



## Endothelin-1 axis fosters YAP-induced chemotherapy escape in ovarian cancer

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### ABSTRACT

The majority of ovarian cancer (OC) patients recur with a platinum-resistant disease. OC cells activate adaptive resistance mechanisms that are only partially described. Here we show that OC cells can adapt to chemotherapy through a positive-feedback loop that favors chemoresistance. In platinum-resistant OC cells we document that the endothelin-1 (ET-1)/endothelin A receptor axis intercepts the YAP pathway. This cross-talk occurs through the LATS/RhoA/actin-dependent pathway and contributes to prevent the chemotherapy-induced apoptosis. Mechanistically, β-arrestin1 (β-arr1) and YAP form a complex shaping TEAD-dependent transcriptional activity on the promoters of YAP target genes, including EDN1, which fuels a feed-forward signaling circuit that sustains a platinum-tolerant state. The FDA approved dual ET-1 receptor antagonist macitentan in co-therapy with cisplatin sensitizes resistant cells to the platinum-based therapy, reducing their metastatic potential. Furthermore, high ET<sub>A</sub>R/YAP gene expression signature is associated with a poor platinum-response in OC patients. Collectively, our findings identify in the networking between ET-1 and YAP pathways an escape strategy from chemotherapy. ET-1 receptor blockade interferes with such adaptive network and enhances platinum-induced apoptosis, representing a promising therapeutic opportunity to restore drug sensitivity in OC patients.

### 1. Introduction

In ovarian cancer (OC) the diagnosis at advanced clinical stages together with disease recurrence due to the failure of first-line platinum-based chemotherapy, which hardly leads to whole tumor eradication, represent the main reason for a poor survival rate [1]. Chemotherapy resistance relies on the dynamic cooperation of converging adaptive signaling pathways which build up precise transcriptional profiles [2]. Dissecting the signaling traits whose activities are tightly coupled is necessary to improve the comprehension of the onset of drug resistance in OC. The identification of new actionable vulnerabilities may be decoded into treatment advances, which may be combined to chemotherapy, for recovering, or at least prolong, the response to treatment in platinum-resistant OC patients. The endothelin-1 (ET-1) signaling, acting through two G protein coupled receptors (GPCR), the ET<sub>A</sub> receptor (ET<sub>A</sub>R) and the ET<sub>B</sub> receptor (ET<sub>B</sub>R), contributes to multiple aspects of tumor progression in many tumor settings, including OC [3].

Clinical analyses conducted on platinum-sensitive and -resistant OC tumors show that the platinum-resistant subgroup expresses higher levels of ET<sub>A</sub>R which are associated with a worst prognosis, validating the unfavorable prognostic role of ET<sub>A</sub>R [4]. The ET-1 signaling activation due to ET-1 binding to ET-1R promotes ET-1R conformational changes ensuring the multi-phase program of GPCR activation signaling. This includes G-protein activation and GPCR kinases (GRKs)-dependent GPCR phosphorylation of serine residues, which triggers β-arrestin1 (β-arr1) or β-arrestin2 (β-arr2) recruitment; thereby preventing G-protein coupling and impeding G-protein signaling. The GPCR become desensitized, internalized and trafficked [5]. Despite the reported β-arr isoforms structure similarity, these do not show entirely redundant activities in regulating GPCR signaling. The β-arr functional divergence may be ascribable to their differential interactions with other proteins or to the different β-arr subcellular localization [6,7]. The multi-task β-arr isoforms, acting as signal transducer of GPCR, facilitate an intricate signaling interchange that rules different cellular effects in malignant

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disease [6–12].  $\beta$ -arr1 and  $\beta$ -arr2 act as major hubs controlling not only many GPCR functions, but also the activity of other class of non GPCR, such as receptor tyrosine kinases (RTK), including the insulin-like growth factor type 1 receptor (IGF-1R) [5], the epidermal growth factor receptor (EGFR) [3], and the insulin receptor [13], as well as integrins [14]. In this perspective, an increasing body of evidence proves how  $\beta$ -arrrs bridge the  $ET_A$ R signaling to other pathways [3,8–12,15–22], fostering several  $ET_A$ R-dependent signaling traits related to OC cell survival, invasion, migration, neovascularization and metastatic progression. Although OC cells express both  $\beta$ -arrrs [15], the two isoforms exhibit differential subcellular distribution:  $\beta$ -arr2, carrying a nuclear export signal in its C-terminus which hinders its nuclear retention, is confined solely to the cytoplasm, conversely  $\beta$ -arr1 may shuttle from the cytoplasm to the nucleus [19]. Indeed, it has been clearly delineated the existence of a  $\beta$ -arr1 nuclear pool of interactors which include transcription factors, co-factors and epigenetic regulators that provide a selective advantage to OC cells [19,23]. Previous preclinical studies highlighted that the  $ET$ -1R/ $\beta$ -arr1 axis may favor the entering of OC cells into a chemotherapy-tolerant state, enabling cells to acquire an epithelial-to-mesenchymal transition (EMT) and to survive to treatment contributing to the onset of the chemoresistance in OC cells [3,4,24]. Among the signaling pathways connected by  $ET$ -1R/ $\beta$ -arr1, we have previously reported the existence of the functional integration between the  $ET$ -1 axis and the RhoA signaling [25]. Interestingly, RhoA expression is significantly associated with advanced stages in OC and RhoA GTPase inhibition enhances the sensitivity of OC cells to cisplatin-induced apoptosis, identifying RhoA GTPase as a central pathway involved in the chemoresistant onset of OC cells [26,27]. Despite the available evidences, the effects of platinum-based therapy on the adaptive signaling mechanisms activated in the treatment-escaping of OC cells remain elusive.

YAP and TAZ, the transcriptional regulators of the Hippo pathway [28–30], that are known to act as signal transducers of the GPCR-initiated signaling routes [31,32], have been recently shown to dampen the efficacy of treatment in several tumor context [29,33–35]. In OC, activated YAP signaling empowers resistance to platinum-based chemotherapy, such as cisplatin, and its expression is associated with a poor prognosis [36–41]. In addition, we recently disclosed a cross-talk between  $ET_A$ R/ $\beta$ -arr1 axis and YAP/TAZ in high-grade serous ovarian cancer (HG-SOC) cells and in breast cancer cell lines harboring TP53 mutations that, fostering the YAP/TAZ-dependent transcriptional program confers to tumor cells an invasive behavior [42]. Interestingly, in uveal melanoma cells YAP acting as a downstream effector of  $ET_B$ R, modulates the response to MEK inhibitors-based therapies [43]. However, the additional perspective of YAP regulation by  $ET$ -1R in cancer cell apoptosis evasion has just begun to be recognized.

In a search of the regulatory signaling network involved in cisplatin-based therapy adaptation, we identify the  $ET_A$ R/ $\beta$ -arr1/RhoA-driven YAP signaling route that allows the instigation of the YAP/TEAD-committed transcription, enabling OC cells to evade the apoptosis and survive. The disruption of such adaptive signaling circuit, through the simultaneous pharmacological co-targeting of  $ET_A$ R axis and YAP signaling, by using the FDA-approved dual  $ET$ -1R antagonist macitentan [3], may be considered as a valid companion for platinum-based therapy to prolong treatment responses in resistant OC patients, overexpressing  $ET_A$ R and YAP.

## 2. Materials and methods

### 2.1. Cell cultures and reagents

The human ovarian cancer cell lines 2008 and its cisplatin resistant subclone, 2008 C13 (CIS) are established from patients with serous cystadenocarcinoma of the ovary [44,45]. The human ovarian carcinoma cell lines A2780 and its cisplatin resistant subclone, A2780 CIS, were obtained from European Collection of Cell Cultures. To retain

platinum resistance, 1  $\mu$ mol/L cisplatin was added to the culture medium every 2 passages [24]. 2008, 2008 CIS, A2780 and A2780 CIS, cultured as previously described [24], were passed in our laboratory for fewer than 3 months after resuscitation and were tested routinely for cell proliferation as well as mycoplasma contamination, and they showed similar growth rate and negative mycoplasma during the experiments. Cell lines were validated by short tandem repeat (STR) profiling. Before each experiment, cells were serum starved by incubation in serum-free medium for 24 h.  $ET$ -1 was used at 100 nM and was purchased from Bachem (Bachem, Bubendorf, Torrance, Switzerland). Macitentan, also called ACT-064992 or N-(5-[4-bromophenyl]-6-{2-[5-bromopyrimidin-2-yloxy]ethoxy}pyrimidin-4-yl)-N'-propylsulfamide, was added 30 min before  $ET$ -1 at a dose of 1  $\mu$ M and was kindly provided by Actelion Pharmaceuticals, Ltd. (Actelion Pharmaceuticals, Allschwil, Switzerland). Latrunculin B (Lat B) and Cytochalasin D (CYTO D) were both purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and respectively used at 2  $\mu$ M for 30 min and 1  $\mu$ M for 4 h. Cisplatin (CIS) was used at 1 mg/ml and was purchased from Teva (TEVA, Petach, Tikva, Israel).

### 2.2. Ectopic expression and silencing

Cells were transfected with wild type or mutated plasmids by using LipofectAMINE 2000 reagent (Life Technologies, Carlsbad, California, USA), following the manufacturer's instructions. Empty vectors pCDNA3 or pQCXIH were used as control (MOCK). Silencing was performed using specific ON-TARGET plus SMART pool siRNAs (Dharmacon, Lafayette, Colorado, USA), or a siRNA negative control (SCR), with Lipofectamine RNAiMAX Reagent (Life Technologies), according to manufacturer's instructions. For more details, please see Supplementary Information.

### 2.3. Immunoblotting and immunoprecipitation

NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific, Waltham, Massachusetts, USA) was used to separate cytoplasmic and nuclear fractions. Whole cell lysates were prepared using a modified RIPA buffer. Cell lysates were resolved by SDS/PAGE. For immunoprecipitation, precleared cell lysates were incubated with indicated antibodies (Abs), with anti-rabbit or anti-mouse IgG Isotype Control (Life Technologies), and protein G-agarose beads (Santa Cruz Biotechnology, Dallas, Texas, USA) at 4 °C overnight. For more details, please see Supplementary Information.

### 2.4. Rho GTPase activation assay

Rho-GTP levels were assessed using a Rho-binding domain affinity precipitation assay (Merck Millipore, Milan, Italy). For further details, please see Supplementary Informations.

### 2.5. Immunofluorescence staining

Cells were fixed in 4% formaldehyde, washed with PBS and permeabilized in 0.3% Triton X-100 in PBS. After the blocking with PBS/0.5% BSA, cells were incubated overnight at 4 °C with primary Abs. For more details, please see Supplementary Informations.

### 2.6. Proximity ligation assay (PLA)

For PLA experiments, after incubation with the primary Abs, 2008 CIS cells were washed in PBS and then incubated with Duolink In Situ PLA secondary probe (Sigma-Aldrich, St. Louis, Missouri, USA). For more details, please see Supplementary Informations.

## 2.7. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the Trizol (Life Technologies), according to the manufacturer's protocol. RNA was reverse transcribed using the SuperScript® VILO™ cDNA synthesis kit (Life Technologies). The mRNA expression was evaluated in the 7500 Fast Real-Time PCR System Mix (Applied Biosystems, Foster City, California, USA), using Power SYBR Green PCR Master Mix (Applied Biosystems). For more details, please see Supplementary Informations.

## 2.8. Chromatin immunoprecipitation (ChIP)

Chromatin was extracted from 2008 CIS cell lines ( $5 \times 10^6$ ) and ChIP assays were performed as previously described [4]. The differential binding between proteins and promoters DNA was examined by PCR. For further details, please see Supplementary Informations.

## 2.9. Luciferase reporter gene assay

Silenced cells were co-transfected with 1  $\mu$ g of luciferase reporter plasmid and 100 ng pCMV- $\beta$ -galactosidase (Promega, Madison, Wisconsin, USA) vector by using LipofectAMINE 2000 reagent (Invitrogen). After 24 h of transfection, serum-starved cells were stimulated as indicated for additional 24 h. Reporter activity was measured using the Luciferase assay system (Promega) and normalized to  $\beta$ -galactosidase activity. For further details, please see Supplementary Informations.

## 2.10. Cell viability analysis

2008 and 2008 CIS cells were seeded in triplicates, in 24-well plates. The cells were transiently transfected with si-YAP or si-TAZ, or si-TEAD or with a non-targeting siRNA and treated with ET-1, macitentan and cisplatin, alone or in combination. After 48 h cell viability was determined by counting cells, for each time point, using a Neubauer-counting chamber and a bright field microscope. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells. The experiments were performed in triplicates for all conditions described.

## 2.11. Chemoinvasion assay

Chemoinvasion assays were carried out using BioCoat growth factor reduced Matrigel Invasion Chamber (BD Biosciences, USA). Silenced cells ( $3 \times 10^4$ ), were stimulated with serum-free medium alone or with ET-1 and/or macitentan, added to the lower chamber, and left to invade for 16 h at 37 °C. For further details, please see Supplementary Informations.

## 2.12. Tubule-like structure formation

2008 and 2008 CIS transfected cells ( $3 \times 10^4$ ) were seeded in a 96-well culture plate precoated with 50  $\mu$ l/well of growth factor reduced Cultrex (Trevigen) and stimulated with ET-1 or MAC for 24 h. For further details, please see Supplementary Informations.

## 2.13. Ovarian cancer xenograft studies

For metastasis assays  $2.5 \times 10^6$  viable 2008, 2008 CIS, A2780 and A2780 CIS cells were intraperitoneally injected into female athymic (nu+/nu+) nude mice, 5- to 6-week of age (Charles River Laboratories, Milan, Italy). All the animal experiments were performed in accordance with the Italian Ministry of Health guidelines after approval by the Animal Welfare Body of Regina Elena Cancer Institute of Rome and comply with all relevant ethical regulations. Two weeks after cell injection, mice were randomized into four groups (n = 6 for 2008 and 2008 CIS, n = 10 for A2780 and A2780 CIS), undergoing the following

treatments: CTR (vehicle) versus macitentan (MAC, 30 mg/kg/oral daily) and/or cisplatin (CIS, 8 mg/kg/i.p. once a week) in mono-therapy or in combination therapy. At the end of the treatment (5 weeks), all mice were euthanized and intraperitoneal tumor nodules throughout the peritoneal cavity (including intestine, mesentery, liver and spleen) were detected and analyzed by immunoblotting analysis.

## 2.14. Statistical analysis

Statistical analyses were performed using Student's t-test and the Mann-Whitney U test. All statistical tests were carried out using GraphPad Prism 8. Receiver operating (ROC) curves and areas under the ROC curve (AUC) were generated by using the ROC plotter tool ([rocplot.org](http://rocplot.org)) [46]. Starting from a cohort of 1022 OC patients from 8 GEO/TCGA datasets with available pathological complete response data, we filtered for OC patients with serous histology subtype and grade 3 who received platinum therapy to generate box plot diagram and ROC curves. Optimal cut-off points were determined by the ROC analyses based on the best balance of sensitivity and specificity.

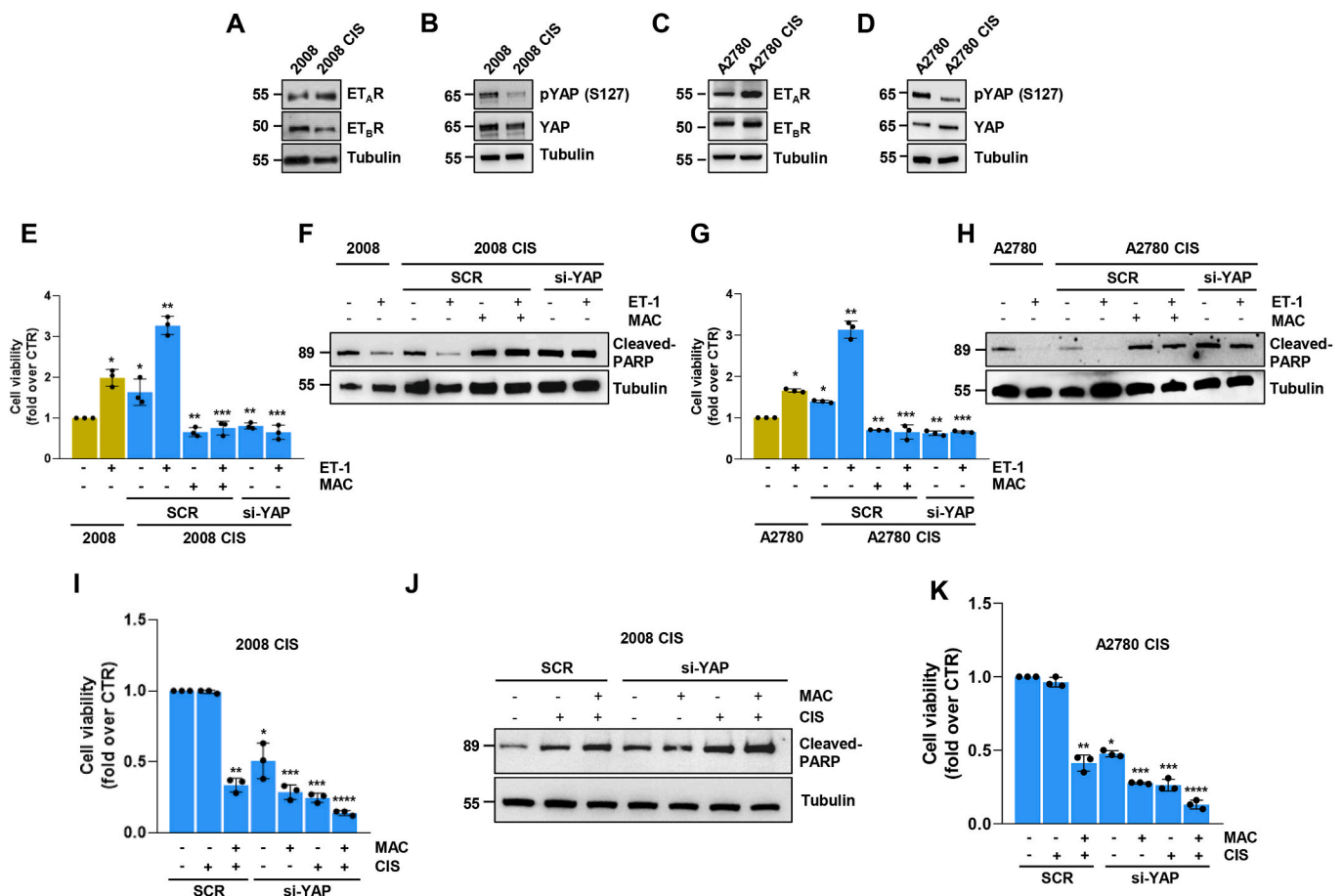
## 3. Results

### 3.1. ET-1/ET<sub>A</sub>R axis mediates the evasion of apoptosis by activating YAP pathway

Given the contribution of YAP to therapy resistance in OC [36, 38–41], and the documented role of ET-1/ET<sub>A</sub>R signaling in inducing OC cell survival [4,24,42], we explored the activity of ET-1/ET<sub>A</sub>R axis in driving YAP activation in OC 2008 and A2780 sensitive and cisplatin (CIS)-resistant cells (2008 CIS and A2780 CIS). Immunoblotting (IB) analysis showed that both 2008 and A2780 sensitive and cisplatin-resistant cells express ET<sub>A</sub>R and ET<sub>B</sub>R (Fig. 1A, C). In particular, 2008 CIS and A2780 CIS cells expressed higher levels of ET<sub>A</sub>R compared to sensitive cells (Fig. 1A, C). Induction of YAP phosphorylation prevents its nuclear localization suppressing YAP transcriptional activity [29]. Of interest, 2008 CIS and A2780 CIS cells exhibited lower levels of the inactive pYAP (S127) compared to sensitive cells (Fig. 1B, D). To uncover the adaptive mechanism of platinum-resistance, we evaluated whether downstream of ET-1/ET<sub>A</sub>R axis YAP might deliver survival signals. We found that YAP depletion strongly reduced the ET-1-induced cell growth mostly in the resistant cells, with an effect comparable to that induced by the treatment of OC cells with the dual ET<sub>A</sub>R/ET<sub>B</sub>R antagonist macitentan (Fig. 1E, G). Cleavage of the Poly (ADP ribose) polymerase (PARP) protein showed that macitentan treatment, as well as YAP depletion, increased cell death (Fig. 1F, H). Intriguingly, cisplatin-resistant cells depleted for YAP expression and concomitantly treated with cisplatin in mono-therapy or in combination with macitentan were less viable than the un-transfected cells. This effect was more pronounced in cells treated with macitentan in combination with cisplatin and even more in cells depleted for YAP and co-treated, compared to those treated with the mono-therapy (Fig. 1I, K). In line with these observations, we detected enhanced apoptosis in cisplatin-resistant cells depleted for YAP protein and co-treated with macitentan and cisplatin (Fig. 1J). Altogether, these results suggest that ET-1/ET<sub>A</sub>R axis favors platinum tolerance of chemoresistant OC cells through YAP that sustains the evasion of apoptosis.

### 3.2. ET-1/ET<sub>A</sub>R axis induces YAP/TAZ cytoplasmic-nuclear translocation in platinum-resistant ovarian cancer cells

Next, we evaluated the effect of ET-1/ET<sub>A</sub>R axis activation on YAP subcellular localization in OC cells. We observed that ET-1 stimulus, in a time-dependent manner, decreased pYAP (S127) and pTAZ (S89) (Fig. 2A and Supplementary Fig. S1A) and caused a greater accumulation of YAP/TAZ in the nucleus of cisplatin-resistant cells, compared to the sensitive cells (Fig. 2B–D and Supplementary Fig. S1B, C).



**Fig. 1.** ET-1/ET<sub>A</sub>R axis induces YAP-mediated apoptosis evasion in platinum-resistant ovarian cancer cells. (A, C) Immunoblotting analysis (IB) of ET<sub>A</sub>R and ET<sub>B</sub>R protein expression in total extracts of 2008, 2008 CIS, A2780 and A2780 CIS cells. Tubulin was used as loading control. (B, D) IB of pYAP (S127) and YAP in total extracts of 2008, 2008 CIS, A2780 and A2780 CIS cells. Tubulin was used as loading control. (E, G) Effect on cell growth of 2008, 2008 CIS, A2780 and A2780 CIS cells stimulated with ET-1 and treated with MAC for 48 h or transfected with SCR, or si-YAP. Bars are means ± SD (\*p < 0.03 vs 2008 SCR CTR or A2780 SCR CTR; \*\*p < 0.008 vs 2008 CIS SCR CTR or A2780 CIS SCR CTR; \*\*\*p < 0.0002 vs 2008 CIS SCR ET-1 or A2780 CIS SCR ET-1; n = 3). (F, H) IB analysis for cleaved-PARP protein expression in 2008, 2008 CIS, A2780 and A2780 CIS cells treated as in E, G. Tubulin was used as loading control. (I, K) Effect of treatment with MAC and/or CIS and MAC + CIS for 48 h on cell growth of 2008 CIS and A2780 CIS cells transfected with SCR, or si-YAP. Bars are means ± SD (\*p < 0.003 vs 2008 CIS SCR CTR or A2780 CIS SCR CTR; \*\*p < 0.0002 vs 2008 CIS or A2780 CIS treated with CIS; \*\*\*p < 0.05 vs 2008 CIS YAP silenced cells or A2780 CIS YAP silenced cells; \*\*\*\*p < 0.02 vs 2008 CIS or A2780 CIS YAP silenced cells treated with MAC + CIS; n = 3). (J) IB analysis for cleaved-PARP protein expression in 2008 CIS cells treated as in I. Tubulin was used as loading control.

Intriguingly, YAP/TAZ nuclear translocation was prevented by macitentan (Fig. 2C and D and Supplementary Fig. S1B, C). The ET-1-dependent YAP nuclear re-localization, in the presence or absence of macitentan, was also evidenced by immunofluorescence (IF) analysis (Fig. 2E). Collectively, these data indicate that ET-1 enhances YAP/TAZ de-phosphorylation and nuclear accumulation in chemoresistant OC cells when compared to their responsive counterparts.

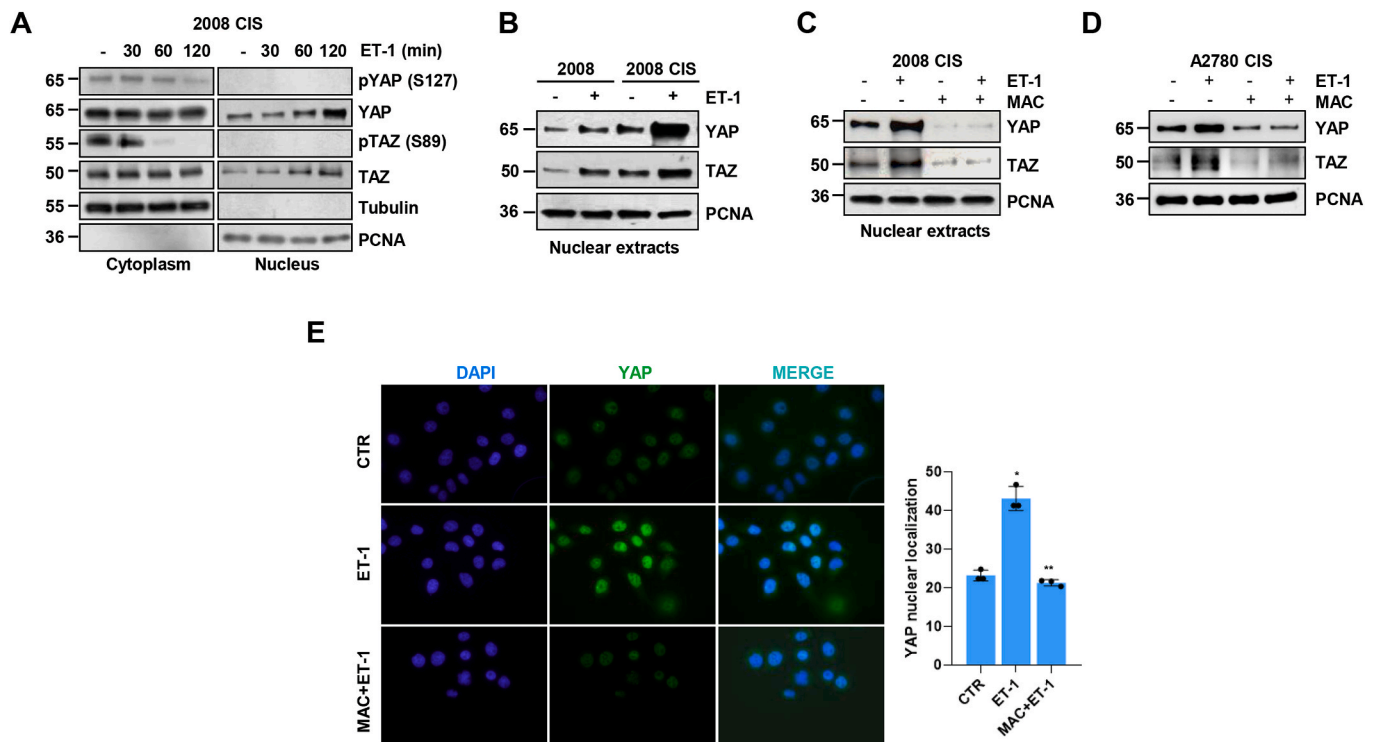
### 3.3. $\beta$ -arrestin1 drives YAP nuclear accumulation in platinum-resistant OC cells

Based on our previous results, we investigated whether ET<sub>A</sub>R/ $\beta$ -arr1 axis might guide YAP nuclear distribution in chemoresistant OC cells. We performed immunoprecipitation (IP) assays in nuclear extracts of sensitive and resistant OC cells observing that upon 90 min of ET-1 stimulus  $\beta$ -arr1 physically binds YAP. This interaction was more evident in resistant cells than in the sensitive counterparts (Fig. 3A). Of note, macitentan treatment led to the  $\beta$ -arr1/YAP nuclear complex disruption (Fig. 3B). In addition, the analysis of nuclear extracts revealed that OC cells ectopically expressing  $\beta$ -arr1-Q394L, in which the nuclear export signal was mutated in OC cells [12], displayed reduced YAP/TAZ nuclear accumulation, compared to un-transfected cells

(Fig. 3C, Supplementary Fig. S2A), highlighting the nuclear function of  $\beta$ -arr1 for YAP/TAZ nuclear translocation. Moreover, we sought to investigate the potential involvement of the G-protein,  $G\alpha_{q/11}$ , in the ET<sub>A</sub>R-dependent YAP nuclear translocation. IB analysis performed on nuclear extracts revealed that  $G\alpha_{q/11}$  depletion did not impact on YAP nuclear accumulation, highlighting that the ET<sub>A</sub>R-induced YAP nuclear enrichment requires by  $\beta$ -arr1 and is independent of  $G\alpha_{q/11}$  (Fig. 3D and Supplementary Fig. S2B).

### 3.4. ET-1/ET<sub>A</sub>R axis activates YAP through LATS/RhoA and actin reorganization

To test the hypothesis that LATS kinases may be part of the ET<sub>A</sub>R/ $\beta$ -arr1-dependent signaling cascade that culminates into higher YAP activation in platinum-resistant OC cells, we first analyzed the effect on YAP nuclear translocation upon LATS depletion in resistant OC cells. We found that LATS depletion prevented the ET-1 and/or macitentan impact on YAP nuclear localization (Fig. 4A and B and Supplementary Fig. S3A). Next, we ectopically expressed a constitutively active YAP mutant (YAP5SA) form resistant to LATS-induced phosphorylation [47] in both sensitive and cisplatin-resistant OC cells. Interestingly, YAP5SA-transfected cells were insensitive to ET-1 and/or macitentan



**Fig. 2.** ET-1 promotes YAP/TAZ cytoplasmic-nuclear translocation in platinum resistant ovarian cancer cells. (A) IB analysis of pYAP (S127), YAP, pTAZ (S89) and TAZ protein expression in the nuclear and cytoplasmic extracts of 2008 CIS cells stimulated with ET-1 (100 nM) for the indicated times. Tubulin and PCNA were used as cytoplasmic and nuclear loading control, respectively. (B) IB analysis of YAP and TAZ protein expression in the nuclear extracts of 2008 and 2008 CIS cells treated or not with ET-1 for 90 min. PCNA was used as loading control. (C, D) IB analysis of YAP and TAZ protein expression in the nuclear extracts of 2008 CIS and A2780 CIS cells upon stimulation with ET-1 and/or macitentan (MAC, 1  $\mu$ M) for 90 min. PCNA was used as loading control. (E) YAP localization evaluated by immunofluorescence (IF) in 2008 CIS cells stimulated with ET-1 and/or MAC for 90 min. Nuclei are stained in blue (DAPI). Scale bar, 10  $\mu$ m (Magnification X64). Right graph represents the quantification of YAP nuclear localization. Bars are means  $\pm$  SD (\* $p$  < 0.0006 vs CTR, \*\* $p$  < 0.0004 vs ET-1;  $n$  = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatment (Fig. 4C and Supplementary Fig. S3B, C). In addition we observed that ET-1 stimulation of OC cells induced not only a reduction in YAP phosphorylation but also the decrease of the phosphorylated active form of LATS1 (pLATS1-T1079) (Supplementary Fig. S3D). Collectively these data prove that LATS takes part to the ET<sub>A</sub>R/ $\beta$ -arr1 signaling pathway and that is required for YAP nuclear accumulation in platinum-resistant cells.

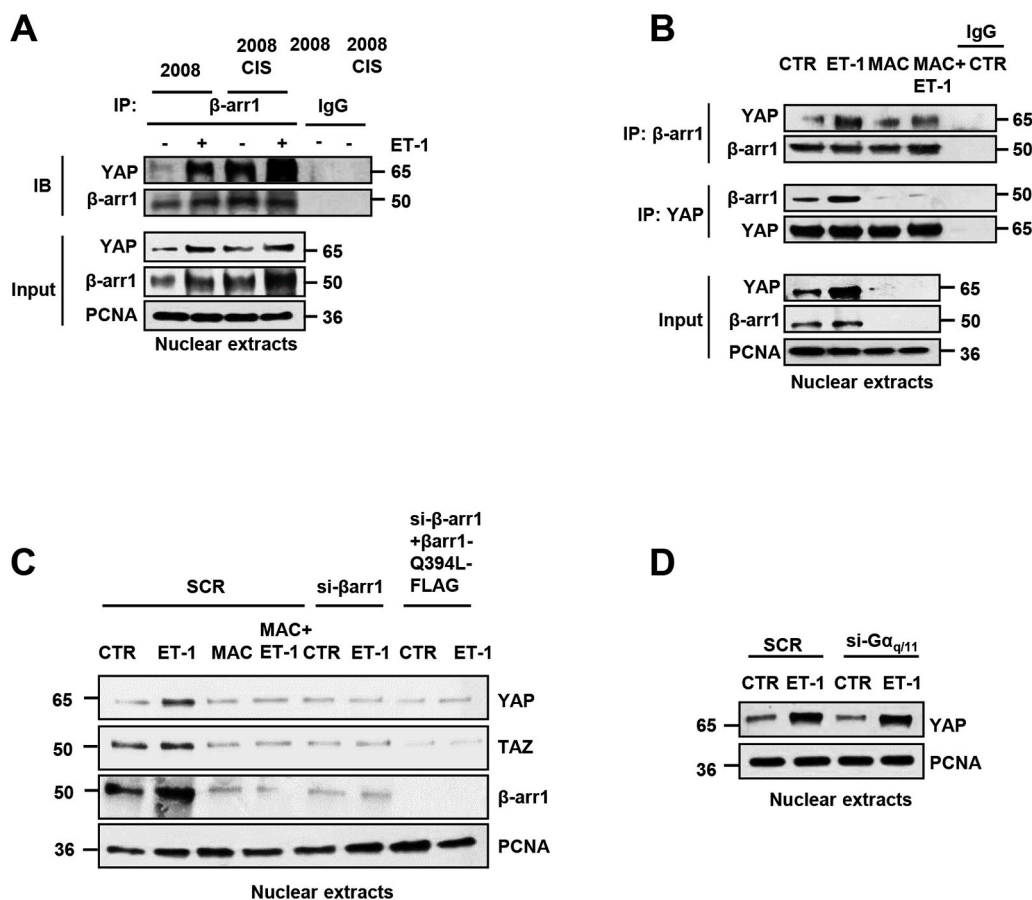
Considering that the RhoA GTPase-induced signaling acts as a determinant of short survival and resistance to drug-induced apoptosis in OC [25,26], and taking into account that the Rho pathway exerts a critical role in YAP/TAZ oncogenic functions [28–30], we examined whether  $\beta$ -arr1 could promote YAP activity through the induction of RhoA GTPase pathway by performing the RhoA pull down assay in 2008 CIS cells. ET-1 stimulation induced RhoA GTPase activation, which was hampered upon macitentan treatment or  $\beta$ -arr1 silencing (Fig. 4D). Interestingly, RhoA GTPase silencing, as well as macitentan treatment, interfered with the ET-1-induced YAP/TAZ nuclear accumulation (Fig. 4E and Supplementary Fig. S3E), indicating that ET<sub>A</sub>R/ $\beta$ -arr1 axis induces YAP/TAZ nuclear enrichment in platinum-resistant OC cells through the RhoA GTPase activity.

Given that the actin-dependent YAP activation appears to have a critical impact on the establishment of a resistant phenotype [28,29,31, 33], we examined whether actin cytoskeleton rearrangements contribute to YAP activation in response to ET-1 stimulation in chemoresistant OC cells. We observed that the pharmacological treatment of cisplatin-resistant OC cells with F-actin disrupting agents, such as Latrunculin B (Lat B) and Cytochalasin D (CYTO D), as well as macitentan treatment, increased the ET-1 repressed YAP phosphorylation, contributing to its inhibition (Fig. 4F). These results suggest that ET<sub>A</sub>R-enhanced actin reorganization affects YAP activation,

contributing to the mechanism of adaptive resistance to cisplatin in OC cells.

### 3.5. Nuclear $\beta$ -arrestin1 mediates the ET<sub>A</sub>R-dependent YAP/TEAD transcriptional program

Binding of YAP to TEAD in the nucleus is essential for YAP/TEAD transcriptional activity [48]. We evaluated the effect of ET-1 on the formation of nuclear YAP/TEAD complexes in cisplatin-resistant OC cells. The interaction between endogenous YAP and TEAD was analyzed by proximity ligation assay (PLA), in which a signal is generated only when the proteins analyzed are in close proximity. PLA analysis revealed an increased interaction between YAP and TEAD in the nuclei of ET-1-stimulated cells. Quantification of PLA spots demonstrated that ET-1 enhanced the number of nuclear YAP/TEAD complexes (Fig. 5A). Having proved that  $\beta$ -arr1 binds YAP by co-IP analysis, we examined such protein-protein interaction by PLA in platinum-resistant OC cells. This analysis showed the direct interaction between  $\beta$ -arr1 and YAP (Fig. 5B) upon ET-1 stimulation, revealing that ET-1 enhances the formation of both  $\beta$ -arr1/YAP and YAP/TEAD complexes in the nucleus of platinum-resistant cells. In line with these observations, chromatin immunoprecipitation (ChIP) analysis revealed that ET-1 induced the concomitant recruitment of  $\beta$ -arr1 and YAP on YAP/TEAD-responsive target gene promoters, such as *CTGF*, *ANKRD1*, and *EDN1* in cisplatin-resistant cells (Fig. 5C). ET-1 induced also the concomitant recruitment of  $\beta$ -arr1 and YAP on the promoter of ET-1 gene (*EDN1*), indicating that ET-1 gene transcription is regulated by  $\beta$ -arr1 and YAP to magnify chemoresistant features in OC cells. Of note, macitentan treatment strongly impaired the recruitment of  $\beta$ -arr1, YAP and TEAD on their target promoters (Fig. 5C). According to these results, the analysis



**Fig. 3.**  $\beta$ -arrestin1 drives YAP nuclear accumulation. (A) Nuclear extracts of 2008 and 2008 CIS cells treated or not with ET-1 for 90 min were immunoprecipitated (IP) for endogenous  $\beta$ -arr1 using *anti*- $\beta$ -arr1, or *anti*-IgG and IB for  $\beta$ -arr1 and YAP. PCNA was used as loading control. (B) Nuclear extracts of 2008 cells treated with ET-1 and/or MAC for 90 min were IP for endogenous  $\beta$ -arr1 using *anti*- $\beta$ -arr1, for endogenous YAP using *anti*-YAP or *anti*-IgG and IB for  $\beta$ -arr1 and YAP. PCNA was used as loading control. (C) IB analysis for YAP, TAZ and  $\beta$ -arr1 upon stimulation with ET-1 and/or MAC for 90 min in nuclear extracts of 2008 cells transfected with SCR, or si- $\beta$ -arr1, or si- $\beta$ -arr1 and mutant  $\beta$ -arr1Q394L-FLAG, unable of nuclear localization. PCNA was used as loading control. (D) IB analysis for YAP in the nuclear extracts of 2008 cells silenced for  $G\alpha_{q/11}$  for 72 h and treated with ET-1 for 90 min. PCNA was used as loading control.

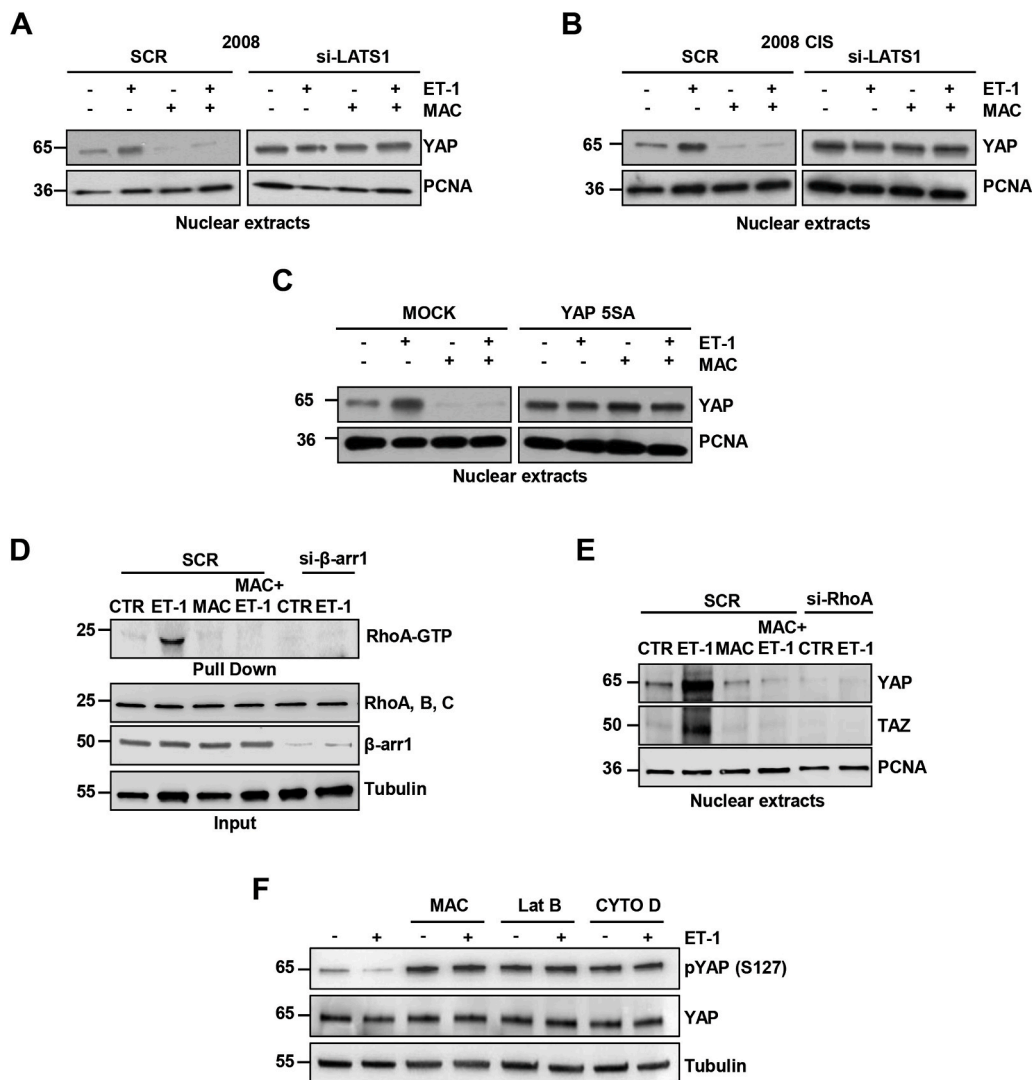
of TEAD transcriptional activity revealed that ET-1 stimulation induced an increase of TEAD transcriptional functions in the resistant cells, compared to the sensitive counterpart (Fig. 5D and Supplementary Fig. S4A-D and S2A). This effect was hampered by macitentan, as well as by  $\beta$ -arr1, YAP and TAZ silencing, or by the ectopic expression of  $\beta$ -arr1-Q394L in  $\beta$ -arr1-depleted cells (Fig. 5D and Supplementary Fig. S4A-D and S2A). Of relevance, TEAD transcriptional activity was restored by the re-expression of  $\beta$ -arr1, but not of  $\beta$ -arr1 mutant (Fig. 5D and Supplementary Fig. S4A-D and S2A). The transcript expression of YAP target genes, *CYR61*, *CTGF*, *ANKRD1* and *EDN1*, increased upon ET-1 stimulation in resistant OC cells. This was reversed by either macitentan treatment or depletion of all the components of this active transcriptional complex and rescued upon the re-expression of  $\beta$ -arr1, but not of the  $\beta$ -arr1-Q394L mutant (Fig. 5E and Supplementary Fig. S4A-C, E and S2A), suggesting that  $\beta$ -arr1 may act as a nuclear tethering platform for YAP to activate the expression of downstream target genes. In line with the above results, ET-1 promoter activity was enhanced in resistant OC cells compared to sensitive cells and was reverted upon treatment with macitentan, as well as upon the depletion of  $\beta$ -arr1, YAP and TAZ or upon the  $\beta$ -arr1-Q394L mutant ectopic expression and rescued by  $\beta$ -arr1 re-expression (Fig. 5F and Supplementary Fig. S4A-C, F and S2A). Altogether, these observations document that  $ET_{AR}/\beta$ -arr1/YAP/TEAD-induced transcriptional machinery is potentiated in the cisplatin-resistant cells. Of note, the subset of YAP/TEAD-induced genes includes *EDN1* whose transcription sustains a self-amplifying circuit able to empower chemoresistant features in platinum-resistant OC cells.

### 3.6. Macitentan-induced suppression of the $ET_{AR}/YAP$ adaptive network, impairs cell plasticity and invasion

Chemoresistant cells acquire an EMT phenotype and display cell plasticity endowing aggressive tumor cells to adapt to therapy [49,50]. Among aggressive traits, platinum-resistant OC cells acquire the capacity to form vascular-like structures in a process known as vasculogenic mimicry [51]. Based on these findings, we aimed to investigate whether the  $ET_{AR}/\beta$ -arr1/YAP adaptive axis might be involved in vascular tubules formation *in vitro*. We found that OC cells were able to form a network of tubule-like structures, and such ability was enhanced by ET-1 treatment (Fig. 6A). Remarkably, quantification analysis showed that the tube length and the number of their nodes were significantly increased in chemoresistant OC cells compared to sensitive cells (Fig. 6B and C). Inversely, cells either treated with macitentan or depleted for YAP, TAZ and TEAD did not form elongated tube structures and exhibited a reduced number of nodes (Fig. 6A–C); thereby suggesting that the  $ET_{AR}/\beta$ -arr1/YAP pathway is required to promote vasculogenic mimicry in chemoresistant OC cells. Moreover, macitentan treatment, YAP, TAZ and TEAD depletion hampered the ET-1-induced OC cell invasive potential (Fig. 6D). These data highlight that the  $ET_{AR}/YAP$  signaling network beyond the ability to sustain OC cell survival, can confer cell plasticity and invasive traits to OC platinum-resistant cells that can be interfered by macitentan.

### 3.7. Macitentan suppresses both $ET_{AR}$ and YAP adaptive signaling pathways and enhances cisplatin efficacy in platinum-resistant OC xenografts

Next, we evaluated whether the therapeutic efficacy of macitentan to control the *in vivo* metastatic dissemination of cisplatin-resistant cells



**Fig. 4.** ET-1/ET<sub>A</sub>R axis induces YAP nuclear accumulation through LATS/RhoA pathway and actin reorganization. (A, B) 2008 (A) and 2008 CIS (B) cells transfected with SCR or si-LATS1 for 72 h and treated with ET-1 and/or MAC for 90 min were IB for YAP. PCNA was used as loading control. (C) 2008 CIS cells transfected with an empty vector (MOCK) or with a vector encoding for YAP constitutively active (YAP 5SA-Myc) for 24 h and stimulated with ET-1 and/or MAC for 90 min were IB for YAP. PCNA was used as loading control. (D) Rhotekin was used to pull down RhoA-GTP from total lysates of 2008 CIS cells transfected with SCR or si-β-arr1 for 72 h and stimulated with ET-1 and/or MAC for 5 min. The GTP pull-down and input were then analyzed by IB. (E) IB analysis for YAP and TAZ in the nuclear extracts of 2008 CIS cells transfected with SCR or si-RhoA for 72 h, or treated with MAC, and stimulated with ET-1 for 90 min. PCNA was used as loading control. (F) IB analysis for pYAP (S127) and YAP in the total extracts of 2008 CIS cells treated or not with ET-1 and additionally treated with MAC or with disruptors of actin cytoskeleton filaments, Latrunculin B (Lat B) or Cytochalasin D (CYTO D). Tubulin was used as loading control.

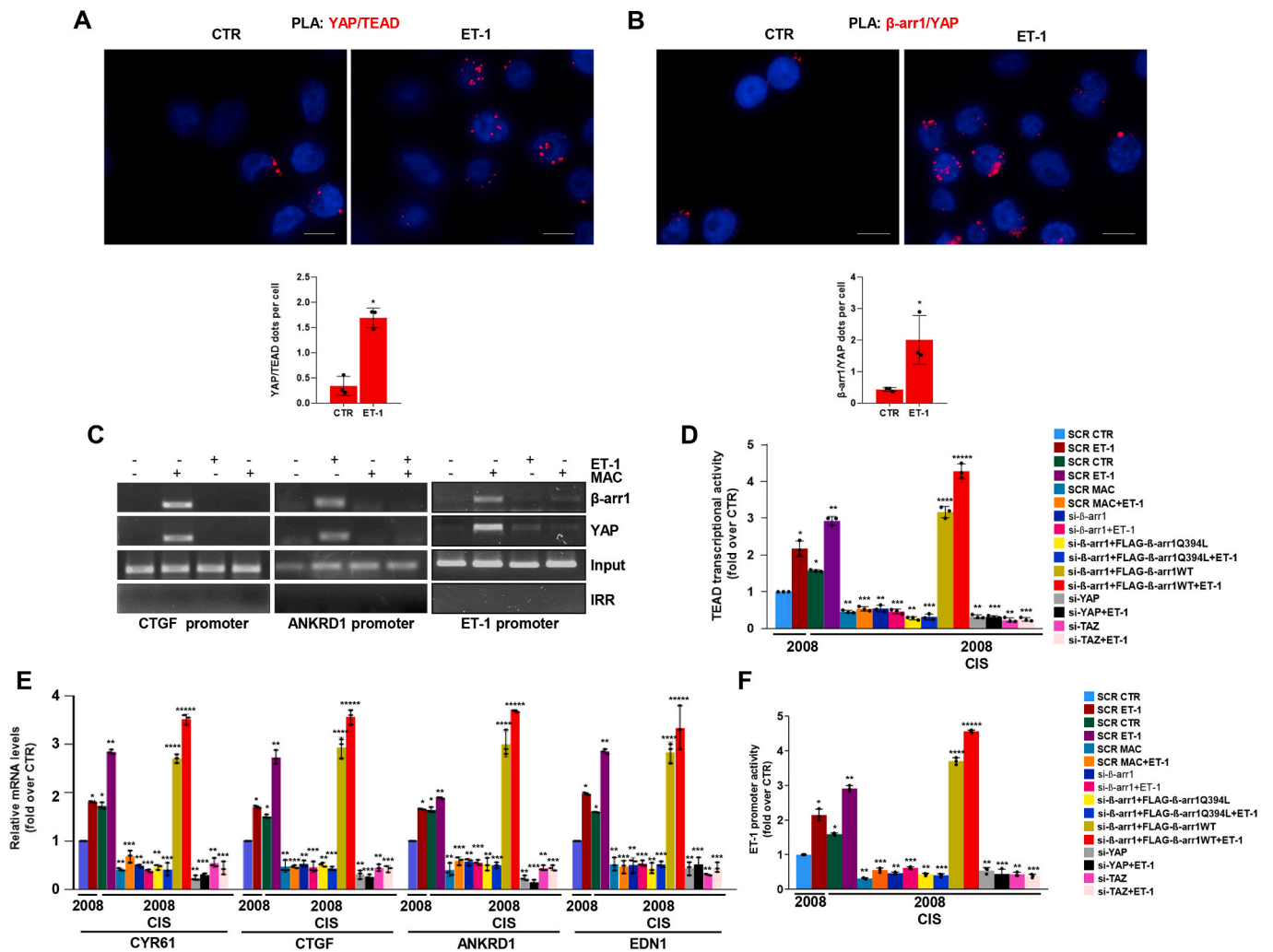
intraperitoneally implanted in nude mice, occurs through the simultaneous suppression of the ET<sub>A</sub>R and YAP adaptive signaling pathways. Mice underwent the following treatments: CTR (treated with vehicle), macitentan (MAC, 30 mg/kg/oral daily) and/or cisplatin (CIS, 8 mg/kg/i.p. once a week) in mono-therapy or in combination therapy. At the end of the treatment (Fig. 7A), 2008 CIS and A2780 CIS xenografted mice metastasized more than mice xenografted with sensitive OC cells (Fig. 7B, D). Macitentan, as well as cisplatin treatment inhibited the *in vivo* metastatic potential of 2008 CIS and A2780 CIS cells. The effect was enhanced in those mice treated with the co-therapy of macitentan with cisplatin (Fig. 7B, D). In accordance with these results, in the cisplatin-resistant xenografts nodules, the combined treatment of macitentan with cisplatin showed that the dual ET-1R antagonist restored the sensitivity to cisplatin administration by inhibiting YAP activity, when compared to the mono-therapies (Fig. 7C, E and Supplementary Figs. S5A and B). Collectively, these findings show that the macitentan-induced ET-1R blockade *in vivo*, concomitantly abrogates both the adaptive ET<sub>A</sub>R and YAP signaling pathways and restores the cisplatin treatment vulnerability. Remarkably, the combined administration of macitentan with cisplatin restores platinum sensitivity in chemoresistant xenografts, hampering OC metastatic potential.

To evaluate the predictive role of ET<sub>A</sub>R/YAP signature in OC patients receiving platinum therapy, we employed the ROC Plotter tool, which is able to identify potential predictive biomarkers by integrating gene expression with cancer response to therapy [46]. The analysis of the

integrated ET<sub>A</sub>R (*EDNRA*) and YAP (*YAP1*) gene expression in serous OC patients subdivided for their response to platinum therapy, showed that this signature is highly expressed in platinum non-responder (N = 46) compared to platinum responder (N = 90) OC patients (Fig. 7F, p = 0.0049). Importantly, ROC plot curves in Fig. 7G indicated that *EDNRA/YAP1* gene expression discriminated between platinum non-responders and platinum responders OC patients (p = 1.9e-03, AUC = 0.65, 95% CI 0.579–0.711), suggesting the potential predictive utility of this signature.

#### 4. Discussion

The inception of integrated signaling networks consisting of simultaneously activated anti-apoptotic pathways that impair the response to chemotherapy may offer a valid tool to bypass the plague of chemotherapy resistance which is the major challenge for successful clinical care of OC patients [1,2]. In this study, using established chemosensitive and chemoresistant OC cell models, we uncover a mechanism through which OC cells gain platinum resistance via the adaptive ET<sub>A</sub>R/RhoA/YAP signaling. The multifaceted protein β-arr1 builds a reciprocal crosstalk involving ET-1 and YAP. Here, we describe how β-arr1 physically and functionally links YAP guiding the YAP/TEAD transcriptional program that sustains cell survival and lowers cisplatin sensitivity in cisplatin-resistant OC cells. Importantly, we found a feed-forward loop that fuels ET-1/ET<sub>A</sub>R/YAP circuit through YAP and ET-1



**Fig. 5.** Nuclear  $\beta$ -arrestin1 mediates  $ET_A$ R-induced YAP/TEAD transcriptional program. (A, B) Representative images of proximity ligation assay (PLA) detection of protein complexes containing YAP and TEAD (A) or  $\beta$ -arr1 and YAP (B) (red signals) in 2008 CIS cells stimulated with ET-1 for 90 min. DAPI staining (blue) highlights the nucleus. Scale bar, 10  $\mu$ m. Bottom graphs represent the quantification of the protein complexes. Bars are means  $\pm$  SD (\* $p$  < 0.03 vs 2008 CIS CTR;  $n$  = 3). (C) 2008 CIS cells were treated with ET-1 and/or MAC for 90 min. The binding of  $\beta$ -arr1 and YAP on *CTGF*, *ANKRD1* and *EDN1* promoters was measured by ChIP analysis. Anti-IgG mouse Ab (IRR) was used as control for all ChIP reactions. (D) TEAD transcriptional activity performed in 2008 and 2008 CIS cells stimulated with ET-1 and treated with MAC for 24 h, and co-transfected with SCR, si- $\beta$ -arr1, si- $\beta$ -arr1 and mutant  $\beta$ -arr1Q394L-FLAG, rescued with  $\beta$ -arr1-FLAG, or in cells transfected with si-YAP, or si-TAZ and TEAD-luc construct for 24 h. Bars are means  $\pm$  SD (\* $p$  < 0.0007 vs 2008 SCR CTR; \*\* $p$  < 0.0002 vs 2008 CIS SCR CTR; \*\*\* $p$  < 0.0002 vs 2008 CIS SCR ET-1; \*\*\*\* $p$  < 0.0002 vs 2008 CIS si- $\beta$ -arr1; \*\*\*\* $p$  < 0.0002 vs 2008 CIS si- $\beta$ -arr1+ET-1;  $n$  = 3). (E) Expression analysis (qRT-PCR) of the indicated YAP/TEAD mRNA target genes in 2008 and 2008 CIS cells stimulated with ET-1 and/or MAC for 24 h and transfected with SCR, si- $\beta$ -arr1, si- $\beta$ -arr1 and mutant  $\beta$ -arr1Q394L-FLAG, rescued with  $\beta$ -arr1-FLAG, or in cells transfected with si-YAP, or si-TAZ for 72 h. Bars are means  $\pm$  SD (\* $p$  < 0.0002 vs 2008 SCR CTR; \*\* $p$  < 0.002 vs 2008 CIS SCR CTR; \*\*\* $p$  < 0.0002 vs 2008 CIS SCR ET-1; \*\*\*\* $p$  < 0.0002 vs 2008 CIS si- $\beta$ -arr1; \*\*\*\* $p$  < 0.0005 vs 2008 CIS si- $\beta$ -arr1+ET-1;  $n$  = 3). (F) ET-1 promoter activity in 2008 and 2008 CIS cells stimulated with ET-1 and treated with MAC for 24 h, and co-transfected with SCR, si- $\beta$ -arr1, si- $\beta$ -arr1 and mutant  $\beta$ -arr1Q394L-FLAG, rescued with  $\beta$ -arr1-FLAG, or in cells transfected with si-YAP, or si-TAZ and ET-1 promoter-luc construct for 24 h. Bars are means  $\pm$  SD (\* $p$  < 0.0004 vs 2008 SCR CTR; \*\* $p$  < 0.0002 vs 2008 CIS SCR CTR; \*\*\* $p$  < 0.0002 vs 2008 CIS SCR ET-1; \*\*\*\* $p$  < 0.0002 vs 2008 CIS si- $\beta$ -arr1; \*\*\*\* $p$  < 0.0002 vs 2008 CIS si- $\beta$ -arr1+ET-1;  $n$  = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

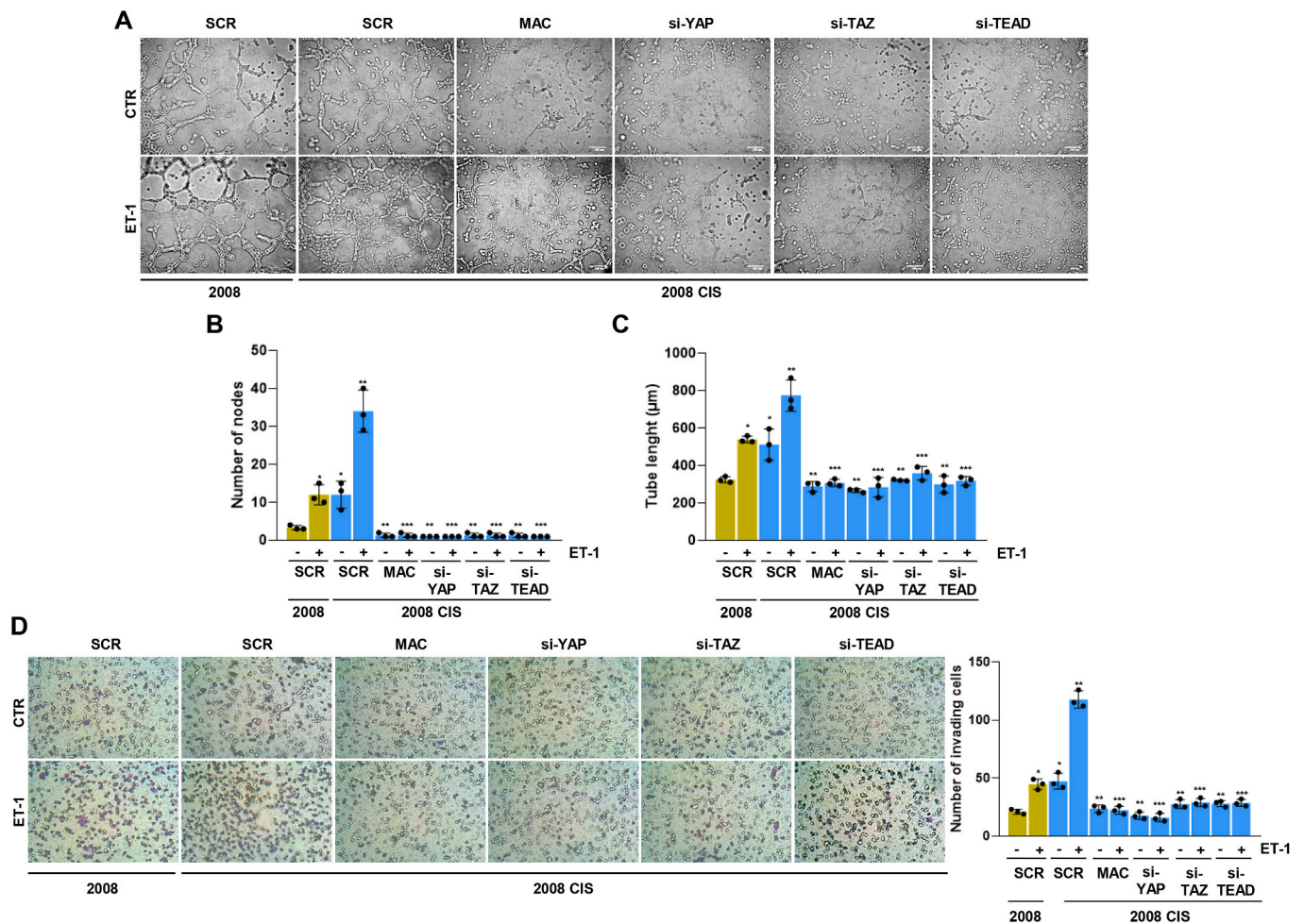
regulation, suggesting that restraining the ET-1/ $ET_A$ R signaling may represent a strategy to confine the YAP-induced cisplatin-tolerant state in OC cells (Fig. 7H).

The role of GPCR as YAP activator in many tumors is mostly centered on the paradigm of the G-proteins signal transduction [31,32]. It has been reported that, either in colon cancer cells, highly expressing  $ET_A$ R [32], or in uveal melanoma cells, overexpressing  $ET_B$ R [43], ET-1R activation leads to YAP/TAZ nuclear accumulation through a G-protein-transduced signaling [32,43]. As alternative route, we have recently documented that in OC cells  $\beta$ -arr1 modulates the crosstalk between the ET-1 axis and YAP. It also favors the formation of a nuclear complex comprising  $\beta$ -arr1, YAP and mutant p53 proteins that

coordinate the transcriptional response to ET-1 [42]. In line with these findings, we now provide novel mechanistic insights to decipher the contribution of  $ET_A$ R/YAP signaling network to the evasion of drug-induced apoptosis as a consequence of the activation of a signaling cascade that sustains cell survival, cell plasticity, and chemoresistance. We also show that in cisplatin-resistant OC cells highly expressing  $ET_A$ R [4], the enhanced YAP nuclear compartmentalization mediated by  $\beta$ -arr1, favors the recruitment of YAP on YAP/TEAD target gene promoters, orchestrating a precise transcriptional reprogramming that may actively consolidate the cancer treatment evasion.

It is clear that the p53 and Hippo tumor-suppressor pathways are closely coordinated through multiple molecular interfaces leading to



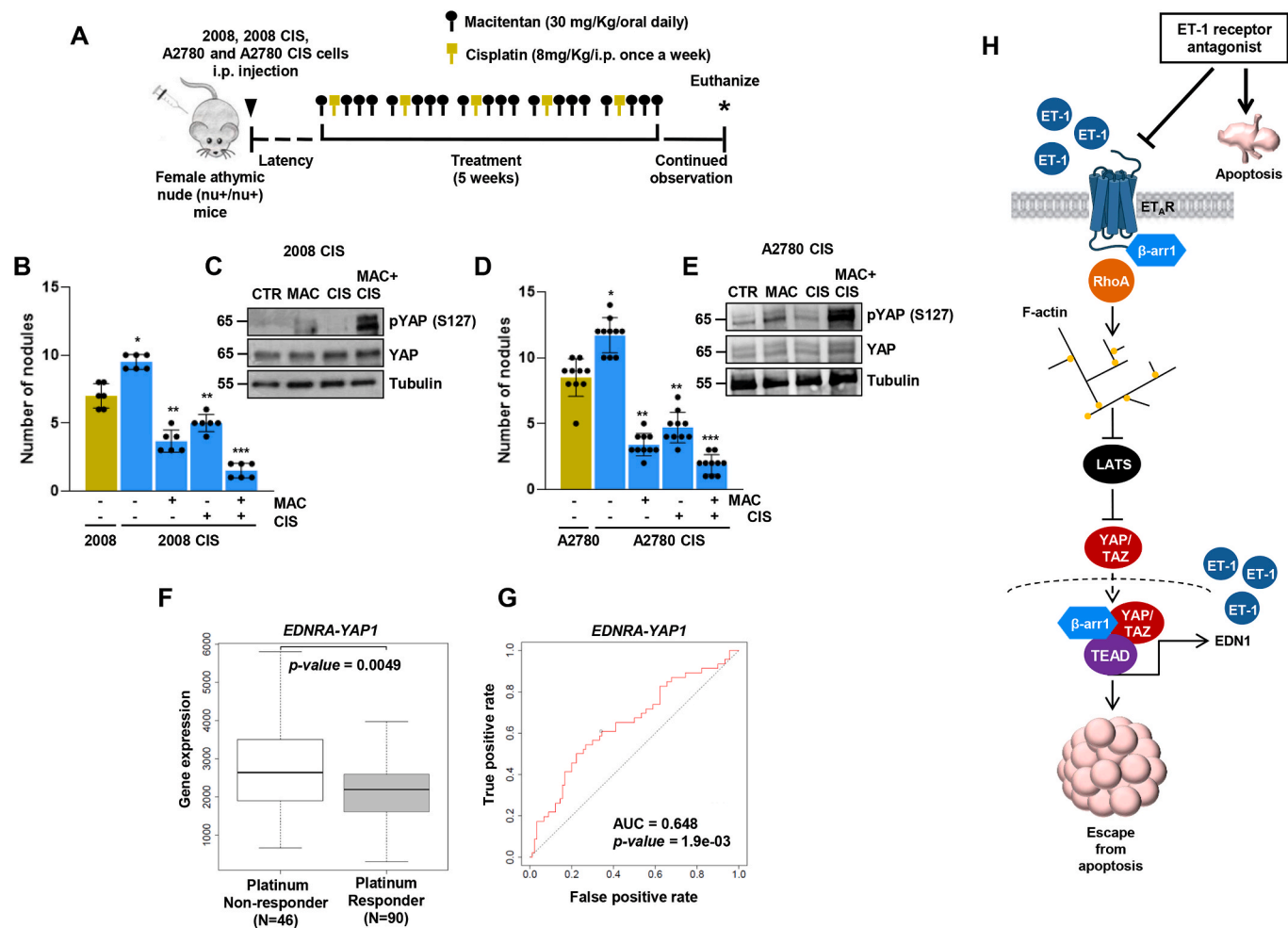


**Fig. 6.** Macitentan impairs ET<sub>A</sub>R/YAP-dependent ovarian cancer cell plasticity and invasion. (A) Tubule-like structure formation ability of 2008 and 2008 CIS cells stimulated or not with ET-1, transfected with SCR, or si-YAP, or si-TAZ, or si-TEAD for 72 h and treated with MAC for 24 h (Magnification X20, scale bar 100 μm). (B, C) Quantification analysis of the number of nodes (B) and tube length (C) referred to the vasculogenic mimicry assay showed in A. Bars are means ± SD (\*p < 0.02 vs 2008 SCR CTR; \*\*p < 0.02 vs 2008 CIS SCR CTR; \*\*\*p < 0.002 vs 2008 CIS SCR ET-1; n = 3). (D) Invasion assays of 2008 and 2008 CIS cells stimulated or not with ET-1, transfected with SCR, or si-YAP, or si-TAZ, or si-TEAD for 72 h and treated with MAC for 24 h. The invasive cells are photographed (Magnification X20) (left) and counted (right). Bars are means ± SD (\*p < 0.004 vs 2008 SCR CTR; \*\*p < 0.02 vs 2008 CIS SCR CTR; \*\*\*p < 0.0002 vs 2008 CIS SCR ET-1; n = 3).

opposite behaviors. In pancreatic cancer cells, p53 guides a tumor-suppressive program that includes induction of the tumor-suppressor gene PTPN14 causing cytoplasmic sequestration and transcriptional inactivation of YAP [52]. Conversely, mutp53, but not wtp53, might bypass Hippo signaling by associating directly with YAP to drive oncogenic transcriptional programs. [42,53–55]. Similarly, β-arr1 and p53 engages a cross-talk that can drive different and apparently opposite functions. Thus, in response to β2-adreno (β2AR) receptor activation, β-arr1 moves to the nucleus where it functions as an adaptor protein to promote the binding and degradation of p53 by the E3-ubiquitin ligase Mdm2, allowing accumulation of DNA damage [56,57]. Conversely, in HG-SOC cells, in response to ET-1R activation, β-arr1 moves to the nucleus where it functions as an adaptor protein to promote the tethering of YAP and mut p53, allowing oncogenic activities of YAP [42]. Overall, the functional output of the cross-talk of β-arr1 and p53 appears crucially related to the conformation of the p53 protein downstream of GPCR signaling.

In accordance with recent studies that identified the RhoA GTPase-induced signaling as a determinant of short survival and drug resistance in OC [26,27], our results show that β-arr1 induces YAP nuclear accumulation through the selective induction of the RhoA GTPase-associated pathway and actin reorganization, portraying a targetable node of YAP regulation during the acquisition of cell

plasticity and chemoresistant phenotype. In OC cells YAP activation has been shown to dampen the efficacy of chemotherapy [36–40] and that the combination of the YAP inhibitor verteporfin with platinum-based therapy enhances chemosensitivity [41]. However, the mechanistic basis for these observations remains largely unexplored. Here, we provide a mechanistic link for these observations and demonstrate that the ET<sub>A</sub>R/β-arr1-dependent YAP activation represents a dominant survival strategy which protects chemoresistant OC cells from cisplatin-induced apoptosis. Furthermore, ET<sub>A</sub>R/YAP acts as a cancer driver that promotes OC progression, by activating cell plasticity and invasiveness. These observations agree with our previous findings highlighting that ET<sub>A</sub>R/β-arr1/YAP expression, as well as the expression of their associated genes, correlates with poor clinical outcomes of OC patients [42]. Of interest, our findings uncover the existence of a self-amplifying circuit by which, downstream of ET<sub>A</sub>R, the β-arr1/YAP/TEAD transcription complex enhances *EDN1* gene transcription that, in turn, favors a persistent ET<sub>A</sub>R/YAP loop activation in platinum-resistant OC cells. These findings expand what has been previously known about the ET<sub>A</sub>R-driven chemoresistant and EMT features [4], outlining a model in which ET<sub>A</sub>R, acting as a guidance receptor, coopts other oncogenic signaling pathways, as Wnt/β-catenin or YAP, providing a selective advantage to OC cells through the amplification of the ET-1 autocrine loop, required for the maintenance of platinum



**Fig. 7.** Macitentan, interfering with  $ET_{A}R$ /YAP activity, enhances cisplatin efficacy in platinum-resistant OC xenografts. (A) Experimental design of the study in 2008, 2008 CIS, A2780 and A2780 CIS xenografts treated with vehicle (CTR) or MAC (30 mg/kg/day, oral daily), cisplatin (CIS, 8 mg/kg/i.p. once a week) in mono-therapy or in co-therapy (MAC + CIS) as indicated in the treatment schedule shown in A. Bars are means  $\pm$  SD of 6 mice for group for 2008 and 2008 CIS and 10 mice for group for A2780 and A2780 CIS (\* $p$  < 0.003 vs vehicle treated 2008 or A2780 injected mice; \*\* $p$  < 0.0002 vs vehicle treated 2008 CIS or A2780 CIS injected mice; \*\*\* $p$  < 0.0006 vs MAC or CIS treated 2008 CIS or A2780 CIS injected mice). (C, E) pYAP (S127) and YAP protein expression in 2008 CIS and A2780 CIS nodules extracts treated as in A and evaluated by IB analysis. Tubulin was used as loading control. (F) Box plot representing the *EDNRA/YAP1* gene expression in platinum non-responder (N = 46) and platinum responder (N = 90) OC patients ( $p$  = 0.0049). (G) ROC curves for *EDNRA/YAP1* gene expression using the same patients as in F ( $p$  = 1.9e-03, AUC = 0.65, 95% CI 0.579–0.711). (H) Proposed model illustrating how the FDA-approved dual ET-1R antagonist macitentan, blunting the  $ET_{A}R$ / $\beta$ -arr1/RhoA/YAP escape signaling pathway, restores drug sensitivity in platinum-resistant OC cells.

tolerance.

The FDA approved ET-1 receptor antagonist macitentan has been described in several cancer settings to exert anti-proliferative and pro-apoptotic activity mainly by down-regulating oncogenic pathways to which cancer cells are addicted [3,4,42,58–62]. In this work, we report that macitentan, interfering with the  $ET_{A}R$ / $\beta$ -arr1/YAP-mediated signaling network, hampers the apoptosis evasion to chemotherapy in cisplatin-resistant OC cells. Of clinical interest, macitentan in combination with cisplatin cooperates to reduce the metastatic progression and re-sensitizes chemoresistant OC xenografts to the treatment, suggesting that macitentan may represent a promising therapeutic opportunity in concert with chemotherapy. Targeting the complex signaling network using the dual ET-1 receptor antagonist to overcome adaptive mechanisms of therapy escape, provides the advantage to simultaneously target OC cells mainly expressing  $ET_{A}R$ , but also the tumor microenvironmental elements, including fibroblasts, blood and lymphatic endothelial cells and immune system cells, which mainly express  $ET_{B}R$  [3,4,42,63,64]. In light of these findings, we can consider macitentan belonging to a new class of molecules able to interfere with

cell survival, EMT and cell plasticity, necessary to combat clinical metastasis formation to enhance patient survival.

Remarkably, the potential predictive role of  $ET_{A}R$ /YAP signature in OC patients non-responders to platinum therapy might aid at the stratification of the unresponsive patients for treatment sensitization through novel combination therapy with macitentan.

Conclusively, the present study recognizes the anti-apoptotic signaling cross-talk between  $ET_{A}R$ / $\beta$ -arr1 and YAP as an OC chemotherapy escape route which embodies a therapeutic vulnerability for the treatment of chemoresistant OC patients, highly expressing  $ET_{A}R$  and YAP. In this perspective, the repurposing of the small molecule macitentan, would overcome the auto-amplifying  $ET_{A}R$ /YAP-induced adaptive mechanism, therefore prolonging treatment responses in chemoresistant OC patients.

**Author contributions**

P.T. performed most of the experiments shown in this work, with the help of R. C., R. S., L; R. and V. D. C., analyzed and discussed the data. G.

B. and A. B. revised critically the study. A. B. conceived and supervised the study and wrote the paper with input from the other authors. All authors provided comments and final approved the version to be submitted.

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## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2020.08.026>.

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