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Title Page

Loss of PKCδ/HuR interaction is necessary to doxorubicin resistance in breast cancer cell lines ^

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Running Title Page

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

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Abstract

The protein kinase C δ interacts and phosphorylates HuR, dictating its functionality. We show here that the genotoxic stimulus induced by doxorubicin triggers PKCδ 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 PKCδ 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, PKCδ and HuR proteins were coordinately downregulated together with the doxorubicin target TOP2A protein, whose mRNA is HuR-regulated. Therefore, we show here that PKCδ, 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 deoxycitidine (dCK) (Costantino *et al.*, 2009) and topoisomerase IIα (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

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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 (PKCδ) 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 PKCδ sustain also HuR aberrant functions in colon cancer cells (Doller *et al.*, 2011). PKCδ 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). PKCδ, 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 PKCδ 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 PKCδ 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:

S221A:FOR:gcagagattcaggttcGCccccatgggcgtcg,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 isoelectrofocused (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

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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 PKCδ 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).

$12x10^6$ MCF-7 cells cultured in the different experimental conditions were syringed by an U-100 insulin needle in 500 µl lysing NT2 buffer (50 mM Tris-HCl pH 7.7, 150 mM NaCl, 1 mM MgCl2; 0.05% NP40, 1 U/µl 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 µl slurry beads/500 µl lysate). 150 µl of the pre-cleared lysate were put to interact with anti-HuR protein A, anti-PKCδ antibody or control IgG coated agarose beads, conjugated for 2 hours at room temperature, then washed twice in NT2 buffer. 20 µl protein A-coated slurry agarose beads were conjugated with 4 µg antibody at room temperature for 2 hours, washed and equilibrated in NT2 lysis buffer before use. Samples were analyzed by western blotting.

Pull down and Dot-blot

HuR Mutant and wild type plasmids were transiently transfected in MCF7 cells treated or not with 10 μM doxorubicin for 3 hours after 48 hours PKCδ 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 μM), 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.

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Results

Doxorubicin triggers the interaction between HuR and PKCδ.

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 PKCδ (Gschwendt *et al.*, 1994). Moreover PKCδ 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 PKCδ in the process, we investigated if PKCδ was activated and induced to bind to HuR by doxorubicin. As shown in Figure 1A, HuR and PKCδ coimmunoprecipitated in the presence of doxorubicin. PKCδ 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 PKCδ and HuR coimmunoprecipitation. In order to assess if PKCδ is pivotal in mediating doxorubicin-induced HuR phosphorylation, we performed siRNA-mediated silencing of PKCδ followed by a two dimensional western blotting against HuR. As shown in Figure 1B, during PKCδ silencing, HuR is posttranslationally modified in cells grown in serum while it is not during starvation. Nevertheless, in the absence of PKCδ, treatment with doxorubicin reduced the number of HuR phosphorylations compared to the control condition (Figure 1B) indicating that PKCδ is responsible of most of them

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during doxorubicin administration. Importantly, doxorubicin induced HuR cytoplasmic accumulation was abolished (Figure 1C) during silencing of PKCδ, and doxorubicin treatment induced a milder apoptotic response than in control condition, as already observed (Panaretakis *et al.*, 2005) (Figure 1D). Therefore PKCδ 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).

PKCδ mediates doxorubicin efficacy by phosphorylating HuR on serine 221 and 318

The two HuR target residues of PKCδ 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 PKCδ mediated and doxorubicin induced HuR phosphorylations we generated two HuR mutants, HuR-S221A and HuR-S318A, that are non-phosphorylatable mutants by PKCδ (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 PKCδ blocked doxorubicin induced HuR phosphorylations and since no further phosphorylations were detected in non-phosphorylatable mutants S221A and S318A we can conclude that PKCδ mediates the doxorubicin induced signaling cascade phosphorylating HuR on these two protein residues. Taken together, these results indicate that the interaction between PKCδ and HuR during doxorubicin administration brings to the phosphorylation in serine 221 and 318 of HuR (Figure 2).

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PKCδ 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 PKCδ 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 PKCδ (Figure 3A). These data indicate that PKCδ 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 PKCδ 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 PKCδ and HuR, leading to HuR phosphorylation and cytosolic localization, is also responsible of the HuR post-transcriptional activity, during doxorubicin-induced cell stress.

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

Given the importance of PKCδ 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 PKCδ 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 PKCδ and WT-HuR in doxoR cell populations. As shown in figure 5A ectopic expression of PKCδ 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 PKCδ interaction and subsequent translocation into the cytoplasm. These

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 (PKCδ, HuR) by post-transcriptional regulation of downstream genes.

Discussion

Within this manuscript we showed that doxorubicin triggers the interaction of the proapoptotic kinase PKCδ 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 PKCδ and HuR to fully exert its toxicity: PKCδ 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 PKCδ 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 PKCδ 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 PKCδ silencing. We then evaluated if doxorubicin sensitivity, PKCδ/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

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 PKCδ, 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 PKCδ/HuR axis, leading to the early signaling cascade to apoptosis, is repressed and that the rescue of the apoptotic response upon ectopic expression of PKCδ witnesses its importance of the response. At the same time, inhibition of the PKCδ/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 PKCδ/HuR axis is therefore a key pivotal factor in the doxorubicin induced activity and pharmacoresistance in breast cancer cell lines.

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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.

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Footnote

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Legends for Figures

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

Figure 1A. **HuR and PKCδ co-immunoprecipitate upon doxorubicin administration.** Coimmunoprecipitation assessing the HuR and PKCδ protein–protein binding in the presence of doxorubicin 10 μM 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-PKCδ immunoprecipitate (PKC) were analized by western blotting probing with anti-HuR or PKCδ antibody. HuR binds to PKCδ following doxorubicin treatment and Rottlerin inhibits this interaction.

Figure 1B. **PKCδ silencing blocks doxorubicin induced HuR phosphorylations.** 2D western blotting probed for HuR on MCF-7 cells treated with doxorubicin (10 μM for 3 hours) in the presence of PKCδ silencing (Si). In the lower part of the panel a representative western blotting anti-PKCδ showing the protein level in the different samples: standard growing (FBS), starvation (Starv) and doxorubicin treated (Doxo) is shown. AP: alkaline phosphatase.

Figure 1C. **PKCδ 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 μM for 3 hours (Doxo) or not (NTC) in the presence of PKCδ silencing (Si PKCδ). Probing for histone H3 (H3) and lactate dehydrogenase (LDH) was used as control for proper biochemical fraction separation. Silencing of PKCδ hampers doxorubicin HuR cytoplasmic translocation.

Figure 1D. **PKCδ is necessary to elicit the full apopotic 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 μM doxorubicin (Doxo) and after 24 hours of PKCδ silencing (Si PKCδ). PKCδ 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 PKCδ. 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 siPKCδ and in the presence of or absence of doxorubicin treatment (10 μM 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 PKCδ. Mutants rHuR were not phosphorylated during doxorubicin treatment. Lower panel: representative western blot showing the level of silencing of PKCδ.

Figure 3 PKCδ modulates HuR post-transcriptional functionality.

Figure 3A. PKCδ 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

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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 µM after starvation, after genetic ablation of PKCδ and starvation or starvation and treatment with doxorubicin 10 µM. 18S is the loading and immunoprecipitation control

Figure 3B. PKCδ 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 PKCδ. 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 PKCδ and HuR are coordinately regulated to develop full doxorubicin resistance.

Figure 4A. **PKCδ, 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-PKCδ, 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, PKCδ and ABCG2 protein levels were measured and normalized to the actin level. ABCG2 is

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

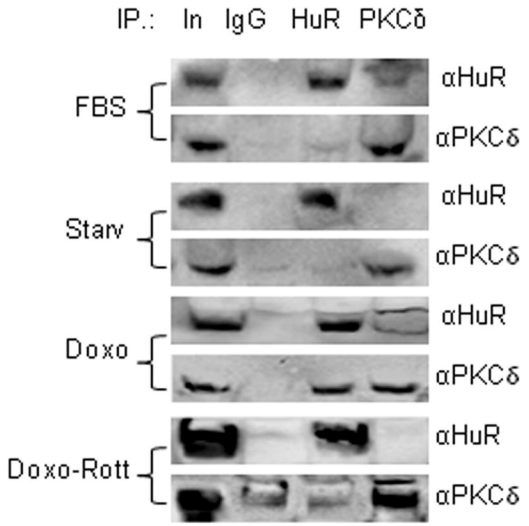
Figure 5. PKCδ and HuR reactivates the doxorubicin induced apoptotic response in MCF-7 doxorubicin resistant cells.

Figure 5A. Ectopic expression of PKCδ 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 μM doxorubicin (Doxo). DR cells were transfected with empty vector, (-), PKCδ, 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 PKCδ in MCF-7 and MCF-7 DR. Representative western blot of the expression level of PKCδ in MCF-7 and MCF-7 DR cells after ectopic expression of the kinase. The blot shows the decrease of the expression level of PKCδ 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



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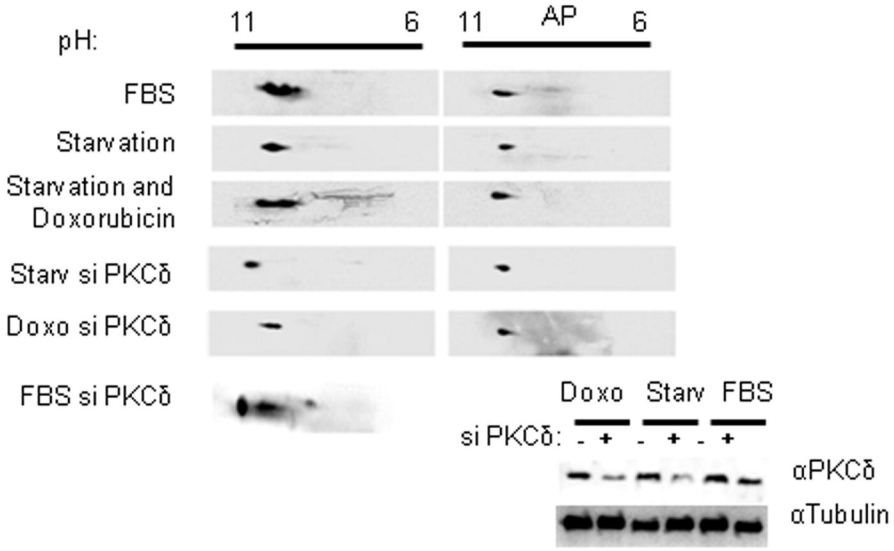

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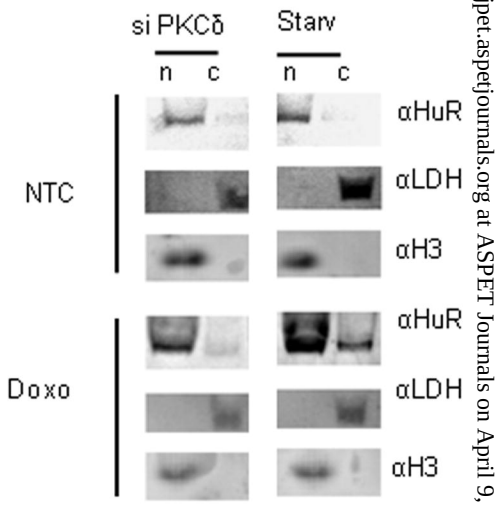

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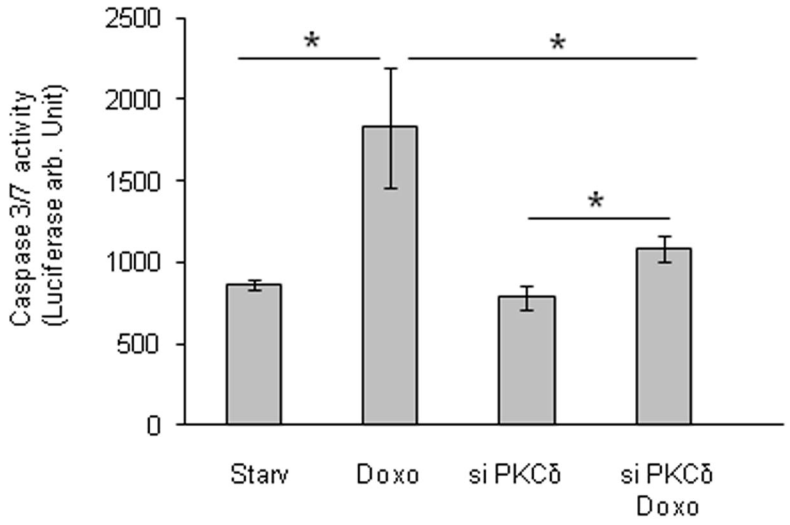

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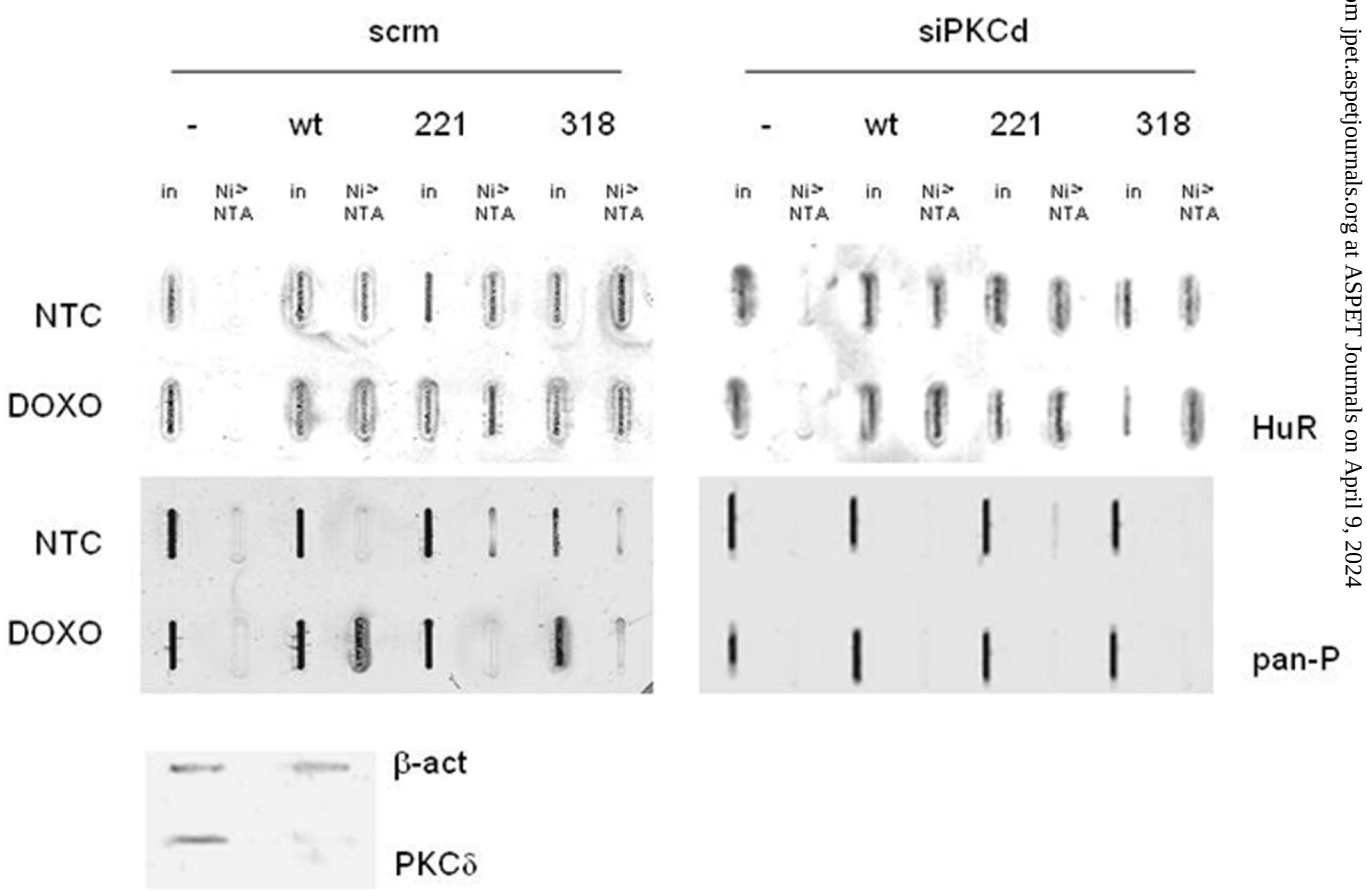

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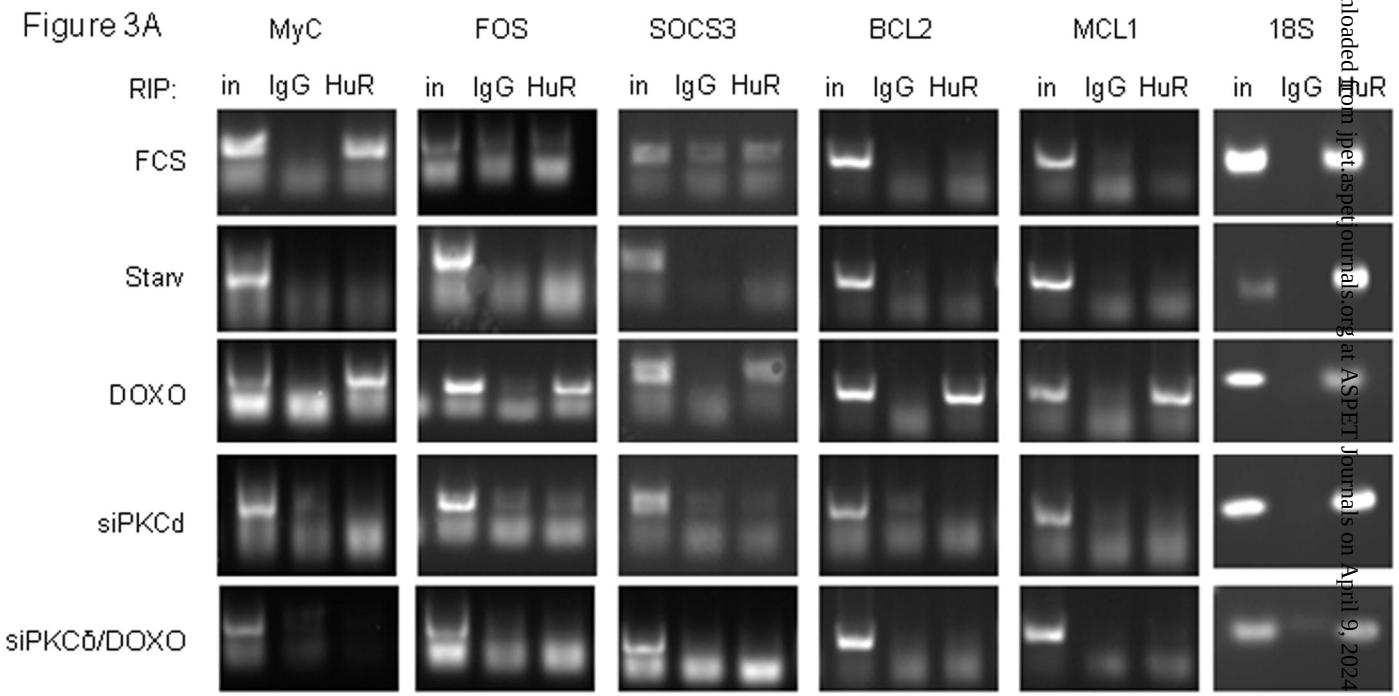

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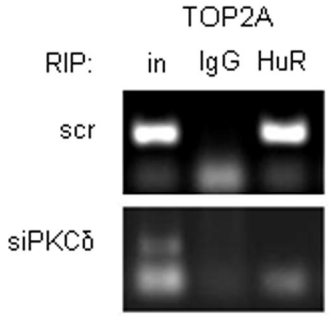

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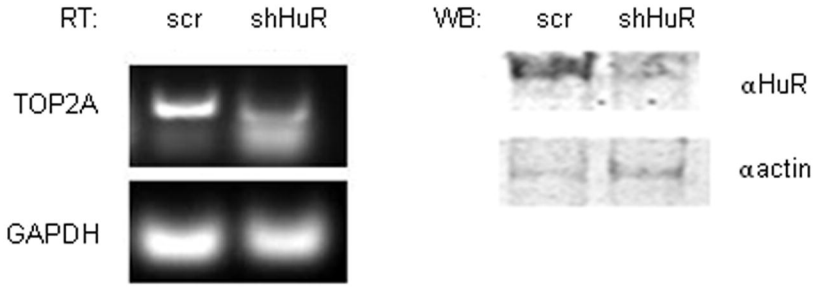

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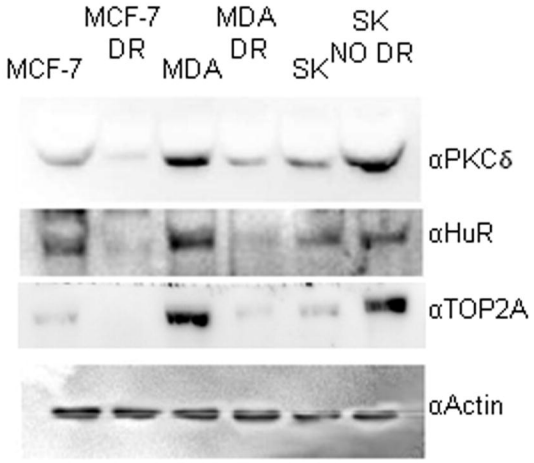

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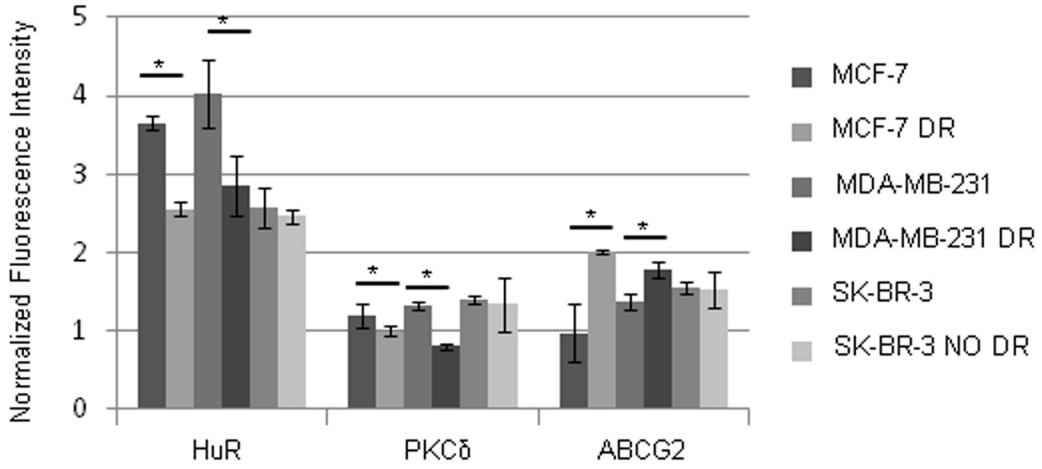

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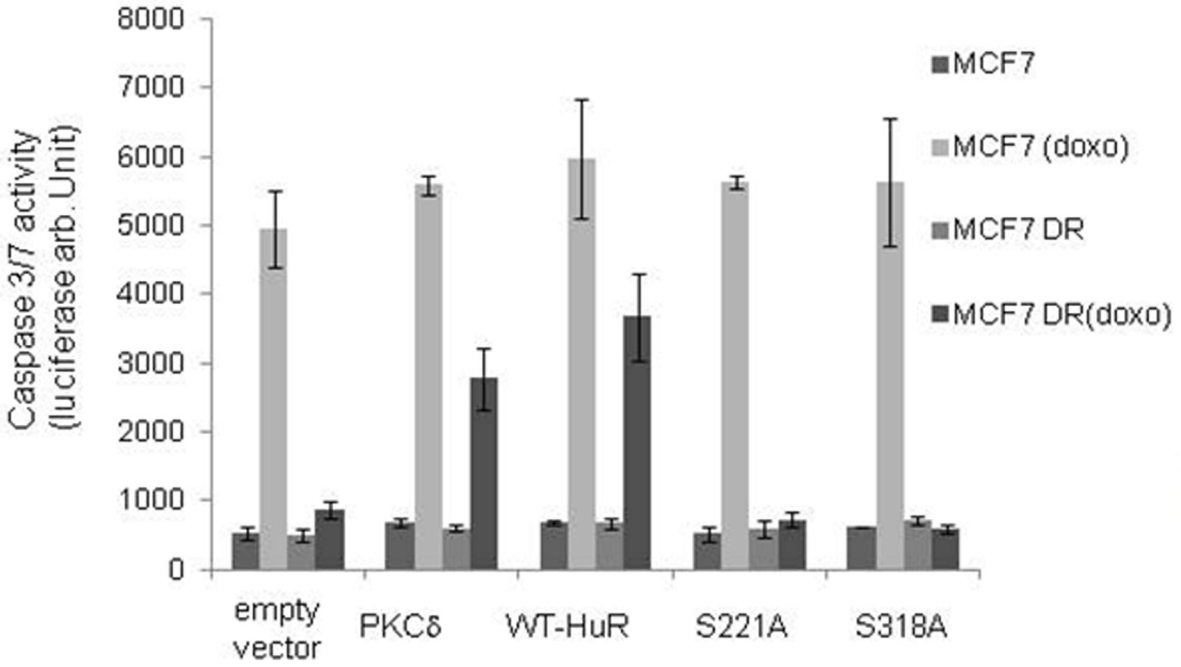

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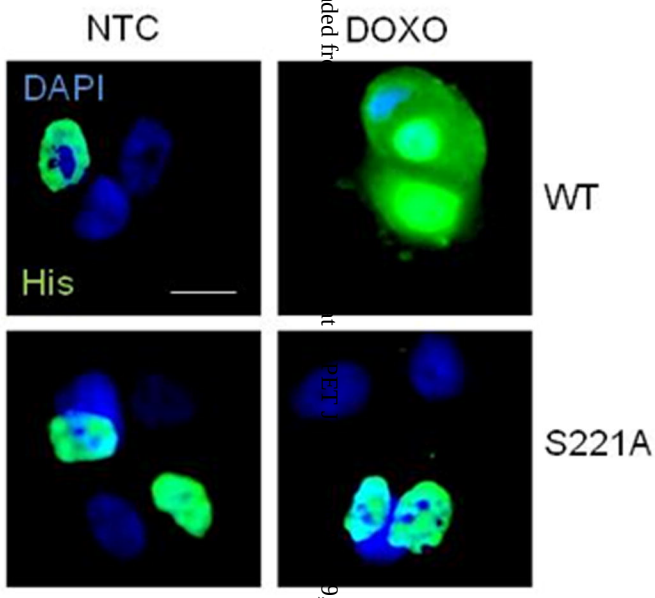

Figure 5B