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Abstract

The intrinsic chemoresistance of pancreatic ductal adenocarcinoma (PDAC) represents the main obstacle in treating this aggressive malignancy. It has been observed that high antioxidant levels and upregulated Nrf2 and the YAP protein expression can be involved in PDAC chemoresistance. The mechanisms of Nrf2 and YAP increase need to be clarified.

We chose a panel of PDAC cell lines with diverse sensitivity to cisplatin and gemcitabine. In PANC-1 chemoresistant cells, we found a low level of oxidative stress and high levels of Nrf2 and YAP protein expressions and their respective targets. On the contrary, in CFPAC-1 chemosensitive cells, we found high levels of oxidative stress and low level of these two proteins, as well as their respective targets. In MiaPaCa-2 cells with a middle chemoresistance, we observed intermediate features. When Nrf2 and YAP were inhibited in PANC-1 cells by Ailanthone, a plant extract, we observed a reduction of viability, thus sustaining the role of these two proteins in maintaining the PDAC chemoresistance.

We then delved into the mechanisms of the Nrf2 and YAP protein upregulation in chemoresistance, discovering that it was at a post-translational level since the mRNA expressions did not match the protein levels. Treatments of PANC-1 cells with the proteasome inhibitor MG-132 and the protein synthesis inhibitor cycloheximide further confirmed this observation.

The expression of DUB3 and OTUD1 deubiquitinases, involved in the control of Nrf2 and YAP protein level, respectively, was also investigated. Both protein expressions were higher in PANC-1 cells, intermediate in MiaPaCa-2 cells, and lower in CFPAC-1 cells. When DUB3 or OTUD1 were silenced, both Nrf2 and YAP expressions were downregulated.

Importantly, in deubiquitinase-silenced cells, we observed a great reduction of proliferation and a higher sensitivity to gemcitabine treatment, suggesting that DUB3 and OTUD1 can represent a suitable target to overcome chemoresistance in PDAC cells.

Keywords: PDAC cells, chemoresistance, oxidative stress, Nrf2, YAP, deubiquitinases.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy that represents the fourth leading cause of cancer deaths in the western world [1]. Despite the improved clinical outcomes, the treatment of PDAC is often ineffective due to its invasive nature and its intrinsic chemoresistance. A mountain of evidence has suggested that the regulation of redox status plays an important role in cancer cell survival to the therapy [2,3]. Indeed, some cancer cells in the advanced stages of the disease up-regulated their antioxidant systems becoming highly adapted to intrinsic or drug-induced oxidative stress [4]. The Keap1-Nrf2 pathway plays a pivotal role in the maintenance of normal tissue structure and organ protection from oxidative stress. Under physiological conditions, Nrf2 is present in the cytoplasm where it is bound by Keap1 (Kelch-like ECH-associated protein), which drives Nrf2 to ubiquitination and proteasomal degradation. An increase of oxidative stress causes a conformational change of Keap1 that prevents Nrf2 ubiquitination. As a consequence, Nrf2 can translocate into the nucleus where it heterodimerizes with small Maf proteins and, through the binding with the antioxidant response element (ARE)/electrophile response element (EpRE), activates target genes for cytoprotection [5].

Along with other mutations, more than 90% of the pancreatic cancers harbor activating K-ras mutations [6]. Interestingly, upregulation of Nrf2 is, at least in part, K-Ras oncogene-driven and contributes to pancreatic cancer proliferation and chemoresistance [7]. Moreover, it has been demonstrated that the deletion of Nrf2 in mouse models with mutant K-ras–driven pancreatic cancer resulted in reduced formation of pancreatic lesions. Thus, these authors proposed Nrf2 as a novel therapeutic target of pancreatic cancer [8].

Yes-associated protein (YAP) is another important player involved in pancreatic cancer progression and metastasis [9]. YAP is a key component of the Hippo tumor-suppressor pathway [10]. The Hippo pathway phosphorylates YAP on Ser127 leading to its cytoplasm sequestration or on Ser 381 that leads YAP to ubiquitination and proteasomal degradation [11]. Conversely, unphosphorylated YAP can translocate into the nucleus and, through the binding with the TEAD family of transcription factors, triggers the expression of several downstream target genes such as FOXM1, Cyr61 and survivin, involved in organ size control, cell proliferation, migration and survival, [12]. YAP can also interact with the FoxO1 transcription factor which stimulates the transcription of the catalase and manganese superoxide dismutase (MnSOD) antioxidant genes, thus contributing to the maintenance of the antioxidant status of the cell [13].

Both Nrf2 and YAP proteins have been involved in the chemoresistance of PDAC cancers [14-16], and their expressions are upregulated in cancers harboring Kras mutations [17-19]. Despite several recent studies indicating the importance of Nrf2 or YAP silencing in reducing the chemoresistance

of cancer cells from diverse origins, much remains to be understood, in particular regarding the post-translational modification of Nrf2 and YAP in pancreatic cancer cells. In recent years the importance of deubiquitinases (DUBs), as druggable targets in pancreatic cancer cells has been reported [20, 21], but no data are available about deubiquitinases targeting Nrf2 or YAP in PDAC cells. In this study we examined, in three lines of chemoresistant or chemo-sensitive pancreatic cancer cells, displaying diverse levels of oxidative stress, Nrf2 and YAP expression and activity, the expression of specific DUBs targeting Nrf2 and YAP proteins. Moreover, we examined, in chemoresistant cells, the consequence of DUB silencing on cell growth and chemoresistance.

Materials and Methods

Cells, culture conditions and treatments

Pancreatic cancer cells, namely PANC-1, MiaPaCa-2, and CFPAC-1, were purchased from ATCC (Manassas, VA, USA). These cells were cultured in DMEM high glucose, supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO_2, 37 °C incubator. Cells were treated with diverse concentrations of CDDP, gemcitabine (Sigma–Aldrich) or ailanthone (Aila) (Baoji Herbest, Bio-Tech Co., Ltd., Baoji city Shannxi, Provence China) and used for the MTT analysis.

MTT assay

MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich) assay was performed by seeding the cells (800–1500 cells/well) in 100 µl of serum-supplemented medium and treated with different concentrations of CDDP or gemcitabine in a range of concentrations ranging from 0.1 to 4 µg/ml for CDDP and from 0.01 to 4 µg/ml for gemcitabine. PANC-1 cells were treated with Aila concentrations ranging from 0.1-2 µg/ml. Untreated cells were used as control. DUB-silenced PANC-1 cells were treated with gemcitabine concentrations ranging from 0.05 to 4 µg/ml. After 72 hrs, the viability was assessed by adding MTT to control and treated cells to a final concentration of 0.5 mg/ml for 2 hrs. At the end of this period, the medium was removed, 100 µl of DMSO were added and the absorbance was recorded at 530 nm by a 96-well-plate ELISA reader.

Measurement of the cell redox status

The oxidative stress level in the cells (200,000 cells/well) was analyzed through incubation of 30 min with 1 µM 2′-7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen, Carlsbad, CA, USA), and by measuring the amount of fluorescent product 2',7'-dichlorofluorescein (DCF) before the cytofluorimetric analysis (Becton Dickinson Accuri).

Lysate preparation and western blot analysis

Lysate preparation and western blot analysis were performed as previously described [5]. Antibodies used were as follows: β-actin (sc-47778), YAP (sc-376830), Nrf2 (sc-365949), Keap1 (sc-33569), GSTA4 (SAB 1401164-100UG, Sigma Aldrich), survivin (D-8, sc-17779), p62/SQSTM1 (AB-81677 Immunological Sciences), heme oxygenase-1 (HO-1) (F-4 sc-390991), FOXM1 (sc-271746), DUB3 (WHO 377630M1-100UG, Sigma Aldrich), OTUD1 (SAB 2108986-100UL, Sigma Aldrich). The detection of the bands was carried out after reaction with chemiluminescence reagents (Western Lightning™ Chemiluminescence Reagent Plus ECL, PerkinElmer NEL105001EA) through film (Santa Cruz Biotechnology sc-201697) autoradiography, or they were visualized using a Bio-Rad visualizer (Bio-Rad Molecular imager ChemiDoc XRS+).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

For reverse transcription, 1 μg of total RNA from each cell line, 25 μM random hexamers and 100U of Reverse Transcriptase (ThermoFisherScientific, USA) were used. The levels of gene expression were measured by quantitative real-time PCR (qRT-PCR) in an iCycler (Bio-Rad Laboratories, USA). Abelson (Abl) gene was utilized as a housekeeping control. The following TaqMan® Gene Expression Assay (Thermo Fisher Scientific, USA) were used: Hs00975960_m1 for Nrf2 gene; Hs00371735_m1, Life Technologies for YAP gene and Hs00245445_m1 for Abl gene, respectively.

PCR reaction, for each cell line, was performed as follows: 50 ng of cDNA was added to the PCR reaction mix containing 1× TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, USA), 1× TaqMan® Gene Expression Assay (Thermo Fisher Scientific, USA) and distilled water to a final volume of 10 μl. All analyses were carried out in duplicate; results showing a discrepancy greater than one cycle threshold in one of the wells were excluded. The results were analyzed using the ΔΔCt method [22].

MG132 treatment

To confirm that the control of Nrf2 and YAP levels in our pancreatic cell lines mainly depended on proteasomal degradation, MG132 (carbobenzoxy-Leu-Leu-leucina) was used. MG132 is a peptide aldehyde, able to inhibit the proteolytic activity of the 26S proteasome complex. MG132 Ready Made Solution was purchased from Sigma. Cells ($2x10^5$ cells/well) were seeded in a 6 well plate in the presence or absence of 5 μM MG132 for 24 hrs. At the end of the treatment, cells were

harvested, washed with PBS, and frozen at -80 °C until protein extraction and the analysis of Nrf2 and YAP expression.

Cycloheximide assay

Cycloheximide (C 7698, Sigma Aldrich) 'was added into culture medium with the final concentration ranging from 100 μg/ml. For Nrf2 expression analysis, PANC-1 and CFPAC-1 cells were collected at 5, 10, and 20 min after the treatment with cycloheximide, since the Nrf2 half-life was about 20 min [23]. Instead, since the YAP half-life was about 12-15 hrs [24], the YAP expression analysis was performed after 15 and 24 hrs from cycloheximide treatment.

Deubiquitinase total activity.

Deubiquitinase total activity in PANC-1, MiaPaCa-2, and CFPAC-1 cells was determine by using the Deubiquitinase Assay Kit (ab 241002-100 test, Prodotti Gianni).

Cell transfection with siRNA against DUB3 and OTUD1 deubiquitinases.

Cells were transfected with DUB3 siRNA (sc-143189) and siRNA OTUD1 (sc-151939) by using the siRNA Transfection Reagent (sc-29528) with a protocol indicated by the manufacturer. Briefly, cells were seeded onto 6 well tissue culture plates in the culture medium containing serum but not antibiotics. After 24 hrs, siRNA and the Transfection Reagent were diluted in siRNA Transfection Medium (sc-36868) and incubated for 15 min at room temperature to allow the complexation between the siRNA and the Transfection Reagent. Afterward, siRNA transfection was carried out in the culture medium. To allow complexation between the siRNAs and the Transfection reagent, both were diluted in siRNA Transfection Medium (sc-36868) and incubated for 15 min at room temperature. Complexes were added drop-wise onto the cells, according to the manufacturer's instruction.

Statistical analysis

Differences between experimental groups were evaluated by one-way ANOVA followed by the Bonferroni post-test (GraphPad InStat software (San Diego, CA, USA). We considered statistically significant values of $p \leq 0.05$.

Results

CDDP and gemcitabine sensitivity and the oxidative stress level in PDAC cells

PANC-1, MiaPaCa-2, and CFPAC-1 cells were treated with diverse concentrations of CDDP or gemcitabine, and their viability was evaluated through MTT assay 72 hrs after the treatment (Fig.1).

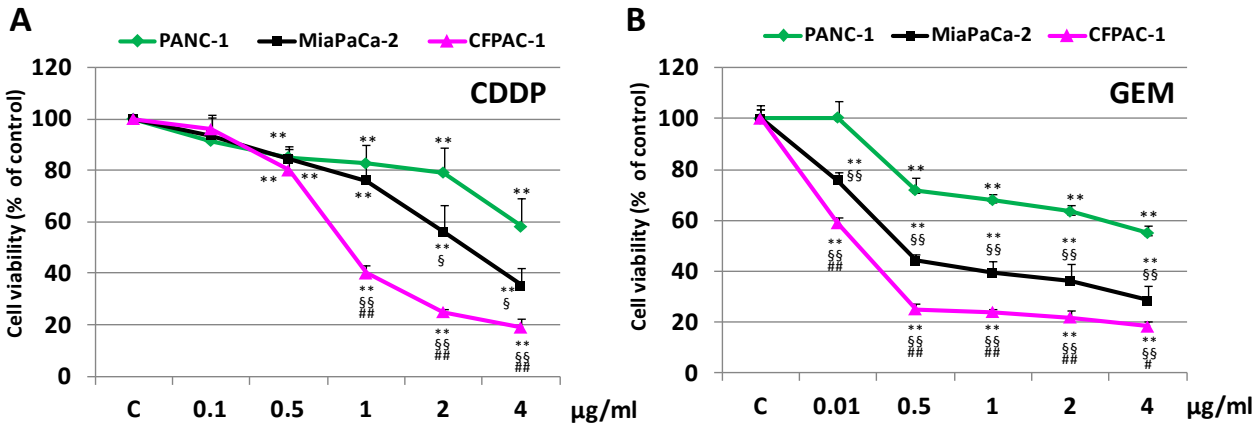

Fig. 1. Viability (MTT assay) in PANC-1, MiaPaCa-2, and CFPAC-1 cells untreated (C) or treated with cisplatin (CDDP) (**panel A**) or gemcitabine (GEM) (**panel B**) at the indicated concentrations 72 hrs after the treatment. Results are expressed as percent of control and are the mean ± SD of three separate experiments. **p<0.01 vs. C; §p<0.05 and §§p<0.01 vs. PANC-1; #p<0.05 and ##p<0.01 vs. MiaPaCa-1.

PANC-1 cells were the more resistant cell line toward the two drugs, MiaPaCa-2 cells had an intermediate response and CFPAC-1 cells were the more sensitive. Confirming previous observations regarding the higher oxidative stress in chemo-sensitive cells [2], the intracellular oxidative stress level was higher in CFPAC-1 cells, middle in MiaPaCa-2 cells and lower in PANC-1 cells (Fig. 2).

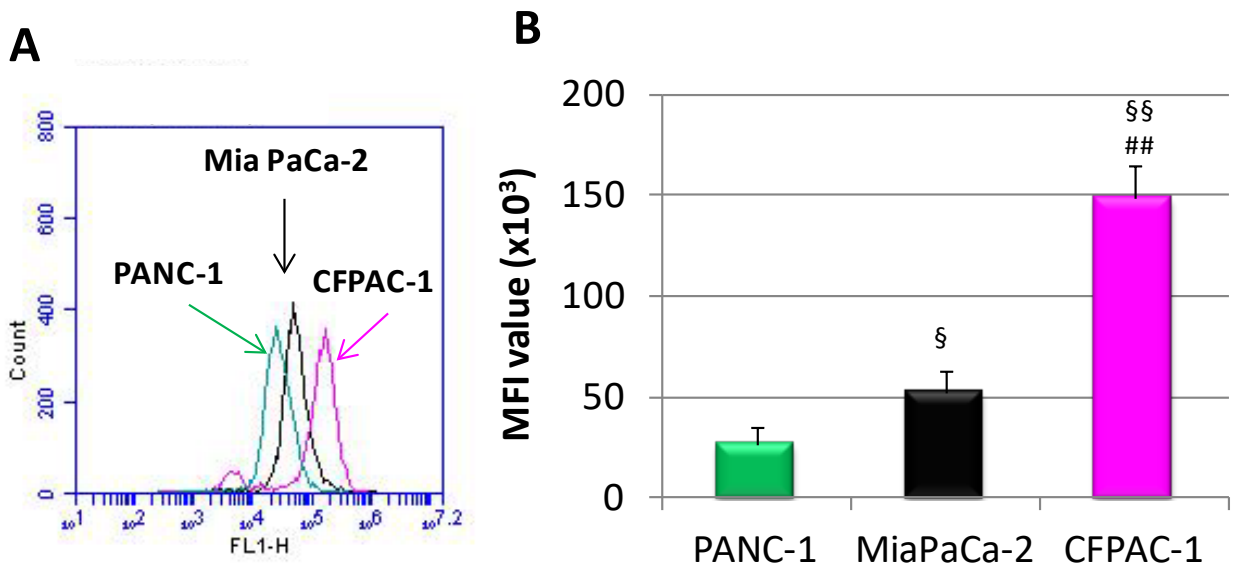

Fig. 2. The intracellular oxidative stress level in PANC-1, MiaPaCa-2, and CFPAC-1 untreated cells, measured by incubating cells with dichlorodihydrofluorescein diacetate (DCF-DA). The amount of fluorescent product (2,7-dichlorodihydrofluorescein, DCF) was measured by the FACScan cytometer (Becton Dickinson Accuri). **Panel A**: representative histogram from flow cytometric analysis. **Panel B:** bar graph showing median fluorescence intensity (MFI) values, expressed as means ± SD. §p<0.05 and §§ p<0.01 vs. PANC-1; ##p<0.01 vs. MiaPaCa-1.

Nrf2 and YAP expressions and activities in PDAC cells

In accordance with the low oxidative stress level, Nrf2 protein expression was higher in PANC-1 cells with respect to MiaPaCa-2 and CFPAC-1 cells, as well as the Nrf2 target, GSTA4. The expression of the other Nrf2 targets, HO-1 and p62, was higher in PANC-1 and MiaPaCa-2 and lower in CFPAC-1 cells. The expression of Keap-1, the Nrf2 inhibitor, was similar in all cell lines (Fig. 3).

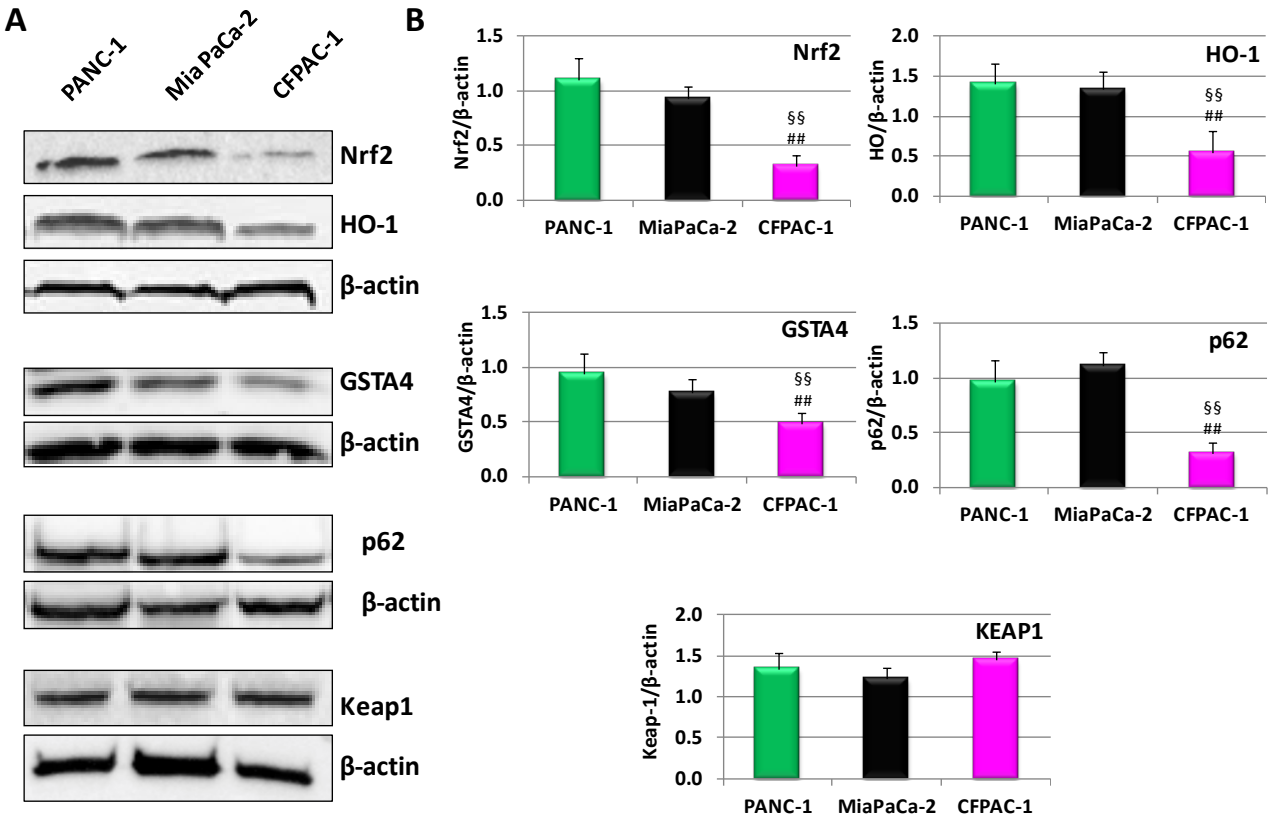

Fig. 3. Protein expression analysis of Nrf2, the Nrf2 target genes, and Keap1 in PANC-1, MiaPaCa-2, and CFPAC-1 untreated cells. **Panel A:** Western blot analysis of Nrf2, HO-1, GSTA4, p62, and Keap1 expressions. **Panel B:** densitometric analysis of the protein expression, normalized using the β-actin signal. Data are the mean ± SD of three independent experiments. §§ p<0.01 vs. PANC-1; ##p<0.01 vs. MiaPaCa-1.

Analogously to Nrf2 expression, YAP expression was higher in PANC-1 cells with respect to MiaPaCa-2 and CFPAC-1 cells. Expressions of two YAP/TEAD targets, survivin and FOXM1, showed a similar trend: they were higher in PANC-1 cells, middle in MiaPaCa-2 cells and lower in CFPAC-1 cells (Fig. 4).

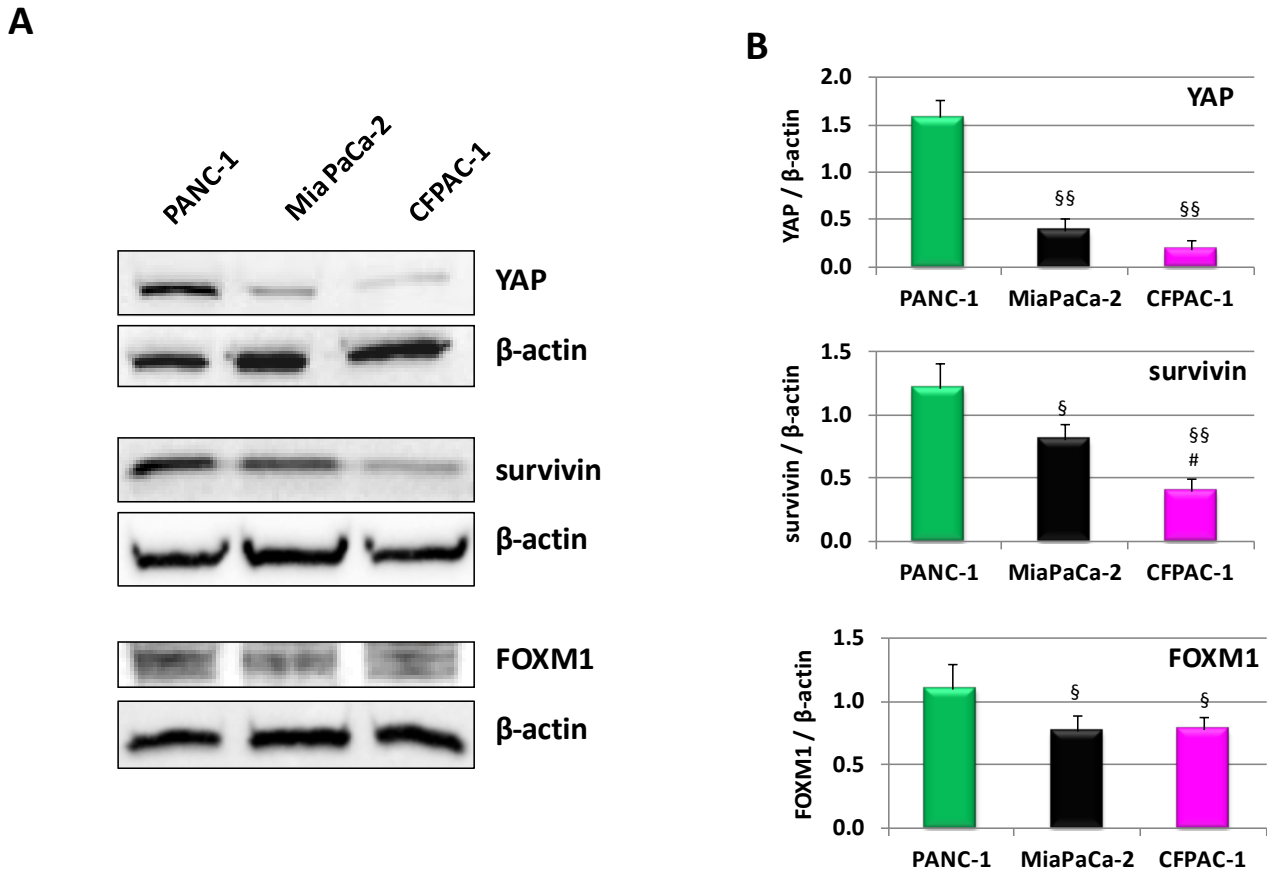

Fig. 4. Protein expression analysis of YAP and YAP target genes in PANC-1, MiaPaCa-2, and CFPAC-1 untreated cells. **Panel A:** Western blot analysis of YAP, survivin and FOXM1 expressions. **Panel B:** densitometric analysis of the protein expression, normalized using the β-actin signal. Data are the mean ± SD of three independent experiments. §$p<0.05$ and §§ $p<0.01$ vs. PANC-1; #$p<0.05$ vs. MiaPaCa-1.

Inhibition of Nrf2 and YAP expression reduces cell growth of chemoresistant PANC-1 cells.

To assess whether in chemoresistant cells, which presented an elevated Nrf2 and YAP expression, the inhibition of these two protein expressions could result in a reduction of cell growth, we treated PANC-1 cells with Aila, a chemical previously demonstrated able to inhibit Nrf2 and YAP expression in diverse cell models [25, 26]. Results demonstrated that the Aila treatment led to the reduction of Nrf2 and YAP protein expression, as well as of the respective targets, GSTA4 and survivin, respectively. The protein expression inhibition was accompanied by a strong reduction of cell growth starting from 0.1 μg/ml Aila (Fig. 5). These results confirmed that the reduction of Nrf2

and YAP expressions was accompanied by a reduction of the proliferative potential in intrinsic chemoresistant PANC-1 cells.

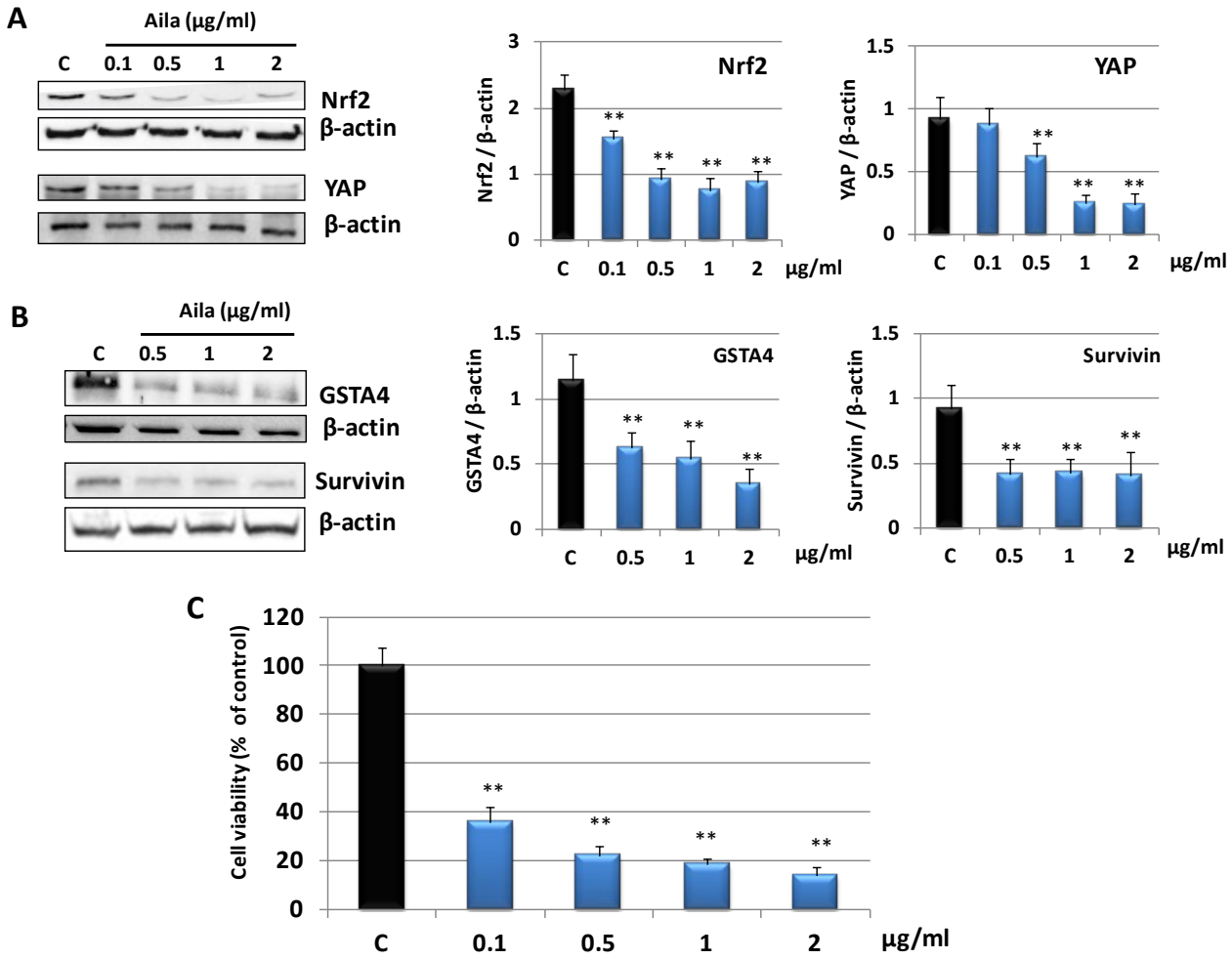

Fig 5. Ailanthone effects in PANC-1 cells. **Panel A, left:** Western blot analysis of Nrf2 and YAP in PANC-1 cells, untreated (control, C) or treated with Ailanthone (Aila) at the indicated concentrations, 24 hrs after the treatment; **right:** densitometric analysis of the protein expression, normalized using the β-actin signal. Data are the mean ± SD of three independent experiments; **p<0.01 vs. C. **Panel B, left:** Western blot analysis of Nrf2 target, GSTA4, and YAP/TEAD target, survivin in untreated (control, C) or treated with Aila at the indicated concentrations, 24 hrs after the treatment; **right** densitometric analysis of the protein expression, normalized using the β-actin signal. Data are the mean ± SD of three independent experiments; ** p<0.01 vs. C. **Panel C**: Viability (MTT assay) in PANC-1 cells, untreated (C) or treated with Aila the indicated concentrations, 72 hrs after the treatment. Results are expressed as percent of control and are the mean ± SD of three separate experiments; **p<0.01 vs. C.

The control of Nrf2 and YAP expression was at the post-translational level.

To verify whether Nrf2 and YAP protein expression was related to the level of mRNA transcription, the Nrf2 and YAP mRNA expression was quantified by qRT-PCR (Fig. 6). Our results demonstrated that, both Nrf2 and YAP mRNA expression did not follow the trend of protein

expression. Indeed, Nrf2 mRNA expression was lower in PANC-1 than in CFPAC-1 cells, whereas it was lowest in MiaPaCa-2 cells; YAP mRNA expression was similar in PANC-1 and CFPAC-1 cells and lower in MiaPaCa-2 cells. These results suggested that in PDAC cells the regulation of Nrf2 and YAP protein expression did not depend on the mRNA synthesis but was instead at post-translational level.

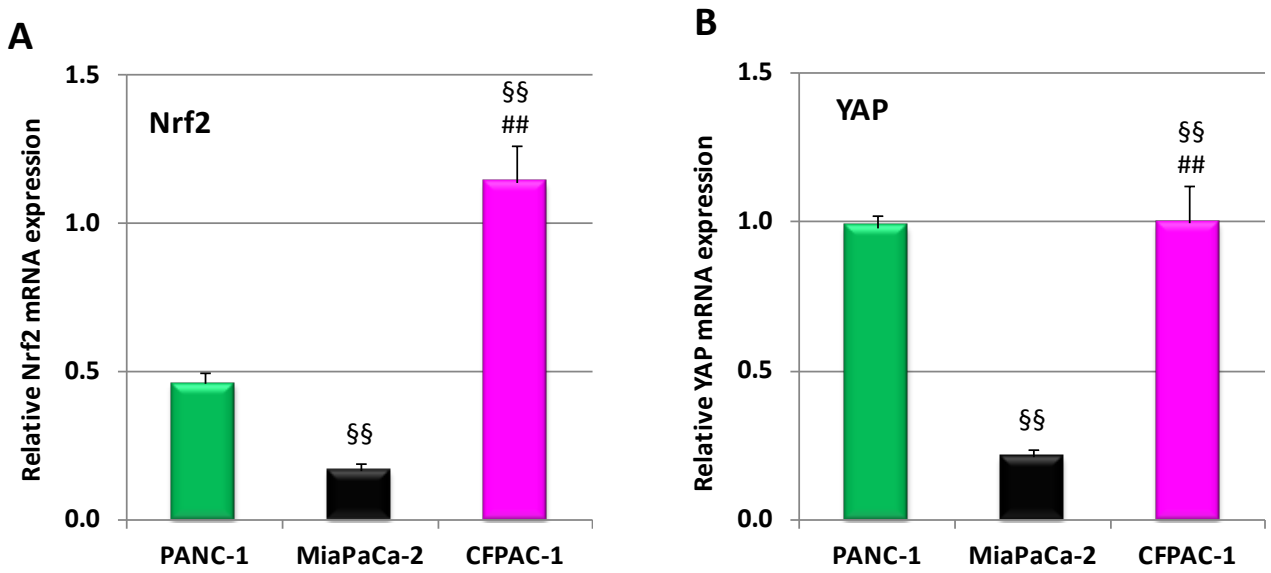

Fig. 6. Nrf2 (**panel A**) and YAP (**panel B**) mRNA expression in PANC-1, MiaPaCa-2, and CFPAC-1, untreated cells. mRNA expression was evaluated by qRT-PCR. Abelson (Abl) gene was utilized as housekeeping control. All analyses were carried out in triplicate; results showing a discrepancy greater than one cycle threshold in one of the wells were excluded. The results were analyzed using the ΔΔCt method. §§ $p<0.01$ vs. PANC-1; ##$p<0.01$ vs. MiaPaCa-1.

In accordance with this observation, the treatment with MG132, a proteasome inhibitor, increased Nrf2 and YAP expression in all PDAC cell lines (Fig. 7).

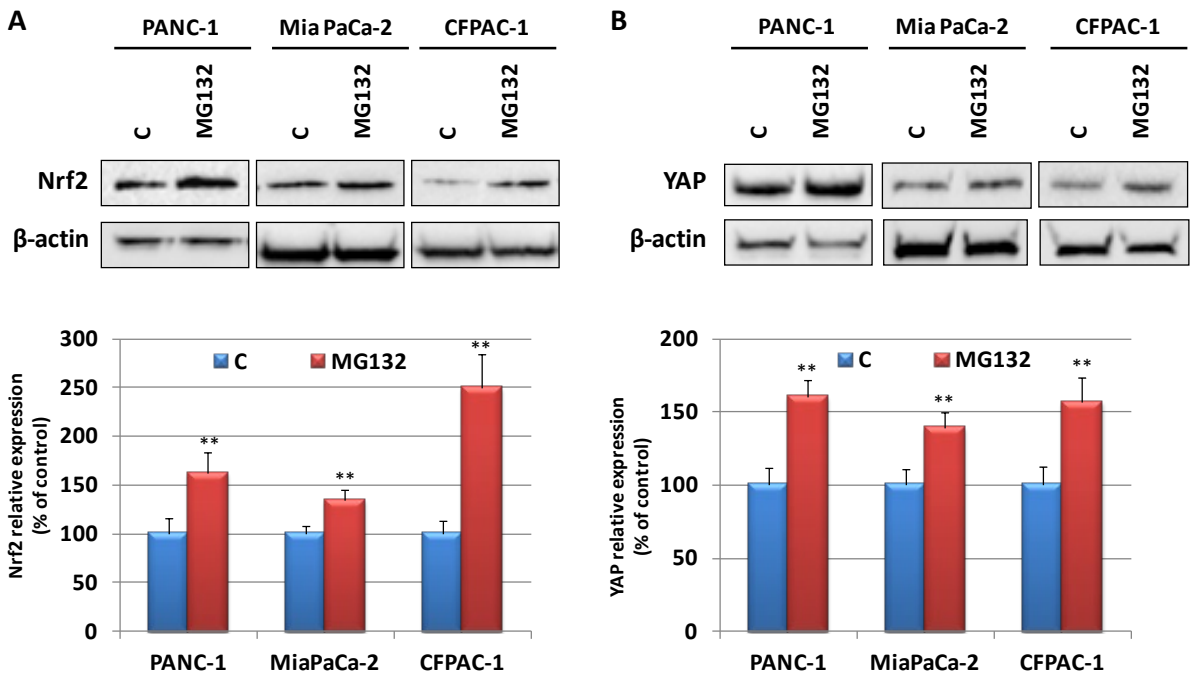

Fig. 7. WB analysis of NRf2 (**panel A**) and YAP (**panel B**) in untreated (control, C) or treated PANC-1, Mia PaCa-2, CFPAC-1 cells with 5 μM MG132, collected 24 hrs after the treatment. Equal protein loading was confirmed by exposure of the membranes to the anti-β-actin antibody. Below are shown the densitometric analysis of the protein expression, normalized using the β-actin signal. Data are indicated as the mean ± SD of three independent experiments. ** p-value ≤0.01 vs. respective C.

The cycloheximide treatment of PANC-1 and CFPAC-1 cells, chosen since they presented different chemosensitivity and Nrf2 and YAP protein expression, demonstrated that the rate of Nrf2 and YAP degradation was higher in CFPAC-1 cells compared with PANC-1 cells (Fig. 8).

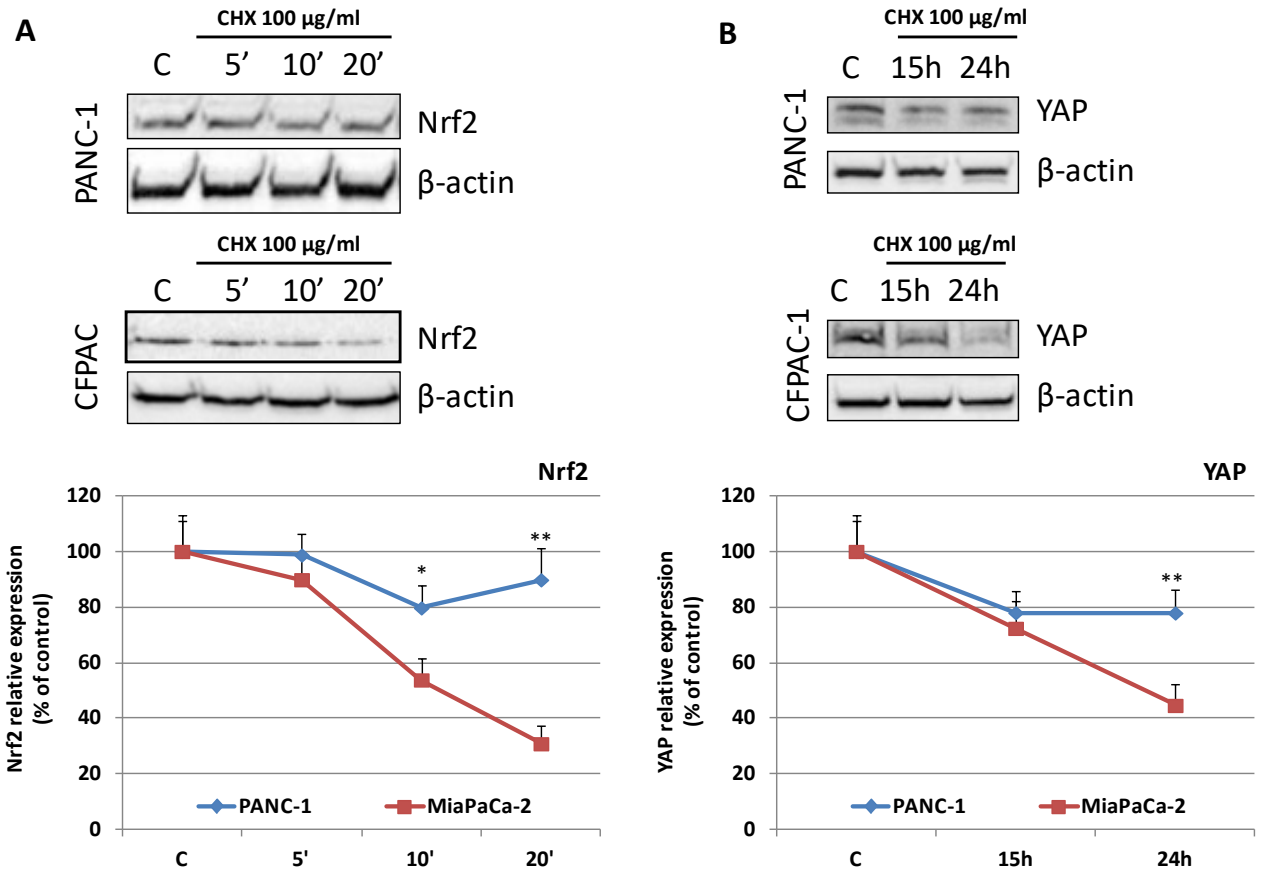

Fig. 8. WB analysis of NRf2 (**Panel A**) and YAP (**Panel B**) in PANC-1 and CFPAC-1 cells, untreated (control, C) or treated with cycloheximide (CHX) 100 µg/ml, collected at the indicated times. Equal protein loading was confirmed by exposure of the membranes to the anti-β-actin antibody. Below are shown the densitometric analysis of the protein expressions, normalized using the β-actin signal. Data are indicated as percentage of control values and are the mean ± SD of three independent experiments. ** p-value ≤0.01 vs. C.

The role of deubiquitinases in controlling Nrf2 and YAP expression.

Both Nrf2 and YAP proteins are degraded by the ubiquitin–proteasome system. However, the ubiquitinylation is a dynamic and reversible process in which deubiquitinating enzymes are crucial. Moreover, it has recently been demonstrated that deubiquitinases play an important role in tumor growth and resistance to therapy [27]. To evaluate whether a difference existed in total deubiquitinase (DUBs) activity, in relation to diverse response to drugs, we analyzed this parameter in all three PDAC cell lines. No differences were observed between PANC-1, MiaPaCa-2 and CFPAC-1 cells (data not shown).

Subsequently, we analyzed the protein expression of DUB3 and OTUD1, two DUBs involved in controlling Nrf2 and YAP ubiquitination, respectively. Results demonstrated that DUB3 was overexpressed in PANC-1 cells, and lower in MiaPaCa-2 and CFPAC-1 cells. Analogously,

OTUD1 was higher in PANC-1 cells, intermediate in MiaPaCa-2 and lower in CFPAC-1 cells (Fig. 9).

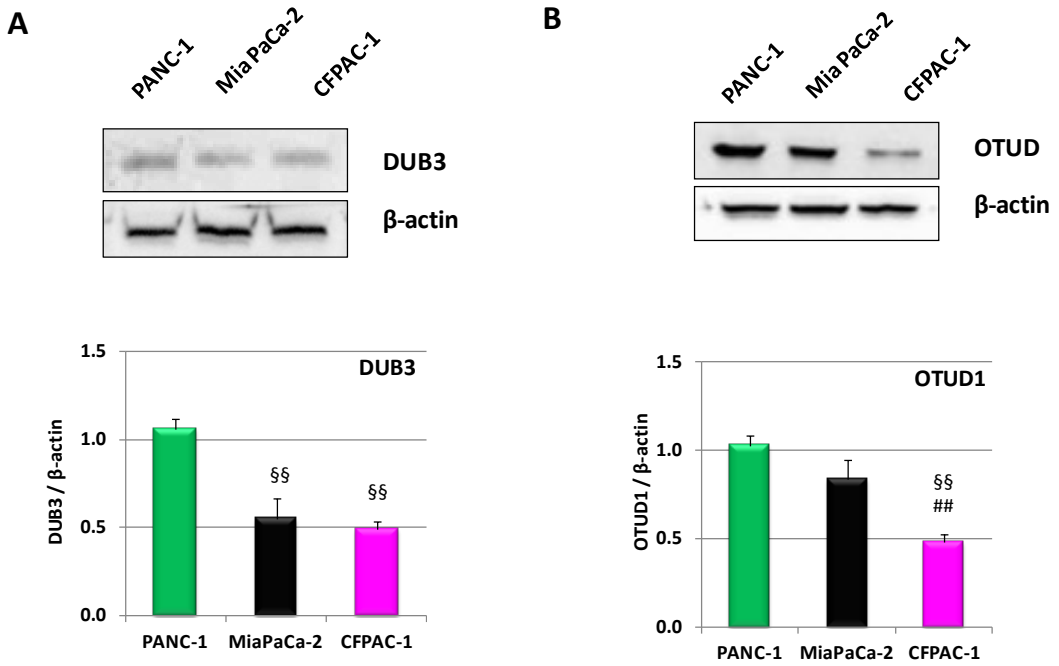

Fig. 9. Western blot analysis of deubiquitinases. DUB3 (**panel A**) and OTUD1 (**panel B**) expressions in PANC-1, MiaPaCa-2, and CFPAC-1 cells. Below are shown the densitometric analysis of the protein expression, normalized using the β-actin signal. Data are the mean ± SD of three independent experiments. §§ $p<0.01$ vs. PANC-1.

This observation led us to investigate, in PANC-1 cells, the more resistant cell line having the higher DUB expression, the effect of DUB silencing on the expression of the respective target, Nrf2 or YAP protein. We found that DUB3 silencing caused a reduction of Nrf2 expression and OTUD1 silencing caused a reduction of YAP expression. Since we previously demonstrated a crosstalk between Nrf2 and YAP [28], we also analyzed in DUB3-silenced cells YAP expression, and in OTUD1-silenced cells Nrf2 expression (Fig.10 A and B). We found that both Nrf2 and YAP expressions were reduced in cell silenced for both deubiquitinases.

Finally, silencing of both deubiquitinases reduced cell growth and makes PANC-1 cells more sensitive to gemcitabine treatment (Fig.10 C and D).

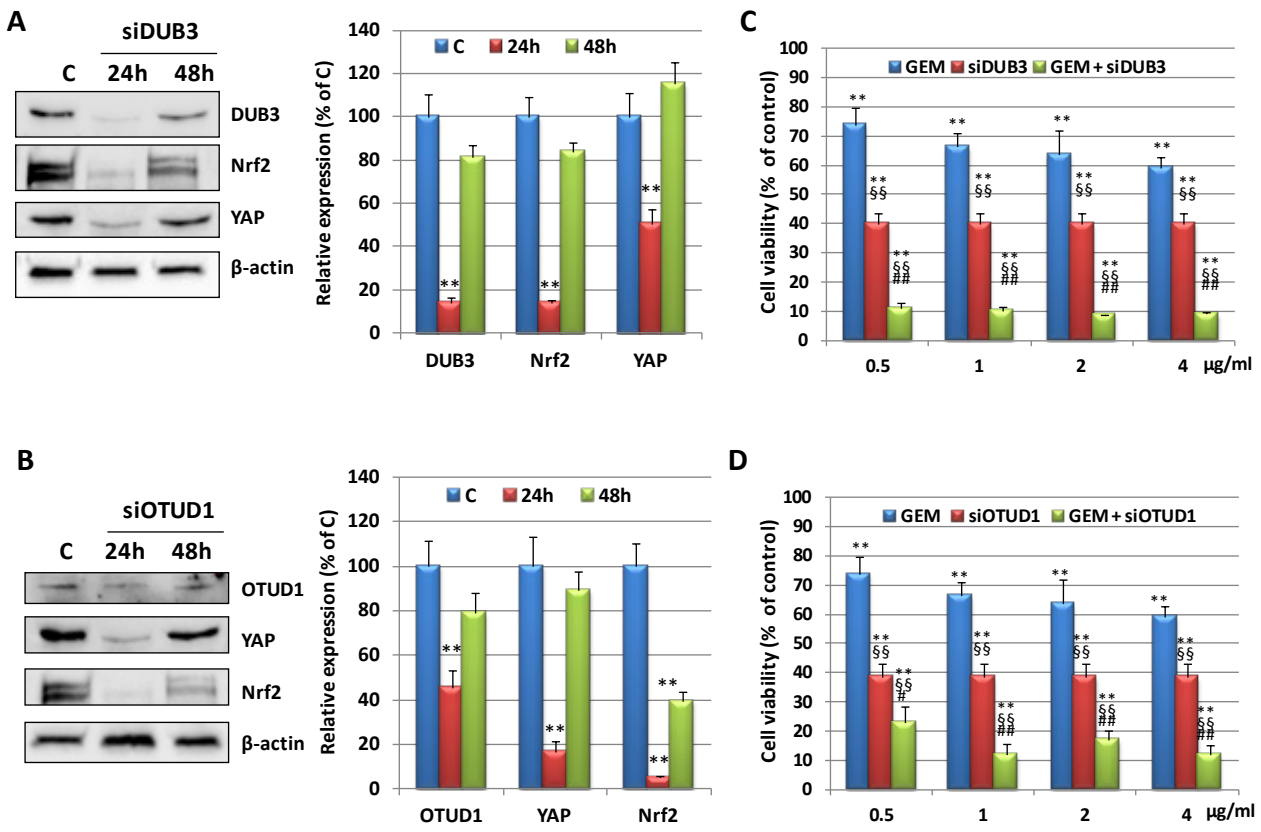

Fig 10. Panel A, Left: Western bot analysis of DUB3, Nrf2, and YAP expressions in PANC-1 cells after 24 and 48 hrs from the treatment with siRNA targeting DUB3 (siDUB3); **right:** densitometric analysis of protein expressions. Data were normalized using the β-actin signal and are indicated in percentage of control values as the mean ± SD of three independent experiments. ** p-value ≤0.01 vs. C. **Panel B, left**: Western bot analysis of OTUD1, YAP, and Nrf2 expressions in PANC-1 cells after 24 hrs and 48 hrs from the treatment with siRNA targeting OTUD1 (siOTUD1); **right**: densitometric analysis of protein expressions. Data were normalized using the β-actin signal and are indicated in percentage of control values as the mean ± SD of three independent experiments. ** p-value ≤0.01 vs. C. **Panel C**: Viability (MTT assay) in PANC-1 cells treated with gemcitabine (GEM) at the indicated concentration, siDUB3, GEM and siDUB3 (GEM+siDUB3) at 72 hrs from the treatment. Results are expressed as percent of control and are the mean ± SD of three separate experiments. **p<0.01 vs. C; §§p<0.01 vs. GEM; ##p<0.01 vs. siDUB3. **Panel D**: Viability (MTT assay) in PANC-1 cells treated with GEM at the indicated concentration, siOTUD1, GEM and siOTUD1 (GEM+siOTUD1) at 72 hrs from the treatment. Results are expressed as percent of control and are the mean ± SD of three separate experiments. **p<0.01 vs. C; §§p<0.01 vs. GEM; ##p<0.01 vs. siOTUD1.

Discussion

In this study, we demonstrated that the intrinsic chemoresistance in our PDAC cells was accompanied by varying levels of oxidative stress, which was higher in chemosensitive cells and lower in chemoresistant cells. These variations were already observed in other types of cancer cells

in which the chemoresistance was induced by drug treatments [2]. In accordance with the low oxidative stress level, PANC-1 chemoresistant cells presented a higher expression of Nrf2 and Nrf2 targets, GSTA4, HO-1 and p62, with respect to the chemosensitive CFPAC-1 cells, whereas the expression of the inhibitor Keap1 was similar in all cell types. Overexpression of p62 could also sustain the Nrf2 protein level. Indeed, p62, an autophagy adaptor, beyond being an Nrf2 target, can bind to Keap1 preventing its binding to Nrf2 and resulting in a constitutive activation of Nrf2 [29]. The role of Nrf2 in pancreatic cancer has been extensively reviewed [7], but the role of deubiquitinases involved in Nrf2 protein stability in pancreatic cancer has not yet been reported.

In recent years, several studies reported the role of YAP in pancreatic cancer demonstrating that it promotes pancreatic cancer cell proliferation, survival and metastasis [31, 32]. In addition, the inhibition of YAP expression by using verteporfin, statins and metformin was suggested as a possible therapeutic strategy [9]. Yap protein was also proposed to promote chemoresistance in PDAC cells by inducing the EMT via activation of the AKT cascade, which can counteract the gemcitabine effect [33]. In agreement with these observations, we found a higher expression of YAP and YAP/TEAD target genes, survivin and FOXM1, in PANC-1 chemoresistant cells, with respect to MiaPaca-2 and CFPAC-1 cells, which were more sensitive to drug treatments, confirming that YAP could be involved in intrinsic chemoresistance of pancreatic cancer cells.

Results obtained by treating PDAC cell lines with MG132 and cycloheximide demonstrated that the Nrf2 and YAP protein expression control was at post-translational level. In particular, the rate of Nrf2 and YAP degradation was reduced in the PANC-1 chemoresistant cell line with respect to CFPAC-1 cells.

The ubiquitin-proteasome system (UPS) plays a pivotal role in the regulation of several processes via control of key protein degradation through ubiquitination or deubiquitination. The addition of ubiquitin is a reversible process: the deubiquitinating enzymes (DUBs) remove ubiquitin from proteins, rescue them from degradation and are involved in cancer progression [34]. The approximately 100 DUB enzymes can regulate protein stability. They can be grouped into five main classes: the cysteine proteases ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Machado-Joseph domain proteases (MJDs) and the metalloproteases JAB1/MPN/MOV34 (JAMM) [35]. Dub3 is a deubiquitinating enzyme highly expressed in tumor-derived cell lines and has an established role in tumor proliferation [27]. It has been demonstrated that DUB3 was involved in the regulation of Nrf2 levels in colorectal cancer [36], and was overexpressed in non-small cell lung cancer (NCSC) where it regulates cell cycle progression by deubiquitinating cyclin A [37]. We found that DUB3 was overexpressed in chemoresistant PANC-1 cells and that its silencing strongly reduced not only Nrf2 expression, but

also YAP expression. Although we cannot exclude that DUB3 can also participate in the regulation of YAP protein expression, this result could be ascribed to the crosstalk between Nrf2 and YAP proteins, previously demonstrated in bladder cancer cells [28]. Importantly, DUB3 silencing significantly reduced cell growth and sensitized PANC-1 cells to gemcitabine treatment, indicating that the DUB3 overexpression was involved in the chemoresistance of PANC-1 cells.

The OTU family of DUBs can act on various proteins, regulating several cell-signaling cascades [38]. The role of OTUD1 in cancer cells is controversial and seems to be related to the cancer cell type. In ovarian cancer cells OTUD1 deubiquitinated the cancer suppressor p53 and it was required for p53 stabilization [39]. In human embryonic kidney 293 cells (HEK239), OTUD1 binds ubiquitinated YAP inhibiting its nuclear localization. [40]. However, no data are available about the role of OTUD1 in PDAC cells. Our results demonstrated that OTUD1 silencing strongly reduced YAP expression, cell growth and gemcitabine chemoresistance in PANC-1 cells. Moreover, analogously to that observed in DUB3 silenced cells, the inhibition of Nrf2 expression was also observed in OTUD1-silenced cells.

Conclusions

Altogether, our results demonstrated that a high level of Nrf2 and YAP proteins is fundamental in maintaining the chemoresistance of PDAC cells and that their expression was related to the expression level of specific deubiquitinases. Although we cannot exclude that, beyond DUB3 and OTUD1, other deubiquitinases could be involved in controlling the expression level of Nrf2 and YAP, our data suggest that DUB3 and OTUD1 can represent a suitable target to repress Nrf2 and YAP expression in PDAC cells. Furthermore, since silencing of DUB3 or OTUD1 has been shown to produce the inhibition of both Nrf2 and YAP expression, the inhibition of one of the two deubiquitinases may be sufficient to obtain an enhancement of response to the chemotherapy drugs.

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Conflict of interest

The authors declare no conflict of interest.

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