$ increase involves mobilization of intracellular Ca^{2+} stores in chemosensitive cells

Although CDDP increased the $[Ca^{2+}]_i$ in OV2008 cells (Figure 4a), the source (intracellular vs extracellular) for the Ca^{2+} mobilization is unknown. The possible contribution of extracellular Ca^{2+} in the CDDP-induced $[Ca^{2+}]_i$ increase was assessed in both normal Ca^{2+} concentration (0.1 g/l) and in a Ca^{2+}-free media. Removal of extracellular Ca^{2+} (for example, Ca^{2+}-free media) had no effect on the CDDP-induced $[Ca^{2+}]_i$ increase, when compared with the response observed with normal Ca^{2+} media (Figure 5a). The CDDP-induced $[Ca^{2+}]_i$ increase in this media declined faster than the regular media that was sustained up to the termination of the experiment.

To provide further evidence that the CDDP-induced $[Ca^{2+}]_i$ increase is mediated via mobilization of intracellular Ca^{2+} stores, internal Ca^{2+} stores in OV2008 were first depleted by CPA before CDDP treatment. Figure 5b demonstrates that CDDP failed to increase the $[Ca^{2+}]_i$ after store depletion in both regular and Ca^{2+}-free media: CDDP-induced $[Ca^{2+}]_i$ increase is dependent on these stores. Whereas CDDP did not enhance CPA-dependent calpain activation and apoptosis in C13* cells, it had a significant effect on these events in OV2008 (Figure 5c). CPA alone could increase $[Ca^{2+}]_i$, activate calpain and induce apoptosis in C13*, whereas CDDP could also activate Ca^{2+}-independent pathways to facilitate these responses in OV2008 cells.

Discussion

The current study demonstrates the important role of Ca^{2+}-mediated, calpain activation in the regulation of p73α function in human ovarian cancer cells, and provides evidence that the dysregulation of this pathway may confer resistance to CDDP-induced apoptosis in these cells. The role of p73 in drug-induced apoptosis in OVCA cells is poorly understood and the few published reports have only considered the effect of exogenous p73 on either the activation of down-stream genes and cell growth or the effect of CDDP on the endogenous level of p73 (Muscolini *et al*., 2008; Righetti *et al*., 2008). We have shown that TAp73α over-expression significantly enhanced CDDP-induced apoptosis in different OVCA cell lines, whereas the effect of ΔNp73α over-expression was variable.

The role of p73α in drug-induced apoptosis is unclear. Although it has been demonstrated that TAp73α is anti-apoptotic and inhibits drug- and p53-induced apoptosis (Grob *et al*., 2001; Vikhanskaya *et al*., 2001; Nyman *et al*., 2005), other studies have indicated that the TA-isoform is a crucial mediator in CDDP-induced apoptosis (Yoshida *et al*., 2008; Sang *et al*., 2009). Our findings of the effect of TAp73α over-expression on CDDP-induced apoptosis in OVCA cells are consistent with a pro-apoptotic effect of TAp73α.

ΔNp73 is a dominant negative regulator of p53- and TAp73-mediated apoptosis in certain cancer cells (Grob *et al*., 2001; Muller *et al*., 2005; Million *et al*., 2006); however, some reports have shown that ΔNp73 can modulate the expression of various genes in a p53-independent manner (Kartasheva *et al*., 2002). Whereas ΔNp73β and ΔNp73γ could be active in transactivation

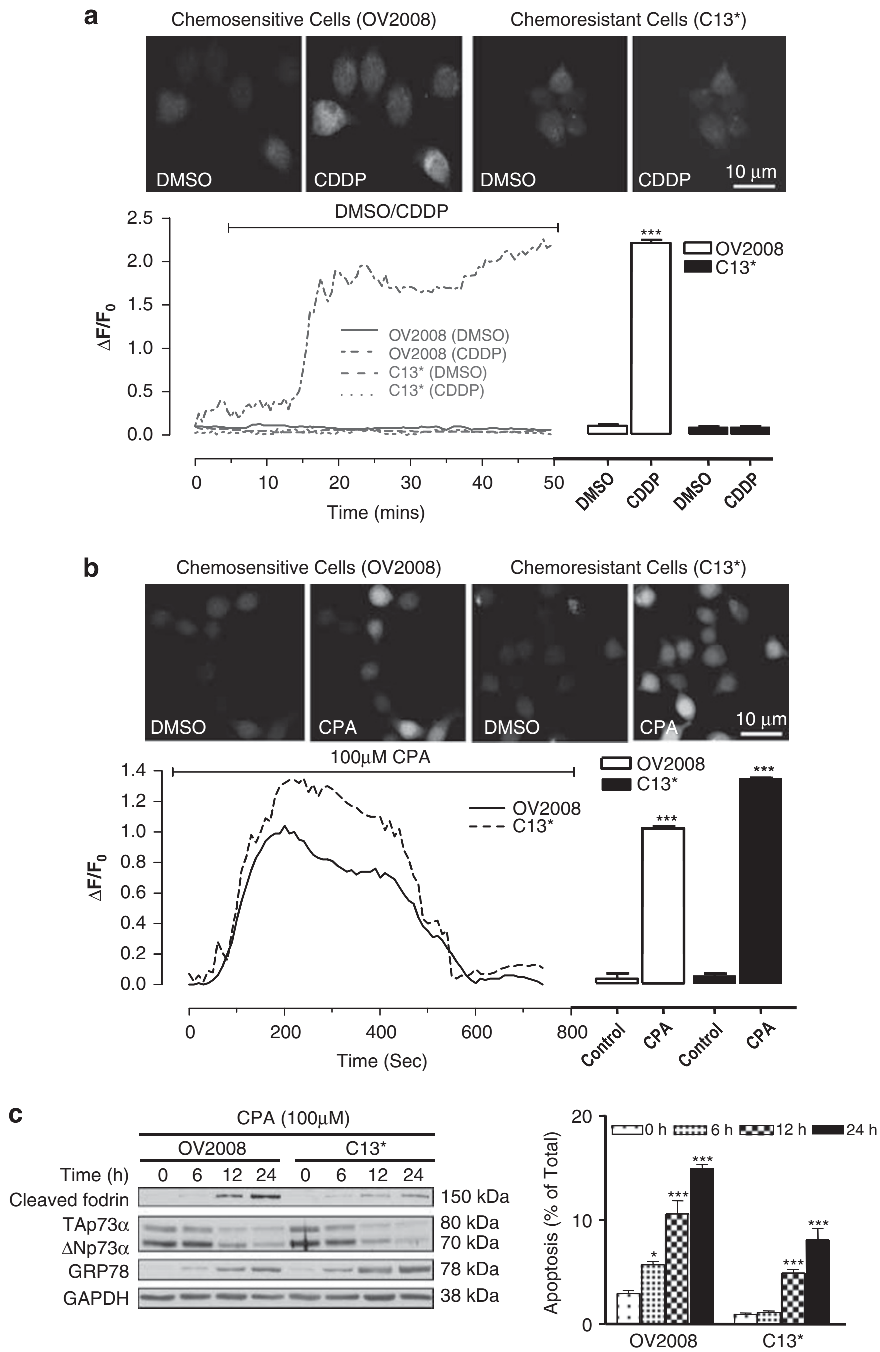

Figure 4 CDDP differentially increases the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in OVCA cells. (**a**) CDDP induced $[Ca^{2+}]_i$ increase in OV2008, but not C13* cells. The effect of CDDP on $[Ca^{2+}]_i$ was assessed by confocal microscopy. CPA induced (**b**) $[Ca^{2+}]_i$ increase, (**c**) calpain activation, TAp73α and ΔNp73α downregulation/cleavage and apoptosis in both OV2008 and C13* cells. Images and figures present the mean ± s.e.m. of three independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

and growth suppression (Liu *et al*., 2004), ΔNp73α does not appear to affect cell growth or alter chemoresistance nor antagonize the pro-apoptotic function of p53 (Marabese *et al*., 2005; Sabatino *et al*., 2007). It is possible that the action of ΔNp73α may be cell-specific: interacting with additional mediators and resulting in differential phenotypes. In our system, the varied response to ΔNp73α over-expression may have been to differences in the genetic background between these cells (that is, Hey cells) and/or the relative importance of the specific regulator of CDDP-induced apoptosis in different cell lines. Specifically, the A2780s cell line does not express TAp73α due to CpG island hypermethylation (Chen *et al*., 2000) and the use of 5-Aza deoxycitidine, a demethylating agent, restored its content (Supplementary Figure 2) whereas ΔNp73α was detected at the

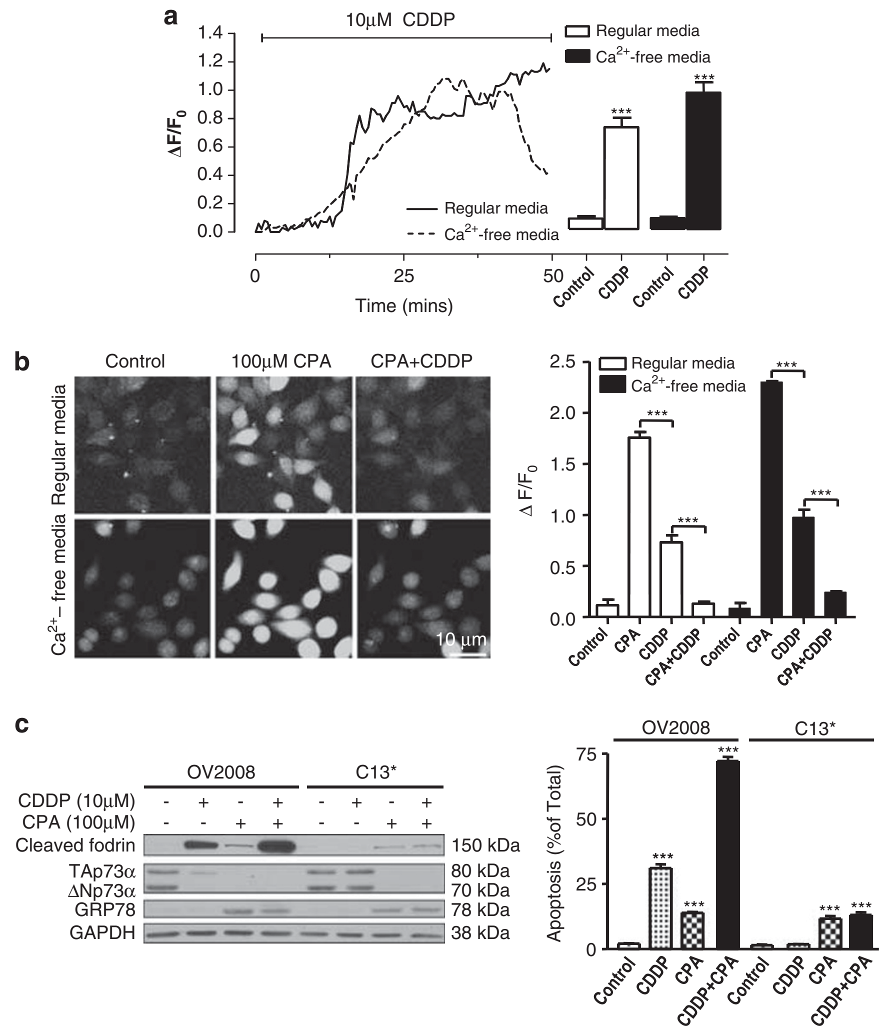

Figure 5 CDDP-induced $[Ca^{2+}]_i$ increase depends upon internal stores. (**a**) CDDP-induced $[Ca^{2+}]_i$ increase was not effected by the removal of extracellular Ca^{2+} (that is, Ca^{2+}-free media). OV2008 cells were cultured in both regular and Ca^{2+}-free media and the effect of CDDP on $[Ca^{2+}]_i$ were measured. (**b**) CDDP-induced $[Ca^{2+}]_i$ increase was ceased after store depletion by CPA in both the regular and the Ca^{2+}-free media. OV2008 internal stores were depleted by CPA prior to CDDP treatment and changes in $[Ca^{2+}]_i$ were assessed. (**c**) CDDP enhanced CPA-dependent calpain activation and apoptosis in OV2008, but not C13* cells. OV2008 and C13* cells were treated with CPA (100 μM; 24 h) and calpain activation, TAp73α and ΔNp73α content and apoptosis were determined. Images and figures present the mean ± s.e.m. of three independent experiments (*$P<0.05$, ***$P<0.001$).

mRNA but not at the protein level (Supplementary Figure 3), suggesting a dysregulation at the translational level. In this case, ΔNp73α might need the presence of TAp73α as well as wild-type p53 in A2780cp cells (which harbor mutant-p53) to enhance CDDP-induced apoptosis. Our finding of ΔNp73 over-expression is consistent with a previous report showing that A2780 clones stably over-expressing ΔNp73α responded to CDDP in a manner similar to their parental cells (Sabatino *et al*., 2007).

The reasons for the discrepancies of the role of both TAp73 and ΔNp73 in drug-induced apoptosis are not immediately apparent. Whether they are attributed to differences in experimental conditions including the type of genotoxic insults (for example, Staurosporine and Etoposide), cell type or origin, inherent biochemical characteristics of the cells (for example, relative distribution of p73 isoforms, interaction with other p73 family members (p53 and p63), and selective activation by the up-stream activators) requires further investigations (Nyman *et al*., 2005; Holcakova *et al*., 2008). In addition, other interactors could modulate the function of p73, as it has already been described, for example, for Wwox (Aqeilan *et al*., 2004) and for the ASPP family (Bergamaschi *et al*., 2004; Sullivan and Lu, 2007). Nonetheless, our results provide new insights into the role of p73α in CDDP-induced apoptosis in OVCA cells. Using two pair of OVCA cell lines for the over-expression experiment and specific cDNA for each isoform allowed us to differentially assess their effect on CDDP-induced apoptosis. Furthermore, the present studies on p73α downregulation provided further evidence that endogenous p73α is a determinant of the sensitivity of OV2008 cell to CDDP. Development of specific antibodies and/or inhibitors to each isoform should be helpful to determine the precise role of p73α in CDDP-induced apoptosis in ovarian cancer cells.

Endogenous 73α could be involved in CDDP-induced apoptosis in OVCA cells. The p53-responsive genes PUMA and NOXA could be its down-stream effectors in the pro-apoptotic function (Melino *et al*., 2004; Muller *et al*., 2006). PUMA expression is also mediated by CDDP-induced, p53-dependent apoptosis in OVCA cells (Fraser *et al*., 2008). Our observations raise the possibility that CDDP-induced PUMA expression in OVCA cells is regulated by both p53-dependent and -independent pathways.

Our findings are the first demonstration that calpain regulates TAp73α and ΔNp73α content in the CDDP-induced apoptosis in cancer cells, although calpain-mediated cleavage of p73 isoforms at both the N- and the C-terminus has been demonstrated (Munarriz *et al*., 2005). Calpain inhibition by a specific inhibitor (Calpeptin) and/or by RNA silencing substantiates the involvement of calpain in the CDDP-induced, TAp73α and ΔNp73α processing in OVCA cells. It also demonstrates, for the first time, that calpain is required for CDDP-induced apoptosis in OVCA cells, where its inhibition decreased OVCA cell sensitivity to CDDP. The novel finding that calpain and p73α interact and co-localize in these cells provided further support for such involvement.

Although calpain can cleave p73 at both the N- and the C-terminus, the consensus sites of calpain cleavage in the p73 sequence are unknown. Heterogeneity of the p73 isoforms at both termini, where the calpain cleavage sites are present, was problematic for the construction of a mutant p73 that lack these functional sites. It is not known whether calpain-mediated cleavage would result in p73α degradation, as in the case of Xiap (Kobayashi *et al*., 2002) or enhanced Bax function (Toyota *et al*., 2003). The occurrence of calpain-mediated p73α cleavage in chemosensitive, and not in the chemoresistant cells, is consistent with our concept that p73α cleavage and activity is involved in the regulation of chemosensitivity. This hypothesis is further supported by the demonstrated role of TAp73α as a pro-apoptotic protein in CDDP-induced apoptosis.

Ca^{2+} homeostasis is critical for regulation of cellular function. We found that CDDP induces $[Ca^{2+}]_i$ increase in OV2008, but not in C13* cells. Although a recent study showed that CDDP increased $[Ca^{2+}]_i$ in chemosensitive HeLa cells, but not in chemoresistant osteosarcoma (U2-OS) cells (Splettstoesser *et al*., 2007), whether the difference in response was cell-origin specific or due to inherent differences in the mechanism of CDDP resistance could not be determined, as the two cell lines were unrelated. We were able to confirm such differences by employing isogenic cell lines pairs. Using both calcium-free media and depleting the internal stores by CPA, we demonstrated that the increase in $[Ca^{2+}]_i$ by CDDP is dependent on the internal stores and the deregulation of these stores might confer CDDP resistance.

The present study demonstrated a novel role of Ca^{2+}-mediated, calpain activation in regulating p73α function in CDDP-induced apoptosis in human ovarian cancer. We observed that CDDP increases $[Ca^{2+}]_i$, induces activation of calpain, and causes apparent calpain-mediated cleavage of p73α. However, the biological relevance of cleaved p73α remains unclear. One possibility that that cleaved p73α may have a role in the nucleus, as we observed decreased CDDP-induced PUMA and NOXA expression that was not secondary to changes in p53 function. Whether p73α influences nuclear function in response to CDDP—perhaps by translocation into the nucleus itself—remains unknown, and our laboratory is currently investigating this and other hypotheses. A greater understanding of the cellular and molecular mechanisms of chemoresistance may offer new strategies for the treatment of chemo-resistant ovarian cancer and direct further research.

Materials and methods

Reagents

Cis-diaminedichloroplatinum (CDDP), dimethyl sulfoxide (DMSO), Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), aprotinin, cycloheximide and 5-Aza deoxycitidine were purchased from Sigma (St Louis, MO, USA). Proteasome inhibitors (Lactacystin and Epoxomicin) were from Calbiochem (San Diego, CA, USA). Calpeptin was from Enzo Life Science International Inc. (Plymouth Meeting, PA, USA). The siRNA for p73α, calpain and scrambled sequence siRNA (control) were provided by Ambion Inc. (Austin, TX, USA), Santa Cruz Biotechnologies (San Diego, CA, USA) and Dharmacon Inc. (Lafayette, CO, USA), respectively. RiboJuice and Lipofectamine Plus and Fluo4-AM dye were from Novagen Inc. (San Diego, CA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Ca^{2+}-free media (RPMI-1640) was from United States Biological (Swampscott, MA, USA).

The primary antibodies were rabbit polyclonal anti-p73SAM (1:2000; Sayan *et al*., 2005), anti-PARP, anti-α-fodrin and anti-calpain (1:5000; Cell Signalling Technology, Beverly, CA, USA), anti-PUMA (1:1000; Santa Cruz Biotechnologies) and anti-Xiap (1:1000; Trevigen, Gaithersburg, MD, USA), mouse monoclonal anti-NOXA (1: 1000, AbCam), anti-GAPDH (ab8245, Abcam, Cambridge, UK) as well as rat anti-HA (1:1000; clone 3F10, Roche, Laval, Quebec, Canada) antibody. Goat anti-p73 and agarose immobilized anti-HA (C-17; Santa Cruz Biotechnologies and Sigma-Aldrich, St Louis, MO, USA) antibodies were used for p73α immunoprecipitation whereas goat anti-calpain 1 antibody (Santa Cruz Biotechnologies) was used for calpain binding. The horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA). Enhanced chemiluminescent reagents and films were from Amersham Biosciences (Buckinghamshire, UK).

Cell culture

Cell lines were a generous gift from Dr B Vanderhyden (University of Ottawa, Ottawa, ON, Canada (Shaw *et al*., 2004) and were tested recently (Abedini *et al*., 2008; Abedini *et al*., 2010). They were maintained at 37 °C and in an atmosphere of 5% CO_2 and 95% air. CDDP-sensitive human OVCA cells (OV2008) and its resistant isogenic counterpart (C13*), as well as Hey cells were maintained in RPMI 1640 medium. CDDP-sensitive (A2780s) and -resistant (A2780cp)

OVCA cells were cultured in DMEM-F12 medium supplemented with fetal bovine serum (10%), streptomycin (50 g/ml), penicillin (50 U/ml), fungizone (0.625 g/ml; Life Technologies, Inc., BRL, Carlsbad, CA, USA), and non-essential amino acids (1%).

Protein extraction and western blotting
Protein extraction and western blotting were performed as described (Abedini *et al*., 2008; Fraser *et al*., 2008). Membranes were incubated overnight at 4 °C with primary antibodies, and detected with horseradish peroxidase-conjugated goat IgG raised against the corresponding species. Peroxidase activity was visualized with an enhanced chemiluminescence (ECL) kit. Signal intensity was determined densitometrically using Scion Image software, version 4.02, from Scion Corporation (Frederick, MD, USA).

Transient transfection
All cells (2.4×10^5) were transfected with 2 μg of pcDNA3.1/CT-GFP vector alone, or pcDNA3.1/CT vectors containing TAp73α or ΔNp73α cDNA (Fraser *et al*., 2008). Cells were treated with CDDP (10 μM; 24 h) or DMSO (vehicle) and then harvested for further analysis.

Adenovirus infection
OV2008 cells were infected with adenoviral Xiap-sense or LacZ control (5 MOI), treated with CDDP and harvested as reported (Fraser *et al*., 2003b).

siRNA transfection
OV2008 cells (2.4×10^5/well) were transfected with siRNA (Abedini *et al*., 2008). Briefly, cells were transfected (48 h) with scrambled sequence siRNA (control) and/or p73α or calpain siRNA. Cells were treated with CDDP and harvested as above.

Determination of apoptosis
Apoptosis was assessed morphologically by Hoechst 33258 dye (6.25 ng/ml). At least 400 cells/treatment groups were counted. Selected fields and blinded slides were determined randomly to avoid experimental bias (Fraser *et al*., 2008).

Reverse transcriptase polymerase–chain reaction (RT–PCR)
RT–PCR was carried out as previously described (Fraser *et al*., 2008). PCR primers from Invitrogen (Burlington, ON, Canada) were as follows: TAp73α sense (5′-GATTCCAGCA TGGACGTCTT-3′), TAp73α antisense (5′-TTCTTCAAGAG CGGGGAGTA-3′); ΔNp73α sense (5′-AAGCGAAAATGCC AACAAAC-3′), ΔNp73α antisense (5′-GTACGTCCAGG TGGCTGACT-3′); β-actin sense (5′-GGACTTCGAGCAAG AGATGG-3′), β-actin antisense (5′-CACCTTCACCGTTCC AGTTT-3′). PCR conditions underwent activation (15 min; 95 °C), denaturation (45 s; 94 °C), annealing (TAp73α and ΔNp73α; 30 s; 52 °C, β-actin; 45 s; 54 °C) and extension (72 °C; 30 s) for 40 and 25 cycles for p73α and β-actin, respectively.

Assessment of protein interactions
P73α in whole cell lysate was immunoprecipitated with goat anti-p73 (C-17, 2 μg), as reported (Fraser *et al*., 2008) and its interaction with calpain was detected by western blot.

Immunocytochemistry
The p73α and calpain co-localization was determined by immunocytochemistry (Abedini *et al*., 2010). Briefly, fixed cells were incubated with goat anti-p73α (1:25) and mouse anti-calpain (1:50) antibodies, subsequently, with donkey anti-goat Cy5-(p73α; 1:100) and anti-mouse FITC-conjugated (calpain; 1:200) antibodies. Florescence images were acquired with an LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany) and a 63 × oil-immersion objective. The images were merged using Adobe Photoshop 7.01 (Adobe, San Jose, CA, USA).

Calcium measurement
OV2008 and C13* cells loaded with the calcium sensitive Fluo4-AM dye (5 μM; 30 min; 37 °C) were washed with media and changes in $[Ca^{2+}]_i$ were observed with a Zeiss 510 laser scanning microscope (Gunes *et al*., 2009). For CDDP experiments, each scan lasted for 2 s in duration, every 30 s for a time period of 1 h. The CPA, images were taken every 15 s for a 20-min period. Cells were excited at 488 nm and the emission was captured at 510 nm using LSM 510 software (Zeiss). Stacks of images were then loaded into ImageJ (http://rsbweb.nih.gov/ij/) for automated analysis. All experiments were performed at room temperature in a constant flow of media (~1 ml/min).

Statistical analysis
Results are expressed as the mean ± s.e.m. of at least three independent experiments and analyzed by two- or three-way ANOVA using PRISM (Version 3.0 GraphPad, San Diego, CA, USA) and Sigma STAT (Version 3.1, Aspire Software International, Ashburn, VA, USA) software program, respectively. Differences between multiple experimental groups were determined by the Bonferroni test. Statistical significance was indicated at $^{***}P < 0.001$, $^{**}P < 0.05$ and $^{*}P < 0.01$.

Conflict of Interest

The authors declare no conflict of interest.

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