

JPET #211839

Title Page

Loss of PKC δ /HuR interaction is necessary to doxorubicin resistance in breast cancer cell lines [^]

Elisa Latorre, Ilaria Castiglioni, Pamela Gatto, Stephana Carelli, Alessandro
Quattrone, Alessandro Provenzani

Laboratory of Genomic Screening, Centre for Integrative Biology, University of Trento,
Trento, Italy EL, IC, AP

Laboratory of Translational Genomics, Centre for Integrative Biology, University of Trento,
Italy PM, AQ

Pharmacology Laboratory, Health Sciences Dept, University of Milan, Italy EL, SC

JPET #211839

Running Title Page

Running Title: PKC δ /HuR is a key interaction in doxorubicin resistant cells

Corresponding author:

Alessandro Provenzani

Laboratory of Genomic Screening, Centre for Integrative Biology (CIBIO)

Via delle Regole 101, 38123 Trento

Tel +390461283094, fax +390461283937

provenzani@science.unitn.it

University of Trento

Number of text page: 27

Number of figures: 5

Number of references: 31

Number of words in the Abstract, Introduction, Results and Discussion: 159, 560,
1211, 581

Non standard abbreviations: protein kinase C δ (PKC δ), RNA binding protein (RBP),
AU-rich elements (AREs), deoxycytidine (dCK), topoisomerase II α (TOP2A)

Recommended section: Chemotherapy, Antibiotics, and Gene Therapy

Abstract

The protein kinase C δ interacts and phosphorylates HuR, dictating its functionality. We show here that the genotoxic stimulus induced by doxorubicin triggers PKC δ interaction with HuR and leads to HuR phosphorylation on serine 221 and 318 and cytoplasmic translocation. This series of events is crucial to elicit the death pathway triggered by doxorubicin and is also necessary to promote HuR function in post-transcriptional regulation of gene expression, since genetic ablation of PKC δ brought to HuR inability to bind its target mRNAs, TOP2A included. In *in vitro* selected doxorubicin resistant human breast cancer cell lines upregulating the multidrug resistance marker ABCG2, PKC δ and HuR proteins were coordinately downregulated together with the doxorubicin target TOP2A protein, whose mRNA is HuR-regulated. Therefore, we show here that PKC δ , HuR and TOP2A constitute a network mediating doxorubicin efficacy in breast cancer cells. The importance of these molecular events in cancer therapy is suggested by their being profoundly suppressed in cells selected for doxorubicin resistance.

Introduction

The efficacy of anticancer therapy is decreased by the onset of resistance mechanisms. This phenomenon holds true either for classical cytotoxic drugs such as 5-fluorouracil, cisplatin or anthracyclines (Broxterman *et al.*, 2009) and for new target-oriented anticancer agents such as tyrosine kinase inhibitors. It takes place through different mechanisms, including point mutation or amplification of the gene coding for the target enzyme and activation of alternative salvage pathways (Ercan *et al.*, 2012). In the case of the anthracycline doxorubicin, cancer cells become refractory to therapy mainly by increasing expression of extrusion pumps such as the ABC transporters (Gros *et al.*, n.d.) and/or decreasing the expression level of the doxorubicin target enzyme topoisomerase II (Fry *et al.*, 1991). HuR/ELAVL1 is a widely studied and ubiquitously expressed RNA binding protein (RBP) playing a pleiotropic role in cells, being involved in regulation of the major cell programs for proliferation, apoptosis and differentiation especially under cell stress conditions (Hinman and Lou, 2008). According to the cellular context, HuR binds to AU-rich elements (AREs) of primarily non coding regions of mRNAs and of pre-mRNAs regulating their splicing, export, stability and translation (Srikantan and Gorospe, 2012). Since HuR coordinates the maturation of thousands of mRNA species (Mukherjee *et al.*, 2009; Dassi *et al.*, 2013), it is not surprising that a deregulation of the activity of this protein is associated with cancer and with inflammatory diseases (Pascale and Govoni, 2012; Srikantan and Gorospe, 2012). Recently HuR has been addressed to mediate the efficacy of gemcitabine and doxorubicin since it regulates post-transcriptionally deoxycytidine (dCK) (Costantino *et al.*, 2009) and topoisomerase II α (TOP2A) (Srikantan *et al.*, 2011), the two enzymes respectively responsible of the effects of these drugs. It has been shown that HuR has a promoting role for chemoresistance in glioblastoma multiforme by increasing

the stability of the mRNA of the antiapoptotic gene BCL-2 (Filippova *et al.*, 2011). We have reported the downregulation of HuR in doxorubicin resistant breast cancer cell lines (Latorre *et al.*, 2012). Post-translational modifications, such as phosphorylation (Eberhardt *et al.*, 2012) and arginine methylation (Vázquez-Chantada *et al.*, 2010), dictate HuR functional plasticity. Protein kinase C δ (PKC δ) is among the proteins demonstrated to interact with HuR and it has been shown to phosphorylate HuR in serine 218 and 321, thereby triggering its cytoplasmic translocation in human mesangial cells (Doller *et al.*, 2010). In this way PKC δ sustain also HuR aberrant functions in colon cancer cells (Doller *et al.*, 2011). PKC δ belongs to the novel subgroup of PKCs, and is regulated by diacylglycerol (DAG) but it is calcium-insensitive (Mellor and Parker, 1998). This kinase is activated by a number of proapoptotic stimuli, such as DNA damaging agents and reactive oxygen species (Anantharam *et al.*, 2002), both in cellular systems (Gonzalez-Guerrico and Kazanietz, 2005) and in mice models (Leitges *et al.*, 2001). PKC δ , the JNK kinase and Caspase-2 are essential to mediate doxorubicin efficacy (Panaretakis *et al.*, 2005; Lasfer *et al.*, 2006) in primary acute lymphoblastic leukemia blasts and Hep3B cells, leading to the activation of the mitochondrial apoptosis pathway.

In this work we identified PKC δ as a key player of HuR activation during doxorubicin induced genotoxic stress, and, by the evaluation of doxorubicin-resistant breast cancer cells, we found that the coordinated downregulation of PKC δ and HuR, with concomitant decrease expression of TOP2A enzyme, is preparatory to the onset of doxorubicin resistance.

Methods

Cell lines.

The MCF-7, MDA-MB-231 and SK-BR-3 breast cancer cell lines, purchased from ICLC (Geona, Italy) were cultured in complete DMEM (Lonza) supplemented with 10% fetal calf serum (FCS, Lonza), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza), or OptiMem (Lonza). MCF-7, MDA-MB-231 and SK-BR-3 doxorubicin resistant cells were derived from the respective parental cell lines by culturing under increasing doxorubicin concentrations, according to Latorre *et al.* (Latorre *et al.*, 2012).

Chemicals, antibodies, siRNA and shRNA.

The following chemicals and reagents were purchased from Sigma Aldrich: Rottlerin (R5648, PubChem Substance ID 24278679), Doxorubicin hydrochloride (D1515, PubChem Substance ID 24893465), protein-A-coated agarose beads. The following antibodies and siRNAs were purchased from Santa Cruz: anti-HuR (sc-71290), anti-PKCδ (sc-937), normal mouse total serum IgG (sc-2025), anti-beta-tubulin (sc-55529), HuR human siRNA (sc-35619), PKCδ human siRNA (sc-36253), scrambled control siRNA-A (sc-37007). Anti-ABCG2 (MAB995) was purchased from R&D systems. CaspaseGlo 3/7 (G8091), and calf intestinal alkaline phosphatase (M1821) were purchased from Promega.

Mutagenesis

HuR mutants were generated by PCR site direct mutagenesis (QuikChange multisite-directed mutagenesis kit Stratagene) as previously described (Bertini *et al.*, 2003) using as template pTrueOrf-HuR-Myc-His (D'Agostino *et al.*, 2013) and with the following primers:

JPET #211839

S221A:FOR:gcagagattcagggttcGCccccatgggcgtcg,REV:CGACGCCCATGGGGGCGAACC
TGAATCTCTGC,S318AFOR:ggacaaaatcttacaggttGccttcaaaaccaacaagtcc,REV:GGACTT
GTTGGTTTTGAAGGCAACCTGTAAGATTTTGTCC

Immunocytochemistry.

Cells were plated on acid-washed glass coverslips and maintained in the appropriate culture medium and experimental conditions. Cells were fixed according to (Latorre *et al.*, 2012) in 3.7% paraformaldehyde (PFA) for 15 minutes at room temperature and permeabilized in sucrose 300 mM and Triton-X 100 0.2% in PBS. Cells were imaged using a Zeiss 100x or 63x/1.4NA Plan Apo oil immersion objective lens on a Zeiss Axio observer Z1 (DAPI: excitation 360/40 nm, emission 457/50 nm; GFP: excitation 490/20 nm, emission 528/38 nm). Each image measured 512x512 pixels, and effective pixel size was 0.106.

2D gel electrophoresis.

Two dimensional gel electrophoresis was performed as previously described, (Latorre *et al.*, 2012). About 250-400 µg of protein from cell total extracts were isoelectrofocussed (IEF) on IPGphor (GE Healthcare) according to the manufacturer recommendations. Second dimension electrophoresis was performed using a Protean II apparatus (Bio-Rad). PKCδ silencing was done by siRNA transient transfection (Mirus TransIT®-LT1 Transfection Reagent) 24 hours before the 2D gel run.

Western blot analysis.

Western blotting was performed with a SDS-PAGE Electrophoresis System (Biorad) as previously described (Latorre *et al.*, 2012). Beta-tubulin was used as a protein loading

JPET #211839

control. “In-cell” Licor protein quantification of MCF-7, MDA-MB-231 and SK-BR-3 cells was performed by seeding cells in standard growing conditions in ViewPlates-96 (Perkin Elmer), fixed in PBS-PFA 4% and permeabilized with sucrose 300 mM and Triton-X100 0.2% in PBS. Cells were incubated with the primary antibody against HuR or PKC δ or ABCG2 together with anti-beta-tubulin as housekeeping for normalization in PBS-BSA 0.2%. Fluorescent labeling and detection was performed as described by the manufacturer. Briefly, the cells were stained with two fluorescent labeled antibodies, IRDye 800CW and 680RD, to mark respectively the housekeeping and the protein to be quantified. The fluorescence signal for each antibody was measured and normalized for the housekeeping signal to plot the graph.

Co-immunoprecipitation (co-IP).

12x10⁶ MCF-7 cells cultured in the different experimental conditions were syringed by an U-100 insulin needle in 500 μ l lysing NT2 buffer (50 mM Tris-HCl pH 7.7, 150 mM NaCl, 1 mM MgCl₂; 0.05% NP40, 1 U/ μ l RNaseIN, 20 mM DTT, 1% BSA, Protease inhibitor cocktail manufacturer’s recommended) chilled at 4°C. The lysate was centrifuged at 10000g for 10 minutes then the supernatant was pre-cleared by interaction with protein-A-coated agarose beads (equilibrated in NT2 buffer) overnight at 4°C in constant shaking (100 μ l slurry beads/500 μ l lysate). 150 μ l of the pre-cleared lysate were put to interact with anti-HuR protein A, anti-PKC δ antibody or control IgG coated agarose beads, conjugated for 2 hours at room temperature, then washed twice in NT2 buffer. 20 μ l protein A-coated slurry agarose beads were conjugated with 4 μ g antibody at room temperature for 2 hours, washed and equilibrated in NT2 lysis buffer before use. Samples were analyzed by western blotting.

JPET #211839

Pull down and Dot-blot

HuR Mutant and wild type plasmids were transiently transfected in MCF7 cells treated or not with 10 μ M doxorubicin for 3 hours after 48 hours PKC δ silencing and 24 hours of starvation. Recombinant HuR protein was purified by affinity chromatography with HisTrap HP resin (GE Healthcare; 17-5248-01) and eluted with imidazole gradient ranging from 62.5 to 500 mM. Samples were blotted onto millipore immobilon PVDF membrane with a PR600 slotblot manifold (Amersham, Arlington Heights, Ill.) and blotting was performed with anti-HuR, anti-Pan-Ac and anti-pan-P antibodies.

Apoptosis detection.

Apoptosis induction was detected by the luminescent Caspase-Glo® 3/7 assay (Promega) after 4 hours of doxorubicin treatment (at 10 μ M), or 1 hour pretreatment with indicated drugs followed by doxorubicin administration according to manufacturer's instructions. Experiments were run in starvation conditions. Briefly, an equal volume of the reagent was added to samples, incubated for 30 minutes and then the luminescence signal of triplicate samples was measured using a Tecan Infinite 200 Reader. Number of cells were identical before and after treatment as measured using Cell titer Glo® (Promega) in separate, within the plate, control wells.

Results

Doxorubicin triggers the interaction between HuR and PKC δ .

We previously showed that HuR was induced to shuttle into the cytoplasm by doxorubicin and was antagonized, in the movement, by a drug, rottlerin. This drug was able to increase doxorubicin induced HuR nuclear retention and to prevent doxorubicin induced HuR phosphorylation in breast cancer cells (Latorre *et al.*, 2012). Despite the action of rottlerin being substantially unspecific (Soltoff, 2007), its first identified molecular target was PKC δ (Gschwendt *et al.*, 1994). Moreover PKC δ has been reported to be involved in the regulation of HuR in different cell systems, although not in the presence of a genotoxic stimulus. Therefore, to understand if the inhibition of doxorubicin-induced phosphorylation of HuR by pretreatment with rottlerin could be explained by a direct involvement of PKC δ in the process, we investigated if PKC δ was activated and induced to bind to HuR by doxorubicin. As shown in Figure 1A, HuR and PKC δ coimmunoprecipitated in the presence of doxorubicin. PKC δ was scarcely present in the pellet derived from HuR immunoprecipitation in cells grown in media containing serum and it was not detectable in cells grown under serum starvation. Notably, the interaction was strongly enriched following addition of doxorubicin to the medium. Moreover, pretreatment with rottlerin hampered doxorubicin-induced PKC δ and HuR coimmunoprecipitation. In order to assess if PKC δ is pivotal in mediating doxorubicin-induced HuR phosphorylation, we performed siRNA-mediated silencing of PKC δ followed by a two dimensional western blotting against HuR. As shown in Figure 1B, during PKC δ silencing, HuR is posttranslationally modified in cells grown in serum while it is not during starvation. Nevertheless, in the absence of PKC δ , treatment with doxorubicin reduced the number of HuR phosphorylations compared to the control condition (Figure 1B) indicating that PKC δ is responsible of most of them

JPET #211839

during doxorubicin administration. Importantly, doxorubicin induced HuR cytoplasmic accumulation was abolished (Figure 1C) during silencing of PKC δ , and doxorubicin treatment induced a milder apoptotic response than in control condition, as already observed (Panaretakis *et al.*, 2005) (Figure 1D). Therefore PKC δ modulates the apoptotic response by inducing phosphorylation and regulating the cell localization of the RNA binding protein HuR, previously identified to mediate doxorubicin cell response upon insurgence of phosphorylations and cytosolic translocation (Latorre *et al.*, 2012).

PKC δ mediates doxorubicin efficacy by phosphorylating HuR on serine 221 and 318

The two HuR target residues of PKC δ have been endowed with different functions encompassing the ability to bind to RNA (S318) and the ability to shuttle between the nucleus and the cytoplasm (S221) (Doller *et al.*, 2010; Schulz *et al.*, 2013). To deeper investigate PKC δ mediated and doxorubicin induced HuR phosphorylations we generated two HuR mutants, HuR-S221A and HuR-S318A, that are non-phosphorylatable mutants by PKC δ (Doller *et al.*, 2010). As shown in Figure 2, upon starvation the basal phosphorylation level of HuR was completely abolished but doxorubicin was still able to induce phosphorylations in HuR in the ectopically expressed protein (WT-HuR) as was the case for the endogenous counterpart (Figure 1B). Silencing of PKC δ blocked doxorubicin induced HuR phosphorylations and since no further phosphorylations were detected in non-phosphorylatable mutants S221A and S318A we can conclude that PKC δ mediates the doxorubicin induced signaling cascade phosphorylating HuR on these two protein residues. Taken together, these results indicate that the interaction between PKC δ and HuR during doxorubicin administration brings to the phosphorylation in serine 221 and 318 of HuR (Figure 2).

PKC δ modulates doxorubicin induced HuR post-transcriptional response.

Since HuR is a major post-transcriptional regulator of stress induced cell responses and regulates the post-transcriptional response to doxorubicin, we investigated if PKC δ would affect HuR binding to specific target mRNA during doxorubicin administration. We chose three already validated targets such as c-MYC, SOCS3 and FOS that are susceptible of doxorubicin induced-HuR mediated post-transcriptional control (Latorre *et al.*, 2012). Moreover we added two anti-apoptotic genes, present in our previous gene list of doxorubicin induced HuR enriched genes, and already known to be target of HuR, such as the antiapoptotic genes MCL-1 and BCL-2 (Filippova *et al.*, 2011). All of these transcripts were preferentially bound by HuR upon doxorubicin administration but not after silencing of PKC δ (Figure 3A). These data indicate that PKC δ affects HuR mRNA binding allowing the association of HuR with specific target mRNA during doxorubicin administration. The HuR/mir-548c-3p post-transcriptional regulation of TOP2A has been addressed to be the responsible of doxorubicin efficacy (Srikantan *et al.*, 2011). During doxorubicin administration in starved cells we could not detect TOP2A mRNA and the formation of a TOP2A mRNA/HuR protein complex (data not shown) being known that TOP2A is expressed only during the G2/M phase (Srikantan *et al.*, 2011). Coherently with the increase of TOP2A mRNA expression during cell cycling, HuR bound to this mRNA and after silencing of PKC δ and without doxorubicin, HuR lost this ability (Figure 3B). As confirmation of the role of HuR in the post-transcriptional control of TOP2A mRNA, in stably HuR silenced cells TOP2A mRNA has a lower expression level compared to wild type cells (Figure 3C). We can therefore conclude that the protein-protein interaction between PKC δ and HuR, leading to HuR phosphorylation and cytosolic localization, is also responsible of the HuR post-transcriptional activity, during doxorubicin-induced cell stress.

PKC δ is downregulated together with HuR in doxorubicin-resistant breast cancer cells.

Given the importance of PKC δ in the cellular response to doxorubicin, we evaluated its expression level in two populations of doxorubicin-resistant breast cancer cells we previously developed (Latorre *et al.*, 2012), MCF-7/doxoR and MDA-MD231/doxoR. In addition we used one population that has been exposed continuously to doxorubicin for at least one month but failed to gain full doxorubicin resistance (SK-BR3/no-doxoR), which therefore well served as negative control. As shown in Figure 4A, in doxoR populations PKC δ was downregulated with respect to the parental doxorubicin-sensitive cells, as was HuR, while it did not happen in the SK-BR3/no-doxoR population. Interestingly, TOP2A protein level decreased significantly in breast cancer resistant cells that showed low HuR expression level (Figure 4A) consistently with what we observed in the stably HuR silenced MCF-7 cells (Figure 3C). Moreover, the ABCG2 transporter, a marker for the doxorubicin resistance phenotype, was found to be overexpressed, compared to parental cells, in the MCF-7/doxoR and in the MDA-MD231/doxoR but not in the SK-BR3/no-doxoR strain (Figure 4B). We then performed rescue of function experiments, by replenishing PKC δ and WT-HuR in doxoR cell populations. As shown in figure 5A ectopic expression of PKC δ allowed recovery of the doxorubicin induced apoptotic response in doxoR cells to the same extent as ectopic expression of HuR did. Interestingly S221A-HuR and S318A-HuR mutants were not able to restart the apoptotic response indicating that the phosphorylation of these two residues is necessary to activate HuR functionality. When we looked at the cell localization of exogenous HuR in doxoR cells, and upon doxorubicin administration during starvation, we observed that, while WT-HuR translocated in the cytoplasm S221A-HuR did not (figure 5C) suggesting that HuR can mediate doxorubicin efficacy upon PKC δ interaction and subsequent translocation into the cytoplasm. These

JPET #211839

results show that long term exposure to doxorubicin brings to the increase of resistance mechanism via a variety of strategies, comprising the typical overexpression of the ABCG2 channel and the downregulation of proteins involved in mediating the apoptotic response (PKC δ , HuR) by post-transcriptional regulation of downstream genes.

Discussion

Within this manuscript we showed that doxorubicin triggers the interaction of the proapoptotic kinase PKC δ with the RBP HuR by determining HuR post-translational modifications in S221 and S318, therefore activating HuR posttranscriptional functionality, apoptotic response and onset of pharmacoresistance. Doxorubicin requires both PKC δ and HuR to fully exert its toxicity: PKC δ is needed in Jurkat cells to activate caspase 2 (Panaretakis *et al.*, 2005) while HuR, in MCF-7 cells, posttranscriptionally regulates the expression level of TOP2A (Srikantan *et al.*, 2011). Here we described the interplay among these factors, showing that the doxorubicin dependent interaction between PKC δ and HuR is functional to the doxorubicin induced apoptotic cell response, since the loss of phosphorylation on HuR residues serine 221 and 318 is critical to elicit the full apoptotic response (Figure 1E, Figure 2, Figure 4C). These observations are in line with consolidated reports that describe the interplay between PKC δ and HuR, but in the absence of genotoxic stress (Doller *et al.*, 2009, 2011). Moreover the doxorubicin induced, HuR mediated, post-transcriptional response, comprising the binding of HuR to the mRNAs of antiapoptotic genes as MCL-1 and BCL-2 and the doxorubicin target encoding TOP2A (Srikantan *et al.*, 2011; Latorre *et al.*, 2012), was lost upon PKC δ silencing. We then evaluated if doxorubicin sensitivity, PKC δ /HuR mediated, could explain the onset of pharmacoresistance, at least in vitro. We previously reported that we observed HuR downregulation in two different breast cancer cell line populations after prolonged exposure to increasing doses of doxorubicin as single agent. Recently (Wang *et al.*, 2013) it has been reported that HuR cytoplasmic accumulation, synonym of high HuR activation, could not predict for neoadjuvant chemotherapy (NACT) sensitivity and efficacy in primary breast cancer patients, but suggested that high HuR cytoplasmic localization can be a negative predictor of the same patients. HuR cytoplasmic localization is therefore a strong

JPET #211839

indicator of the aggressiveness of the tumors and can be ascertained to the massive presence of antiapoptotic, mitogenic and proliferative stimuli that HuR receives and mediates. In this perspective HuR linked pharmacoresistance due to overexpression of BCL-2 family members and sustained by HuR post-transcriptional activity, was observed in glioblastoma cells that carried innate chemoresistance (Nabors 2011) as could be the case for highly expressing HuR breast cancer patients. However it should be noticed the peculiar case of pancreatic adenocarcinoma sensitivity to the single agent gemcitabine where high cytoplasmic HuR clearly stratified the best prognosis patients (Costantino *et al.*, 2009). In this manuscript we also report that, by further characterization of our cell system and for the first time to our knowledge, in acquired doxorubicin-resistant breast cancer cell lines, downregulation of PKC δ , HuR and TOP2A proteins occurs simultaneously, as if resistant cells were corepressing a whole pathway responsible for doxorubicin efficacy. Indeed the net result of HuR post-transcriptional control is complex and depends on the cellular and experimental conditions and possibly even more complicated in the clinical setting. In these resistant cell lines we observed that doxorubicin activation of the PKC δ /HuR axis, leading to the early signaling cascade to apoptosis, is repressed and that the rescue of the apoptotic response upon ectopic expression of PKC δ witnesses its importance of the response. At the same time, inhibition of the PKC δ /HuR dependent post-transcriptional regulation process leads, as reported, to the downregulation of the doxorubicin target TOP2A and to the impairment of the HuR mediated control on other downstream genes. The PKC δ /HuR axis is therefore a key pivotal factor in the doxorubicin induced activity and pharmacoresistance in breast cancer cell lines.

Acknowledgments

JPET #211839

We thank Dr. Devid Meniglio (Biotech center, University of Trento) for support with confocal microscope, Dr. Valentina Adami, Giusy Covello and Laura Vidalino (CIBIO, University of Trento) for helpful discussion.

Authorship contribution

Participated in research design: EL, AP.

Conducted experiments: EL, IC, SC, PG.

Performed data analysis: EL.

Wrote or contributed to the writing of the manuscript: EL, AQ, AP.

JPET #211839

References

- Anantharam V, Kitazawa M, Wagner J, Kaul S, and Kanthasamy AG (2002) Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. *J Neurosci* **22**:1738–51.
- Bertini I, Gelis I, Katsaros N, Luchinat C, and Provenzani A (2003) Tuning the affinity for lanthanides of calcium binding proteins. *Biochemistry* **42**:8011–21.
- Broxterman HJ, Gotink KJ, and Verheul HMW (2009) Understanding the causes of multidrug resistance in cancer: a comparison of doxorubicin and sunitinib. *Drug Resist Updat* **12**:114–26.
- Costantino CL, Witkiewicz AK, Kuwano Y, Cozzitorto JA, Kennedy EP, Dasgupta A, Keen JC, Yeo CJ, Gorospe M, and Brody JR (2009) The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR Up-regulates the expression of the gemcitabine metabolizing enzyme deoxycytidine kinase. *Cancer Res* **69**:4567–72.
- D'Agostino VG, Adami V, and Provenzani A (2013) A novel high throughput biochemical assay to evaluate the HuR protein-RNA complex formation. *PLoS One* **8**:e72426.
- Dassi E, Zuccotti P, Leo S, Provenzani A, Assalg M, D'Onofrio M, Riva P, and Quattrone A (2013) Hyper conserved elements in vertebrate mRNA 3'-UTRs reveal a translational network of RNA-binding proteins controlled by HuR. *Nucleic Acids Res* **41**:3201–3216.

JPET #211839

- Doller A, Gauer S, Sobkowiak E, Geiger H, Pfeilschifter J, and Eberhardt W (2009) Angiotensin II induces renal plasminogen activator inhibitor-1 and cyclooxygenase-2 expression post-transcriptionally via activation of the mRNA-stabilizing factor human-antigen R. *Am J Pathol* **174**:1252–63.
- Doller A, Schlepckow K, Schwalbe H, Pfeilschifter J, and Eberhardt W (2010) Tandem phosphorylation of serines 221 and 318 by protein kinase Cdelta coordinates mRNA binding and nucleocytoplasmic shuttling of HuR. *Mol Cell Biol* **30**:1397–410.
- Doller A, Winkler C, Azrilian I, Schulz S, Hartmann S, Pfeilschifter J, and Eberhardt W (2011) High-constitutive HuR phosphorylation at Ser 318 by PKC{delta} propagates tumor relevant functions in colon carcinoma cells. *Carcinogenesis* **32**:676–85.
- Eberhardt W, Doller A, and Pfeilschifter J (2012) Regulation of the mRNA-Binding Protein HuR by Posttranslational Modification: Spotlight on Phosphorylation. *Curr Protein Pept Sci* **13**:380–90.
- Ercan D, Xu C, Yanagita M, Monast CS, Pratilas CA, Montero J, Butaney M, Shimamura T, Sholl L, Ivanova E V, Tadi M, Rogers A, Repellin C, Capelletti M, Maertens O, Goetz EM, Letai A, Garraway LA, Lazzara MJ, Rosen N, Gray NS, Wong K-K, and Jänne PA (2012) Reactivation of ERK signaling causes resistance to EGFR kinase inhibitors. *Cancer Discov* **2**:934–47.
- Filippova N, Yang X, Wang Y, Gillespie GY, Langford C, King PH, Wheeler C, and Nabors LB (2011) The RNA-binding protein HuR promotes glioma growth and treatment resistance. *Mol Cancer Res* **9**:648–59.

JPET #211839

Fry AM, Chresta CM, Davies SM, Walker MC, Harris AL, Hartley JA, Masters JR, and Hickson ID (1991) Relationship between topoisomerase II level and chemosensitivity in human tumor cell lines. *Cancer Res* **51**:6592–5.

Gonzalez-Guerrico AM, and Kazanietz MG (2005) Phorbol ester-induced apoptosis in prostate cancer cells via autocrine activation of the extrinsic apoptotic cascade: a key role for protein kinase C delta. *J Biol Chem* **280**:38982–91.

Gros P, Ben Neriah YB, Croop JM, and Housman DE (n.d.) Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* **323**:728–31.

Gschwendt M, Müller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, and Marks F (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem Biophys Res Commun* **199**:93–8.

Hinman MN, and Lou H (2008) Diverse molecular functions of Hu proteins. *Cell Mol Life Sci* **65**:3168–3181.

Lasfer M, Davenne L, Vadrot N, Alexia C, Sadji-Ouatas Z, Bringuier A-F, Feldmann G, Pessayre D, and Reyl-Desmars F (2006) Protein kinase PKC delta and c-Abl are required for mitochondrial apoptosis induction by genotoxic stress in the absence of p53, p73 and Fas receptor. *FEBS Lett* **580**:2547–52.

Latorre E, Tebaldi T, Viero G, Spartà AM, Quattrone A, and Provenzani A (2012) Downregulation of HuR as a new mechanism of doxorubicin resistance in breast cancer cells. *Mol Cancer* **11**:13.

JPET #211839

Leitges M, Mayr M, Braun U, Mayr U, Li C, Pfister G, Ghaffari-Tabrizi N, Baier G, Hu Y, and Xu Q (2001) Exacerbated vein graft arteriosclerosis in protein kinase Cdelta-null mice. *J Clin Invest* **108**:1505–12.

Mellor H, and Parker PJ (1998) The extended protein kinase C superfamily. *Biochem J* **332 (Pt 2)**:281–92.

Mukherjee N, Lager PJ, Friedersdorf MB, Thompson M a, and Keene JD (2009) Coordinated posttranscriptional mRNA population dynamics during T-cell activation. *Mol Syst Biol* **5**:288.

Panaretakis T, Laane E, Pokrovskaja K, Björklund A-C, Moustakas A, Zhivotovsky B, Heyman M, Shoshan MC, and Grandér D (2005) Doxorubicin requires the sequential activation of caspase-2, protein kinase Cdelta, and c-Jun NH2-terminal kinase to induce apoptosis. *Mol Biol Cell* **16**:3821–31.

Pascale A, and Govoni S (2012) The complex world of post-transcriptional mechanisms: is their deregulation a common link for diseases? Focus on ELAV-like RNA-binding proteins. *Cell Mol Life Sci* **69**:501–17.

Schulz S, Doller A, Pendini NR, Wilce J a, Pfeilschifter J, and Eberhardt W (2013) Domain-specific phosphomimetic mutation allows dissection of different protein kinase C (PKC) isotype-triggered activities of the RNA binding protein HuR. *Cell Signal* **25**:2485–2495.

Soltoff SP (2007) Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta. *Trends Pharmacol Sci* **28**:453–8.

JPET #211839

Srikantan S, Abdelmohsen K, Lee EK, Tominaga K, Subaran SS, Kuwano Y, Kulshrestha R, Panchakshari R, Kim HH, Yang X, Martindale JL, Marasa BS, Kim MM, Wersto RP, Indig FE, Chowdhury D, and Gorospe M (2011) Translational control of TOP2A influences doxorubicin efficacy. *Mol Cell Biol* **31**:3790–801.

Srikantan S, and Gorospe M (2012) HuR function in disease. *Front Biosci* **17**:189–205.

Vázquez-Chantada M, Fernández-Ramos D, Embade N, Martínez-Lopez N, Varela-Rey M, Woodhoo A, Luka Z, Wagner C, Anglim PP, Finnell RH, Caballería J, Laird-Offringa I a, Gorospe M, Lu SC, Mato JM, and Martínez-Chantar ML (2010) HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation, and carcinogenesis. *Gastroenterology* **138**:1943–53.

Wang J, Li D, Wang B, and Wu Y (2013) Predictive and prognostic significance of cytoplasmic expression of ELAV-like protein HuR in invasive breast cancer treated with neoadjuvant chemotherapy. *Breast Cancer Res Treat* **141**:213–24.

JPET #211839

Footnote

[^] Financial contribution: This work was supported by the Italian Ministry of Health [GR-2008-1135635].

Legends for Figures

Figure 1 PKC δ is involved in the apoptotic response to doxorubicin via complex formation with HuR.

Figure 1A. HuR and PKC δ co-immunoprecipitate upon doxorubicin administration.

Coimmunoprecipitation assessing the HuR and PKC δ protein–protein binding in the presence of doxorubicin 10 μ M for 3 hours (Doxo), in standard growing condition (FBS), in starvation (Starv) and in co-treatment with doxorubicin and rottlerin (Doxo-Rott). Immunoprecipitation samples, input (In), pre-immune immunoglobulins immunoprecipitate (IgG), anti-HuR immunoprecipitate (HuR) and anti-PKC δ immunoprecipitate (PKC) were analyzed by western blotting probing with anti-HuR or PKC δ antibody. HuR binds to PKC δ following doxorubicin treatment and Rottlerin inhibits this interaction.

Figure 1B. PKC δ silencing blocks doxorubicin induced HuR phosphorylations.

2D western blotting probed for HuR on MCF-7 cells treated with doxorubicin (10 μ M for 3 hours) in the presence of PKC δ silencing (Si). In the lower part of the panel a representative western blotting anti-PKC δ showing the protein level in the different samples: standard growing (FBS), starvation (Starv) and doxorubicin treated (Doxo) is shown. AP: alkaline phosphatase.

Figure 1C. PKC δ silencing inhibits doxorubicin induced HuR cytoplasmic shuttling.

Western blots on nuclear (n) and cytoplasmic (c) fractions of MCF-7 cells probed for HuR. Starved MCF-7 cells (Starv) were treated with doxorubicin 10 μ M for 3 hours (Doxo) or not (NTC) in the presence of PKC δ silencing (Si PKC δ). Probing for histone H3 (H3) and lactate dehydrogenase (LDH) was used as control for proper biochemical fraction separation. Silencing of PKC δ hampers doxorubicin HuR cytoplasmic translocation.

JPET #211839

Figure 1D. PKC δ is necessary to elicit the full apoptotic response to doxorubicin.

Histogram showing caspase 3 and 7 activation recorded as chemiluminescence in MCF-7 cells cultured in standard conditions (FCS), in starvation (Starv) after 4 hours of 10 μ M doxorubicin (Doxo) and after 24 hours of PKC δ silencing (Si PKC δ). PKC δ silencing minimizes the doxorubicin induced apoptotic response. * indicates a p-value < 0.05. Representative results out of at least three independent experiments, each experiments was run in technical triplicate. Number of cells was estimated identical all over the experiments by measurement of ATP levels.

Figure 2 HuR S221 and S318 are phosphorylated upon doxorubicin administration via PKC δ .

Dot Blot after pull down of ectopically transfected recombinant, his tagged, HuR protein (rHuR). Empty vector, wild type rHuR, S221A and S318A rHuR have been pulled down after transfection of scramble siRNA (scramble) or siPKC δ and in the presence of or absence of doxorubicin treatment (10 μ M for 3 hours). Membranes were probed with antibody anti HuR to evaluate the rHuR precipitation and with antibody anti-pan phosphorylation to evaluate the level of rHuR phosphorylation. The experiments were run during starvation and exposure to doxorubicin for 4 hours. rHuR was phosphorylated during doxorubicin treatment and in the presence of PKC δ . Mutants rHuR were not phosphorylated during doxorubicin treatment. Lower panel: representative western blot showing the level of silencing of PKC δ .

Figure 3 PKC δ modulates HuR post-transcriptional functionality.

Figure 3A. PKC δ silencing inhibits doxorubicin induced HuR binding to specific messenger RNA.

Semiquantitative RT-PCR on RIP samples immunoprecipitated with anti-HuR antibody (h), whole serum IgG as a negative control (g) and the

JPET #211839

immunoprecipitation input (in). RIP analysis was performed on MCF-7 cells grown in standard condition (FCS), starved for 24 hours (starved), treated with doxorubicin 10 μ M after starvation, after genetic ablation of PKC δ and starvation or starvation and treatment with doxorubicin 10 μ M. 18S is the loading and immunoprecipitation control

Figure 3B. PKC δ silencing inhibits post-transcriptional functionality of HuR on TOP2A target mRNA. Semiquantitative RT-PCR on RIP samples immunoprecipitated with anti-HuR antibody (h), whole serum IgG as a negative control (g) and the immunoprecipitation input (in). RIP analysis was performed on MCF-7 cells grown in standard condition (FCS) or after genetic ablation of PKC δ . Scr: scramble siRNA.

Figure 3C. HuR silencing causes a decrease in the total expression level of TOP2A mRNA. Semiquantitative RT-PCR on total RNA extracted from untreated cycling cells that have been stably silenced for HuR. Scr: scramble siRNA, shHuR: population of MCF-7 cells stably silenced for HuR using Mission Lentiviral Particles (Sigma Aldrich). Side: Representative western blot of the expression level of HuR in shHuR cell population.

Figure 4 PKC δ and HuR are coordinately regulated to develop full doxorubicin resistance.

Figure 4A. PKC δ , HuR and TOP2A are downregulated in doxorubicin resistant breast cancer cells. Representative western blots on whole cell lysates from MCF-7, MDA-MB-231 and SK-BR-3 parental or doxorubicin resistant (DR) cells. Filters are probed with anti-PKC δ , anti-HuR and anti-TOP2A antibodies, actin was used as loading control.

Figure 4B. ABCG2 is upregulated in doxorubicin resistant breast cancer cells. The histogram shows an in well protein quantification recorded by Licor technology on MCF-7, MDA-MB-231 and SK-BR-3 parental or doxorubicin resistant (DR) cells. HuR, PKC δ and ABCG2 protein levels were measured and normalized to the actin level. ABCG2 is

JPET #211839

upregulated in MCF-7 DR and MDA DR cells but not in SK DR cells. HuR and PKC δ are down regulated in DR and MDA DR cells but not in SK DR cells. * indicates a p-value < 0.05.

Figure 5. PKC δ and HuR reactivates the doxorubicin induced apoptotic response in MCF-7 doxorubicin resistant cells.

Figure 5A. Ectopic expression of PKC δ and HuR in MCF-7 DR reactivates the doxorubicin induced apoptotic response. Histogram showing caspase 3 and 7 activation recorded as chemiluminescence in MCF-7 and MCF-7 DR cells in starvation (Starv) and after 4 hours of 10 μ M doxorubicin (Doxo). DR cells were transfected with empty vector, (-), PKC δ , wild type HuR (WT-HuR), S221A and S318A HuR mutants (S221A, S318A). * indicates a p-value < 0.05. Representative results out of at least three independent experiments, each experiments was run in technical triplicate. Number of cells was estimated identical all over the experiments by measurement of ATP levels. Lower panel:

Figure 5B. Ectopic expression of PKC δ in MCF-7 and MCF-7 DR. Representative western blot of the expression level of PKC δ in MCF-7 and MCF-7 DR cells after ectopic expression of the kinase. The blot shows the decrease of the expression level of PKC δ in MCF-7 DR and the efficacy of the ectopic overexpression.

Figure 5C. The S221A HuR mutant can not shuttle into the cytosol during doxorubicin treatment in MCF-7 DR cells. Representative immunofluorescence of wt-HuR and S221A HuR mutant ectopically transfected in MCF-DR cells treated with doxorubicin. WT-HuR can shuttle into the cytoplasm and restart the doxorubicin induced apoptotic response, S221A is confined into the nucleus and is not able to rescue HuR functionality.

Figure 1A

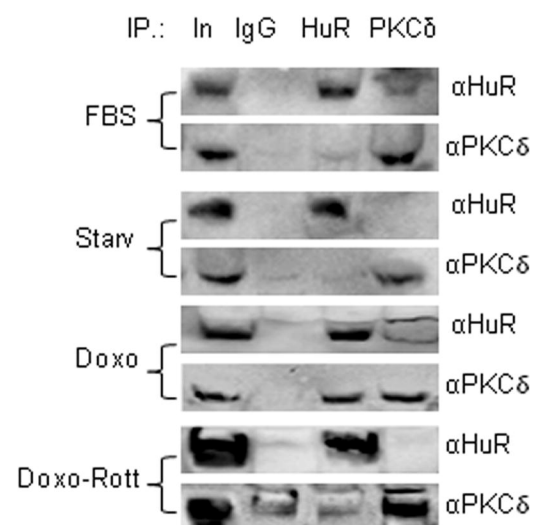


Figure 1C

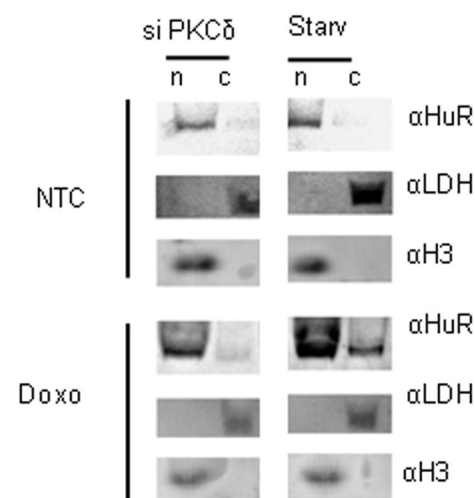


Figure 1B

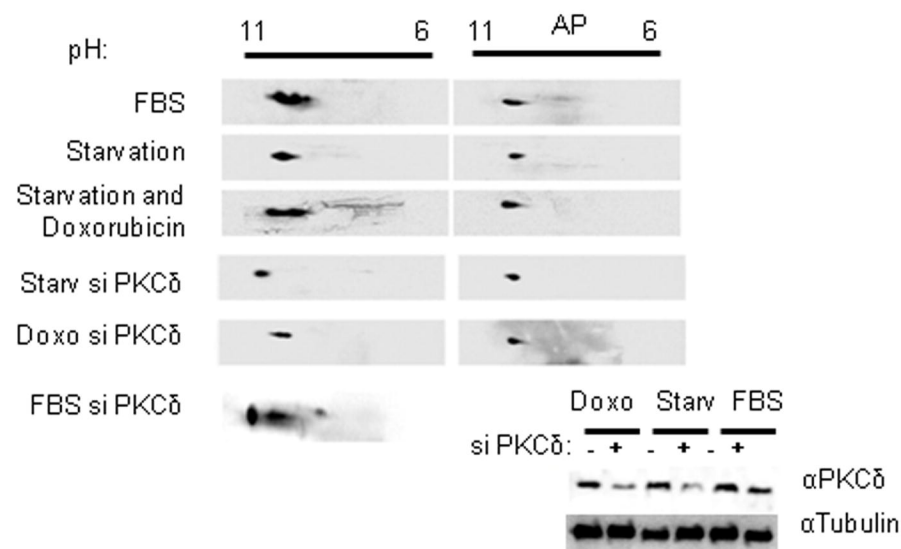


Figure 1D

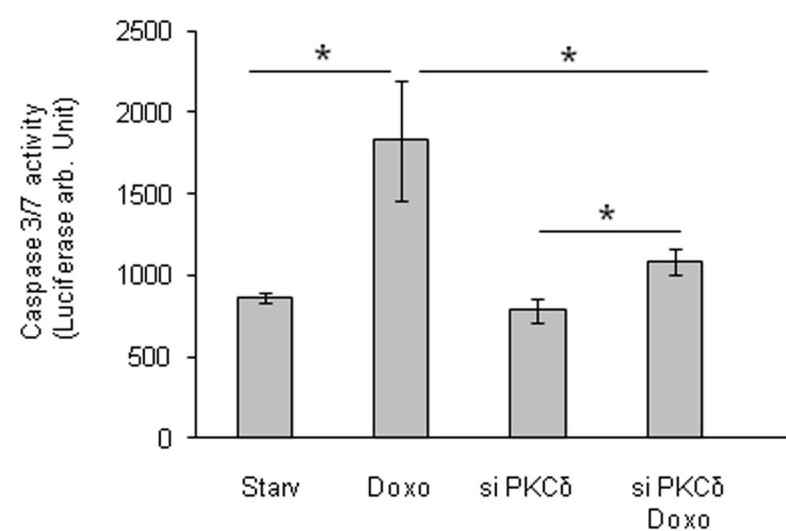


Figure 2

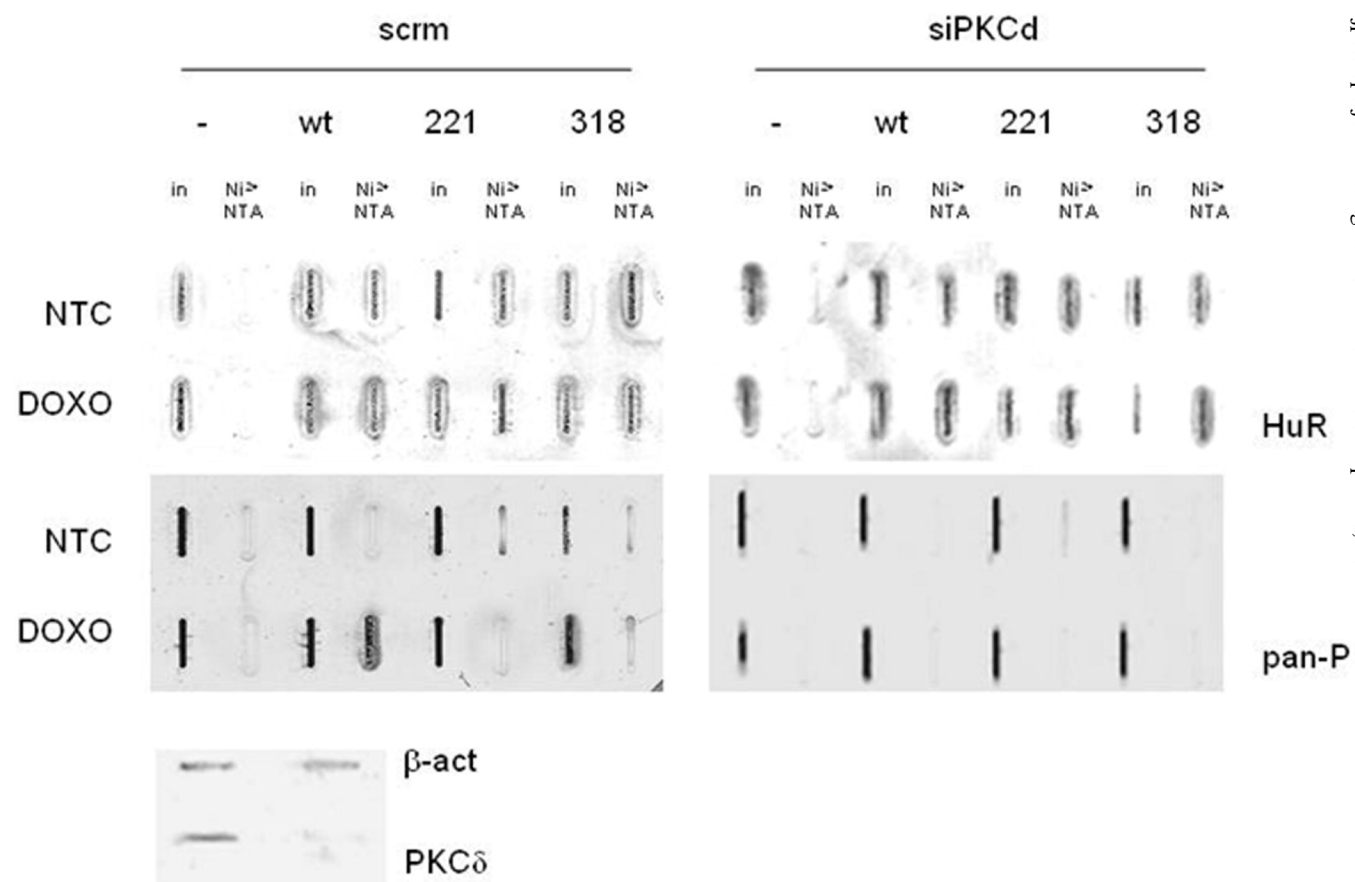


Figure 3A

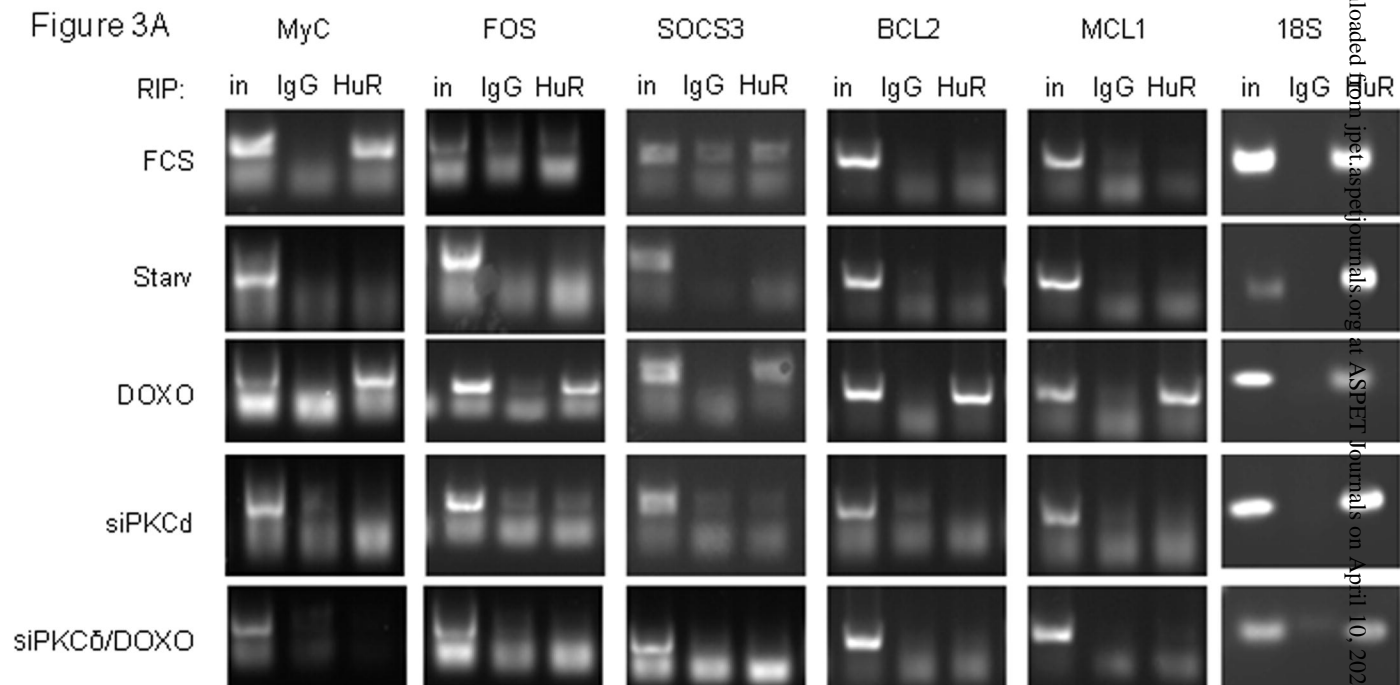


Figure 3B

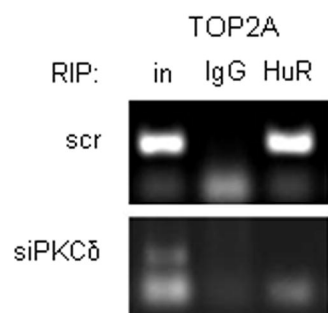


Figure 3C

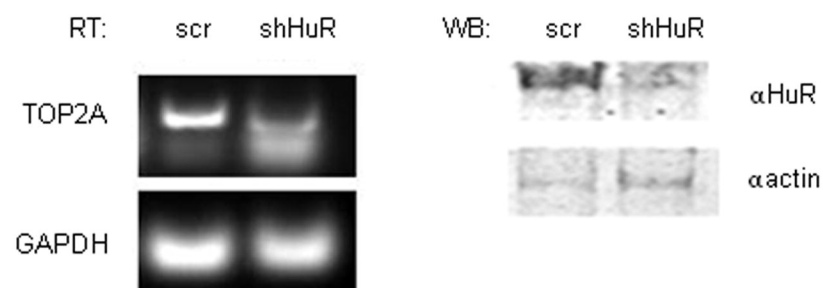


Figure 4A

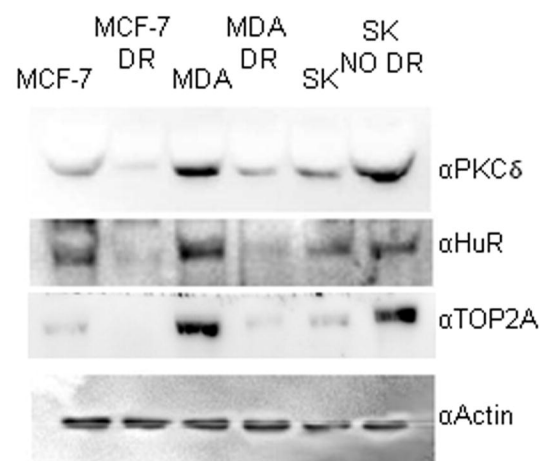


Figure 4B

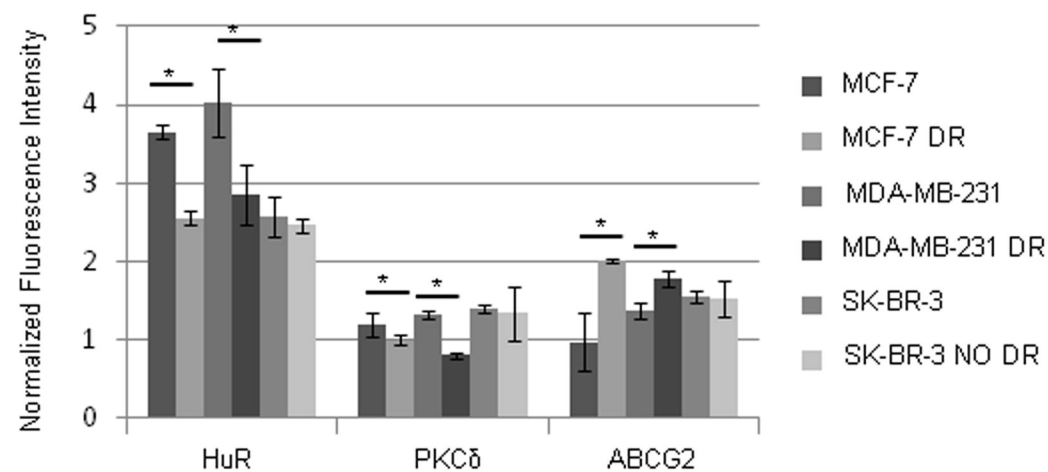


Figure 5A

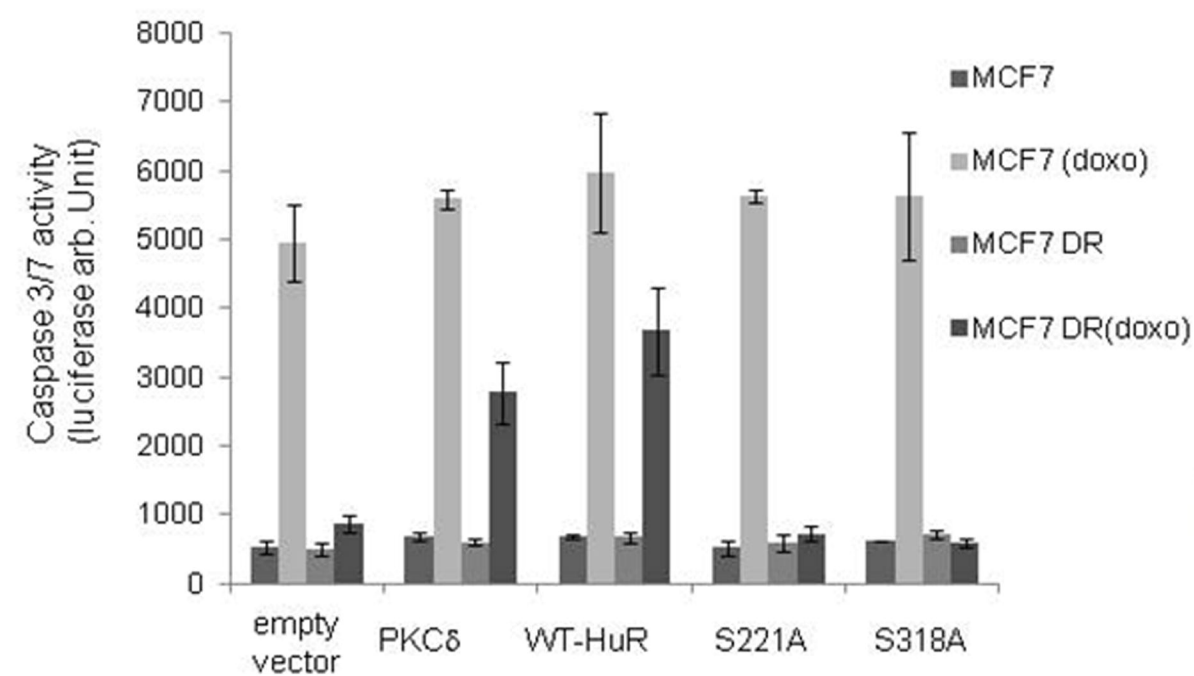


Figure 5B

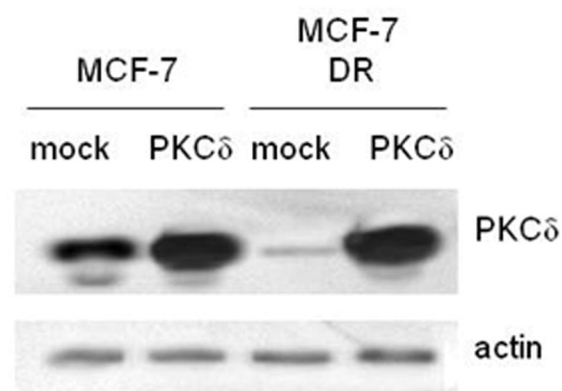


Figure 5C

