Mixed copper (II)-phenanthroline complexes induce cell death of ovarian cancer cells by evoking unfolded protein response

Supplementary methods

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Synthesis of Cu(II) phenanthroline complexes

C0-C3 were synthesized according to previously reported procedures [10, 15]. For **C0**, a one-pot synthesis was chosen so as not to isolate the copper perchlorate, hygroscopic and explosive if dried out. In brief, concentrated perchloric acid was added to a warmed (60°C) ethanolic suspension of $Cu_2(CO_3)(OH)_2$ (involved reaction is $Cu_2(CO_3)(OH)_2 + 4HClO_4 \rightarrow 2Cu^{+2} + 4ClO_4^- + CO_2 + 3H_2O$), the resulting solution was cooled and an ethanolic solution of 1,10- phenanthroline (phen) was added (involved reaction is $2Cu^{+2} + 4ClO_4^- + 2H_2O + 4phen \rightarrow 2[Cu(phen)_2(OH_2)](ClO_4)_2$), the formed precipitate $Cu(phen)_2(OH_2)](ClO_4)_2$ (**C0**) was filtered off, washed with ethanol and dried at room temperature. This compound is stable at room temperature, is soluble in DMSO at 0.1 M concentration level, in CH_3CN at 0.01M, and in pure water at 5 microM. Millimolar concentration of **C0** in water could be reached by dissolving the product in few μl of DMSO and then diluting with water, warming at 40 °C and gently mixing (avoid sonication to prevent the formation of emulsions). Stock solutions of **C0** in DMSO and CH_3CN are stable at 4°C for 4-6 months, stock solutions in DMSO:water (0.5:99.5) prepared as described before are stable at 4°C for max 1 month.

As regards **C1-C3**, an heterogenous phase reaction was used exploiting the different solubility in water of **C0** and the products (approx. 1 μM). **C0** and the proper imidazolidine-2-thione were suspended in distilled water in 1:1 molar ratio and the suspension was stirred for 12 h at room

temperature, after which a green powder was recovered by filtration under vacuum. This reaction ($[Cu(phen)_2(OH_2)](ClO_4)_2 + L \rightarrow [Cu(phen)_2(L)](ClO_4)_2 + H_2O$) occurs faster (2 h) under sonication, however the obtained precipitate is finely dispersed and should be recovered by centrifuge. The final product was washed with water and dried at room temperature. The **C1**-**C3** complexes, as **C0**, are soluble in DMSO (0.1 M) and in CH_3CN (0.01M). Water solutions at 1 mM could be prepared as described before for **C0**. Stock solutions of **C1**-**C3** in DMSO or CH_3CN should be stored in the darkness at 4°C and are stable for 1 month. DMSO:water (0.5:99.5) solutions, stored in the darkness at 4°C, are stable for 2/3 weeks.

The authenticity and purity of reaction products were validated by mass spectrometry, elemental analysis and UV/VIS spectrophotometry.

Fig. S1

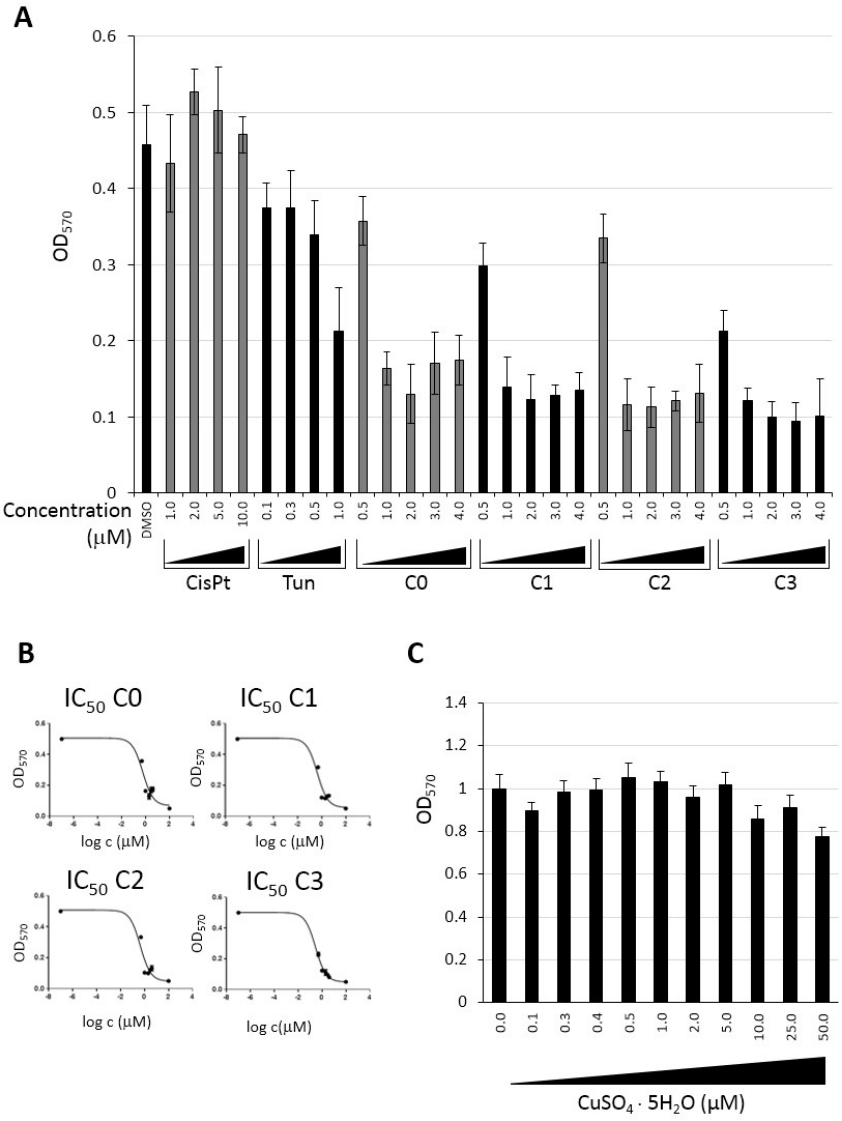

Fig. S1. (**A**) Viability of A2780 cells cultured for 24 hrs. in presence of individual **C0-C3**, cisplatin and tunicamycin at different concentrations, or DMSO as a control. Then, the cell viability was determined by conversion of the 4-nitro blue tetrazolium chloride to formazan followed by measurement of OD at 570nm (MTT assay). The plots represent means ± SD from three independent experiments performed in technical pentaplicates. Asterisk indicates statistical significance at $p < 0.05$. (**B**) IC50 dose of **C0-C3** on A2780 as determined by the MTT assay. (**C**) Viability of A2780 cells cultured in presence of $CuSO_4 \cdot 5H_2O$ for 24 hrs. as determined by the MTT assay.

Fig. S2

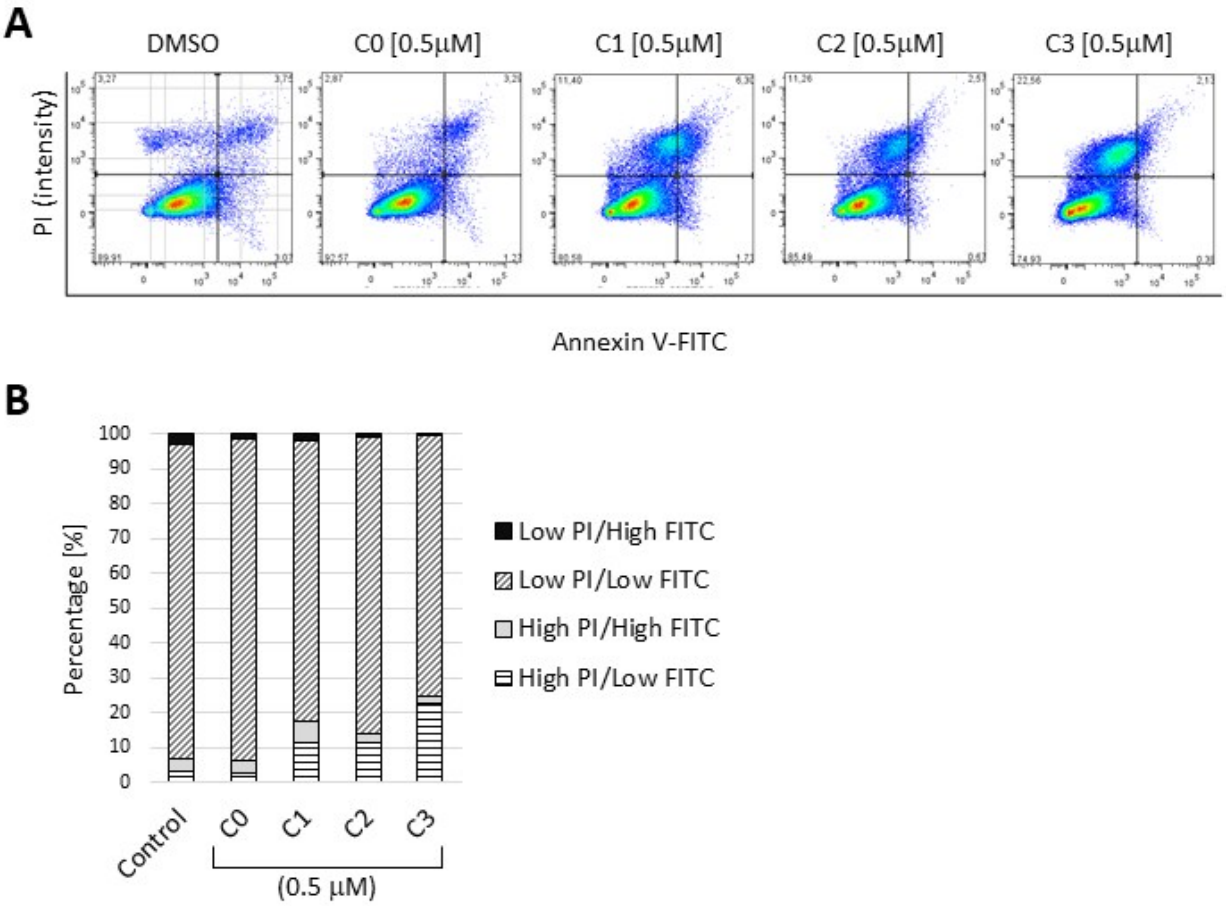

Fig. S2. (**A**) Viability of A2780 cells determined by propidium iodide (PI) and annexin V staining and FACS analysis. Quadrant 1 (Q1) represents PI positive, annexin V negative cells (necrosis); Q2 PI positive, annexin V positive cells (late apoptosis/secondary necrosis); Q3 PI negative, annexin V positive cells (early apoptosis); and Q4 PI negative, annexin V negative cells (viable). (**B**) Quantification of cell death analyzed by propidium iodide (PI) /annexin V staining. Bars represent the percentage of PI/ annexin V negative cells (viable), PI positive (necrosis), annexin V positive (early apoptosis), and PI/ annexin V positive cells (late apoptosis/secondary necrosis).

Fig. S3

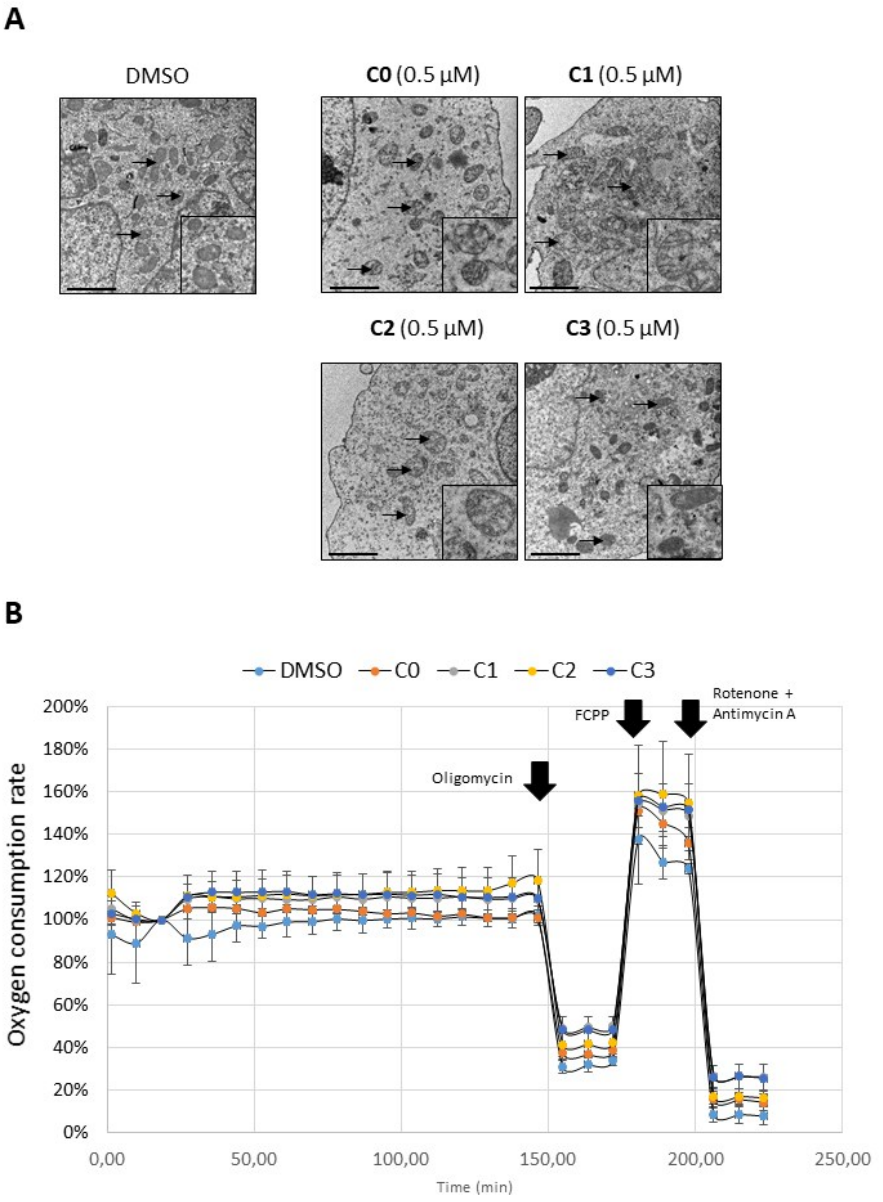

Fig. S3. (**A**) Transmission electron microscopy (TEM) of A2780 cells treated for 24 hrs. with **C0-C3** or DMSO as a control, revealing mitochondria (arrows). Insets show details of mitochondrial structure. Scale bars indicate 2 µm. (**B**) Representative plot (means ± SDs) of four independent measurements in technical tetraplicates documenting mitochondrial respiration of A2780 cells expressed as oxygen consumption rate (%). First the basal respiration of A2780 adhering overnight was measured, then **C0-C3** or DMSO were injected and the respiration was followed for 2 hours Then mitochondrial stress was induced by sequential addition of 1 µM oligomycin, 1 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 1 µM rotenone and antimycin A.

Fig. S4

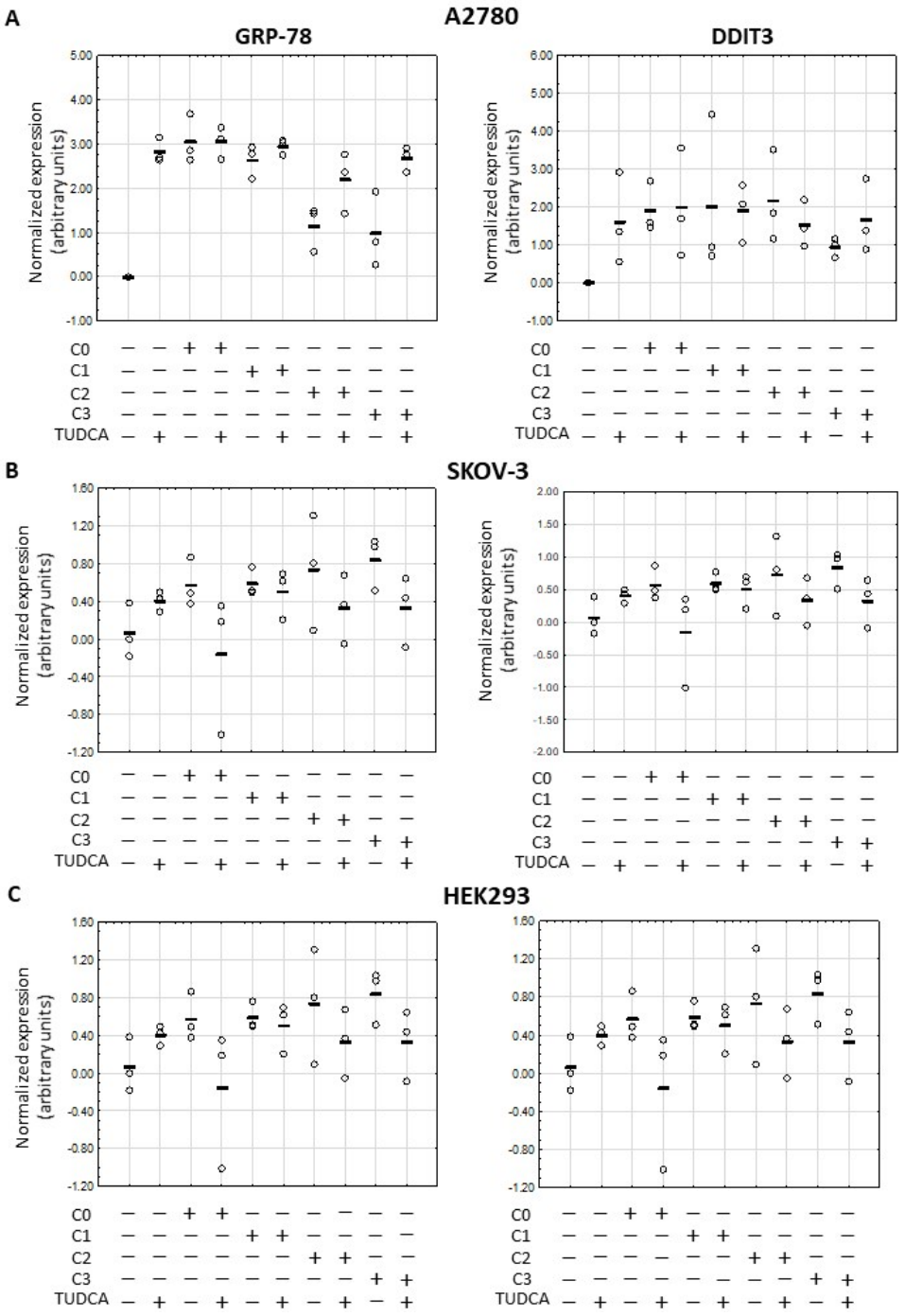

Fig. S4. Expression of GRP-78 and DDIT3 in (**A**) A2780, (**B**) SKOV-3 and (**C**) HEK293 cells. Briefly, cells were treated with **C0-C3** or DMSO and/or TUDCA for 24 hrs. The RNA was extracted, reversely transcribed and used as template for GRP-78 and DDIT3-specific qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene control. GAPDH-normalized expression from three independent experiments (open circles) is expressed in log scale. Line represents a mean value.

Fig. S5

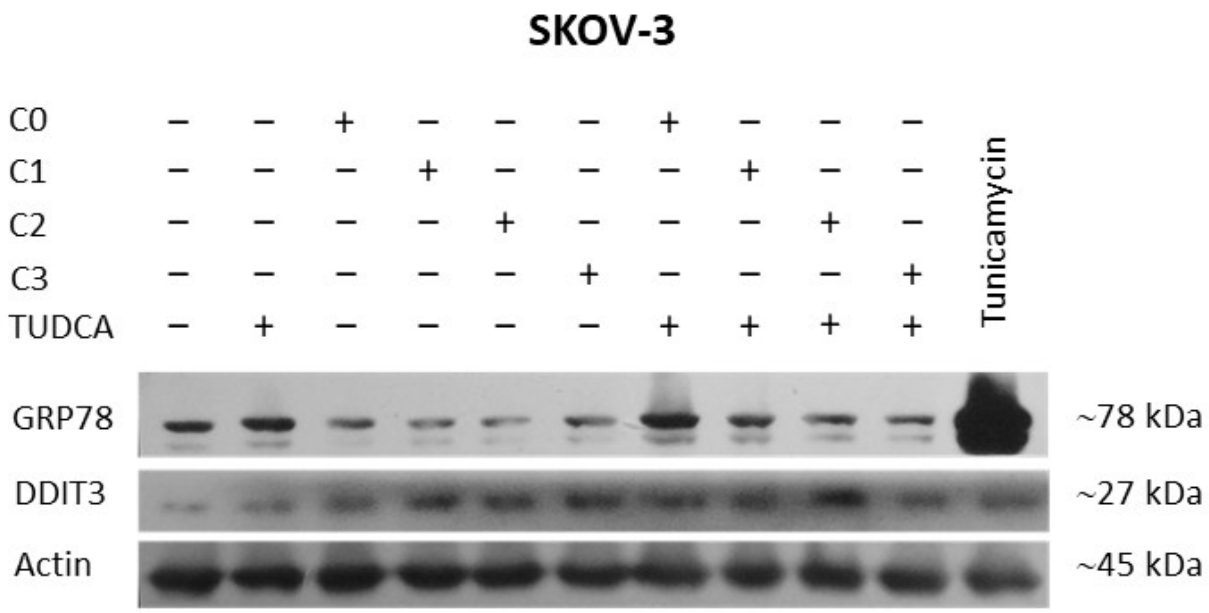

Fig. S5. Protein levels of GRP-78 and DDIT3 in SKOV-3 cells treated for 24 hrs. by **C0-C3** or TUDCA as documented by immunoblotting. Actin was used as a control of equal loading.

Fig. S6.

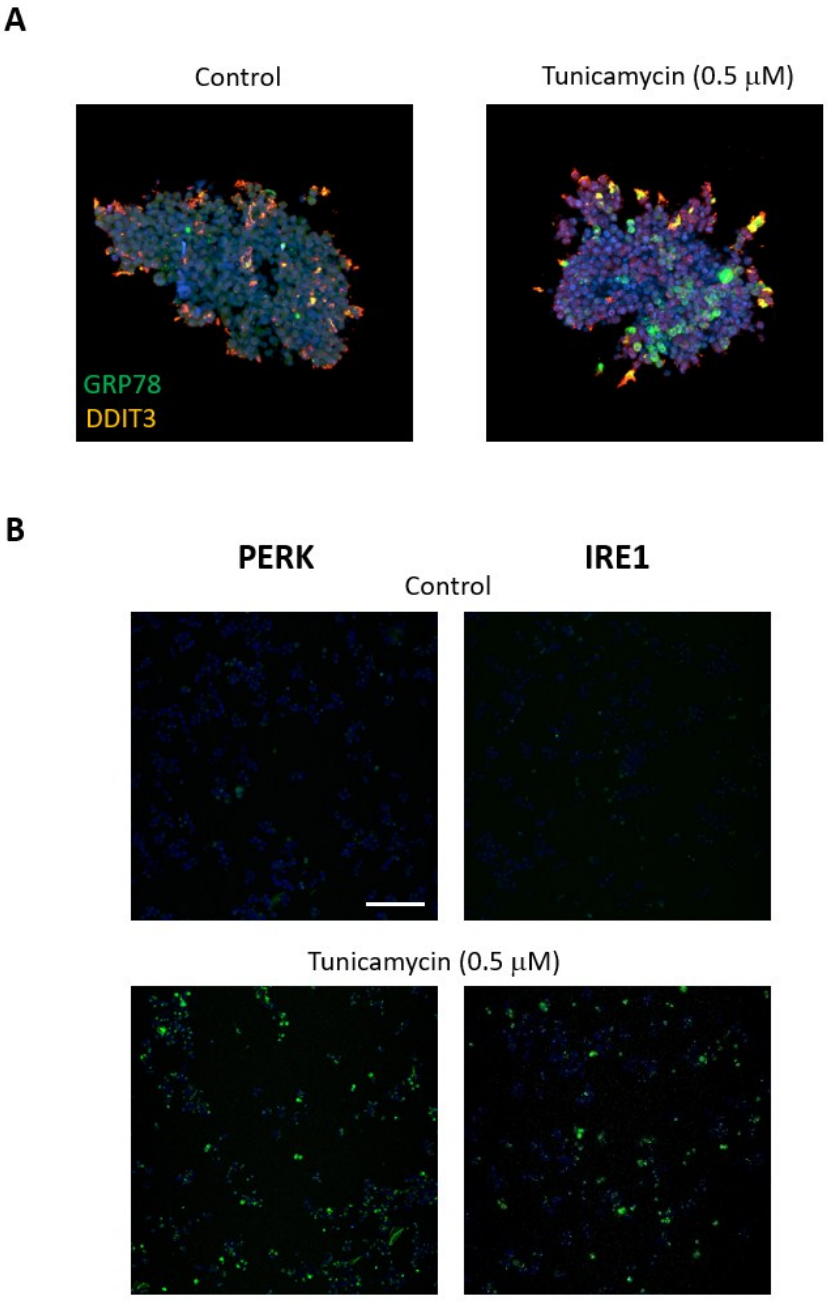

Fig. S6. (**A**) Upregulation of PERK and IRE1 activities in A2780 transfected with PERK and IRE1 fluorescent reporters upon treatment by 0.5μM tunicamycin as documented by fluorescent microscopy. (**B**) 3D spheroids were derived from A2780 cell, treated with 0.5 μM tunicamycin and analyzed by immunofluorescent microscopy for expression of GRP-78 and DDIT3 proteins. GRP-78 and DDIT3 are visualized by the green and red signal, respectively. Nuclei (blue) are visualized by Hoechst staining. The scale bars indicate 200 μm.