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ERp29 regulates response to doxorubicin by a PERK-mediated mechanism

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ABSTRACT

ERp29 is an endoplasmic reticulum (ER) luminal protein with a putative secretion factor/escort chaperone function. Accumulated evidence has implicated ERp29 in the thyroglobulin secretion, polyoma virus transport and recently in carcinogenesis. ERp29 levels were elevated in the tumors of various origins and under the conditions of genotoxic stress, such as ionizing radiation. Here we report the induction of ERp29 during the treatment of cells with doxorubicin, a commonly used antineoplastic agent. Experiments in the p53 −/− cells and p53 knockout mouse revealed that doxorubicin effect on ERp29 is p53 dependent. The increase of ERp29 level appears to activate a negative feedback loop where the elevated amounts of ERp29 augment cell viability as shown by a clonogenic cell survival assay. To elucidate the mechanisms behind the doxorubicin effects we have studied the impact of ERp29 on the interaction with the ER stress-activated eukaryotic translation initiation factor 2-alpha kinase 3 (PERK) that was shown to facilitate tumor cells' resistance to drug toxicity. Co-immunoprecipitation demonstrated physical interaction of ERp29 with PERK and moreover, over-expression of ERp29 enhanced endogenous levels of PERK. Our results identify ERp29 as a novel regulator of PERK and provide evidence for the role of ER resident factors in the regulation of chemotherapeutic efficacy. These findings show that PERK may represent a nodal point between ER stress and chemotherapeutic response.

1. Introduction

ERp29 is a relatively small (29 kDa) protein localized in the luminal compartment of the ER. Despite the structural resemblance to the protein disulfide isomerases (PDI) as manifested by the thioredoxin-like N-terminal domain, ERp29 has no characteristic disulfide isomerase or chaperone activity [1]. It is ubiquitously expressed with the highest expression in the secretory cells [2]. Such expression profile and also involvement in the secretion of thyroglobulin and other secretory proteins suggest a possible secretion factor/escort chaperone function [3]. Recent evidence also implicated ERp29 in pathological processes, such as polyoma virus infection and tumorigenesis, roles that are most likely dependent on its endogenous secretion function [4–6].

The ER is often challenged by the rapid accumulation of misfolded proteins (ER stress) caused by a variety of pathological or physiological impacts. The ER protein folding and secretory capacity is regulated by the adaptive cellular mechanism termed unfolded protein response (UPR). UPR is triggered upon activation of the three ER stress sensor molecules, transcription factor ATF6 and two receptor kinases, IRE1 and eukaryotic translation initiation factor 2-alpha kinase 3 (PERK) [7]. The combined effect of their activation is a temporary inhibition of the protein translation (PERK), induction of molecular chaperones and other prosurvival factors implicated in the protein folding, secretion and even protein degradation (ATF6 and IRE1) [7].

ERp29 was proposed to be involved in the UPR as a factor facilitating transport of newly synthesized secretory proteins. The increased expression of ERp29 was demonstrated in the certain cell types both under the pharmacologically induced UPR and under the physiological conditions (lactation, differentiation of thyroid cells) [6,8]. In addition to UPR, ERp29 expression has been recently shown to be enhanced by yet another disturbance of cell homeostasis, namely genotoxic stress. As shown by Zhang et al. [9] ERp29 levels were elevated in the cells exposed to the ionizing radiation, a known DNA damaging factor. Cells have evolved an adaptive response system to such genotoxic impacts that includes cell cycle arrest, activation of the DNA repair or, in the case of irreparable damage, apoptosis [10]. These mechanisms are also activated upon administration of the widely used antineoplastic agent, doxorubicin (DOX) that acts via the intercalation of DNA and subsequent activation of the tumor suppressor p53 [10]. Interestingly, although UPR is mostly a prosurvival adaptive response, the excessive ER stress may also activate the apoptotic pathway, similar to the genotoxic stress response, and is largely dependent on PERK [11]. PERK activation results in the inhibition of protein translation due to the phosphorylation of a general translation factor eIF2α but also in the initiation of a pro-apoptotic program that includes activation of CHOP/GADD153 transcription factor and Bim [11,12]. In addition, induction of PERK pathway may result in the cell cycle arrest caused by the inhibition of synthesis of cell cycle regulators, such as cyclin D1 [13].

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In the present study we sought to answer the question if ERp29, a protein whose expression is increased under UPR, and which was proposed to play a protective role in genotoxic stress [9], might be involved in a common for these two pathways mechanism(s). Indeed, here we demonstrate that, ERp29 is upregulated in response to the administration of the anticancer agent doxorubicin in a p53-dependent manner, interacts physically with PERK and confers resistance to DOX.

2. Materials and methods

2.1. Cell culture, transfection and treatment with DOX

A549 human lung epithelial adenocarcinoma cells and PC-3 prostate adenocarcinoma cells were maintained in DMEM containing 10% FBS and antibiotics/antimycotics. MEFs were isolated at E11.5 from female p53 heterozygous mice that have been mated with heterozygous males, by using standard procedures and were subsequently genotyped to assess the p53 zygosity status as previously described [14].

For transient transfection experiments, the cells were seeded 24 h prior to transfection and transfected by using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested and analyzed 48 h later, if not otherwise stated. For immunoblot analysis, binding and survival assays, cells were transiently transfected with empty pcDNA 3.1 plasmids (mock) or plasmids encoding for ERp29 (pcDNA3.1-ERp29) [15], pcDNA3.1-ERp29/C157 (a mutant of ERp29 that was suggested to operate as a dominant-negative form of the protein) [15], PERK (pcDNA1-PERK), mutant PERK (pcDNA1-PERK-K618A dominant negative mutant, kindly provided by Dr Antonis Koromilas) [16] or siRNA for ERp29 (sc-60599, Santa Cruz). siRNA transfection was performed using Lipofectamin 2000 reagent (Invitrogen). For p-eIF2α detection A549 cells were transiently transfected with pcDNA3.1-ERp29 and pcDNA3.1-ERp29/D42A (a mutant of ERp29 that fails to dimerize) [17], harvested and analyzed 4 or 8 h later.

For immunoblot analysis, RT-PCR analysis, binding and survival assays, cells were treated with 0.1–0.2 μM DOX (Sigma) or vehicle (DMSO) for 24 h. For UPR induction control, cells were treated with 5 μg/ml Tunicamycin (Sigma) for 4 or 8 h.

2.2. RT-PCR analysis

RNA was isolated using Trizol Reagent (Invitrogen), according to the manufacturer's instructions. cDNA was obtained after a two-step reaction using AMV-RT (Promega). Detailed PCR conditions have been described previously [18].

2.3. Western blot analysis

For immunoblot analysis cells were solubilized with ice-cold RIPA buffer (Thermo Scientific) supplemented with protease inhibitor

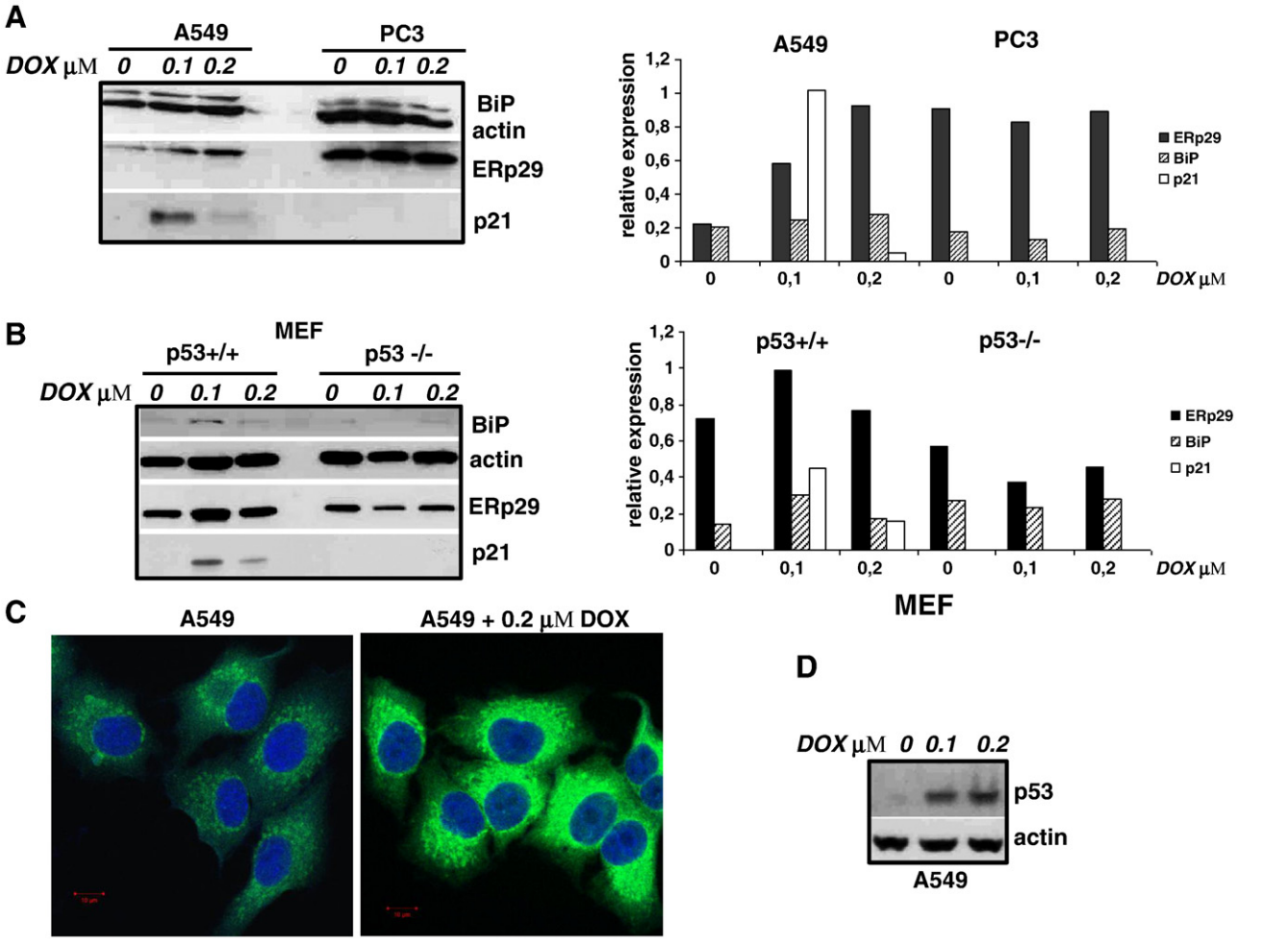

Fig. 1. p53-dependent induction of ERp29 by DOX. DOX induces ERp29 protein levels as detected by immunoblot analysis in the p53 positive cells, such as MEFs and A549 lung cancer cells but not in p53-null cells, such as PC3 prostate adenocarcinoma cells and p53-null MEFs. The densitometric quantification of these results (normalized to the actin levels) is shown adjacent to each immunoblot (A, B, and C). C. ERp29 is shown in green and nuclei were identified by DAPI (blue). D. Induction of p53 expression in A549 cells following DOX exposure.

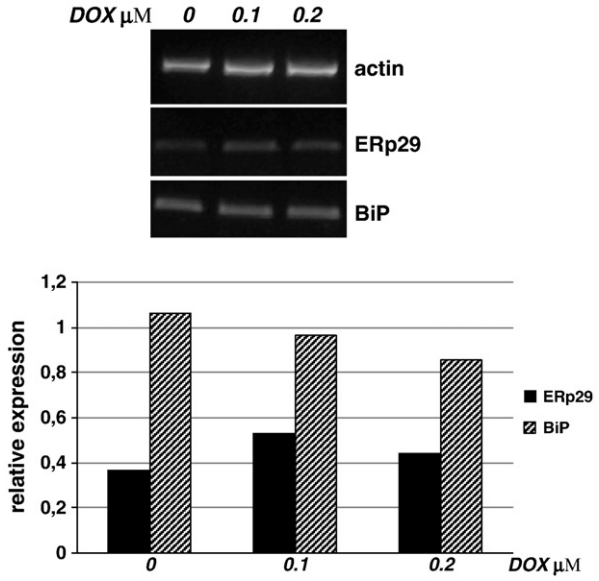

Fig. 2. RT-PCR analysis of ERp29 and BiP mRNA expression after DOX treatment in A549 cells. cDNA from A549 cells was amplified using gene specific primer pairs in 25 cycles. PCR fragments were resolved on EtBr agarose gel (upper panel). Lower panel represents results of the densitometric analysis of ERp29 and BiP PCR fragments after normalization to the actin levels.

cocktail (Thermo Scientific). The protein concentration in the lysates was determined by using Bradford assay (Biorad). Equal amounts of total protein were resolved by SDS-PAGE and immunoblotted with anti-ERp29 [3], anti-BiP (NeoMarkers), anti-p21 (sc-6246; Santa Cruz), anti-PERK (sc-13073; Santa Cruz), anti-p53 (sc-71817; Santa Cruz), anti-p-eIF2α (#33985; Cell Signaling), anti-eIF2α (sc-11386; Santa Cruz) and anti-actin (Sigma). Relative protein amounts were evaluated by a densitometric analysis using ScienceLab software (Fujifilm). Levels of expression are shown in arbitrary units and normalized to the corresponding actin levels, unless otherwise stated. All experiments have been performed at least 3 times and representative results and the corresponding quantification data of one experiment are shown.

2.4. Immunofluorescent microscopy

A549 cells were treated with 0.2 μM DOX or vehicle for 24 h, fixed and stained as described previously [3]. Images were analyzed using LSM T-PMT confocal microscope (Zeiss) and 60X oil immersion objective.

2.5. Immunohistochemistry

Livers were obtained from 8 weeks old wild type or p53-deficient mice that were treated with a single i.p. injection of DOX (4 mg/kg) or vehicle (DMSO) for 24 h. Liver sections (4-μm-thick) from paraffin-embedded blocks were collected onto poly-L-lysine-coated slides and stained with anti-ERp29 [3] by using the SuperPicture HRP Polymer Conjugate Broad Spectrum (DAB) kit (Zymed Laboratories Inc.), according to the manufacturer's instructions and counterstained with hematoxylin.

2.6. Immunoprecipitation

A549 cells or MEFs after treatment with DOX or transfection as described in the Results' section were lysed in a buffer containing 50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 1 mM DTT), protease inhibitor cocktail (Thermo Scientific), spanned down and the supernatant was immunoprecipitatated with anti-PERK (sc-13073; Santa Cruz) for overnight. Protein A/G beads (sc-2003; Santa Cruz) were used to isolate the antibody–protein complexes, which were washed twice with lysis buffer, resuspended in 40 μl of SDS buffer and immunoblotted with anti-ERp29 [3], anti-BiP (NeoMarkers), anti-PERK (sc-13073; Santa Cruz) and anti-actin (Sigma).

2.7. Clonogenic cell survival assay

For clonogenic survival assays cells were transiently transfected with pcDNA3.1-ERp29 and pcDNA1-PERK/K618A. Co-transfection of pcDNA1-PERK/K618A and pcDNA3.1-ERp29 was performed at 3:1 molar ratio. Since no antibiotic resistance is conferred by the pcDNA1 plasmid, co-tranfection with empty vector pcDNA3.1 has been performed in a manner that all experimental sets have received equimolar amount of pcDNA3.1 or pcDNA3.1-ERp29. In order to diminish the consequences of non-specific DNA-mediated toxicity, total DNA for each transfection was adjusted to the final amount of 2 μg by using the plasmid pUC19. 24 h post transfection, cells were treated with 0.2 μM DOX or vehicle (DMSO) for 24 h, selected in the presence of 1 mg/ml of G418 for 10 days and counted using the trypan blue exclusion assay.

3. Results

3.1. p53-dependent induction of ERp29 by doxorubicin

In view of a recent report that identified ERp29 as a radiation-induced gene [9], we have asked if genotoxic stress, such as that elicited by the chemotherapeutic agent DOX [10] may also stimulate ERp29 expression. Indeed, exposure of the human lung cancer cells A549 and wild type MEFs to DOX, resulted in the enhancement of ERp29 both on protein (Fig. 1) and mRNA (Fig. 2) levels. Semi-quantitative RT-PCR analysis in A549 cells suggested that the mRNA increase was not as pronounced as the one observed at the protein level (~0.5-fold and 0.2-fold higher levels than those of the controls as opposed to ~2-fold and 5-fold induction for 0.1 μM and 0.2 μM DOX respectively). This may reflect the existence of the additional post-transcriptional mechanisms of ERp29 regulation (Fig. 2). In order to discriminate between genotoxic stress and UPR we have tested same cells for the expression of the abundant ER chaperone BiP, the primary indicator of UPR [19]. BiP levels remained essentially unaffected (Fig. 1A,B), despite some induction in the MEFs exposed to 0.1 μM doxorubicin (Fig. 1B). As DOX-mediated DNA damage leads to the activation of the major tumor suppressor p53 [10], we have monitored the p53 activity during DOX treatment by

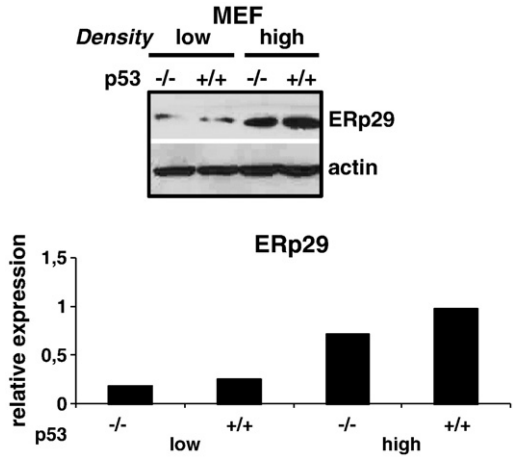

Fig. 3. Cell density mediated regulation of ERp29 expression is independent of p53. Western blot detection of ERp29 expression in the MEFs from wild type or p53-null mice cultured under conditions of low density (lower than 40%) and high density (above 95%).

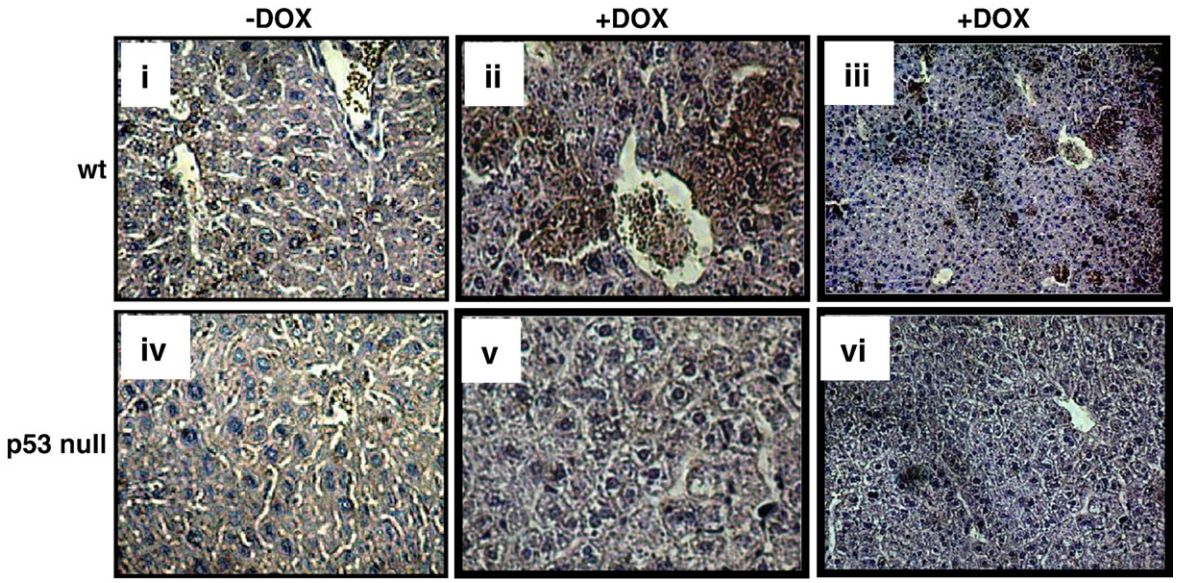

Fig. 4. DOX increases ERp29 expression in the livers of wild type but not the p53 null mice. Liver sections were stained with anti-ERp29 and counterstained with hematoxylin. i,ii,iii, ERp29 immunoreactivity in the livers of 8 weeks old wild type or p53-deficient (iv,v,vi) mice [22]. iii and vi are low resolution microphotographs of sections shown in ii and v. DOX (4 mg/kg) was administered 24 h prior to the sacrifice of mice.

assessing the levels of its downstream target p21 [20]. The increased amounts of p21 (Fig. 1A,B) and p53 (Fig. 1D) confirmed that the cells underwent a canonical DOX-dependent genotoxic stress response with the activation of p53 and its downstream targets. Next we have decided to test whether the DOX-mediated ERp29 expression is dependent on p53. Indeed, in the p53 deficient human PC3 prostate cancer cells [21] and in the MEFs isolated from p53-null mice [22], DOX failed to induce the expression of ERp29 (Fig. 1A,B). It is of note that the patterns of DOX-

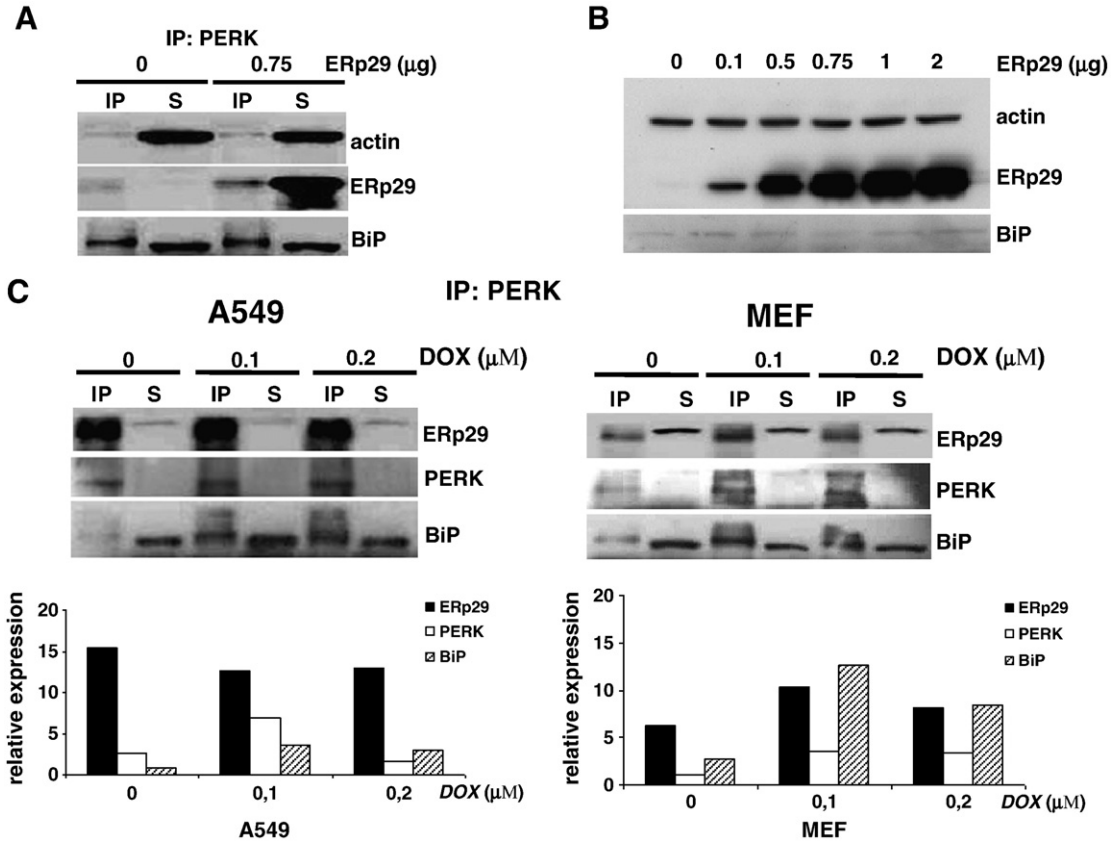

Fig. 5. Physical interaction of PERK and ERp29. **A.** ERp29 co-immunoprecipitates with PERK in A549 cells **B.** Immunoblot analysis of A549 cells for BiP and ERp29 following transfection with increasing amounts of ERp29-expressing plasmids. **C.** Immunoprecipitation of A549 cells and MEFs by anti-PERK antibody following the exposure of cells to DOX at 0.1 and 0.2 μM for 24 h. Amounts of plasmid are shown above the immunoblots. Actin levels were used as a loading control. Densitometric quantification of IP bands is shown at the bottom of each immunoblot image; IP, immunoprecipitate; S, supernatant.

dependent ERp29 and p21 expression are not identical, implying that p53 is apparently not the sole regulator of ERp29 following the DOX exposure.

In addition, these *in vitro* findings were supported by the increased ERp29 immunoreactivity that was detected in the livers of wild type but not of p53-deficient mice after the administration of DOX (Fig. 4). ERp29 immunoreactivity was observed in clusters of hepatocytes, an observation that probably reflects the bioavailability of DOX in the livers of the experimental animals. Thus, an intact p53 response is essential for the upregulation of ERp29 following the genotoxic stress as induced by DOX.

We have shown earlier that ERp29 expression positively correlates with the cell culture density [6]. It was therefore interesting to test whether this effect might also be dependent on p53. However, manipulation of p53 genetic status in MEFs had no effect on ERp29 expression (Fig. 3), reflecting apparently a different role of p53 for the regulation of ERp29 by cell culture density and DOX.

3.2. ERp29 interacts with PERK

As mentioned earlier, it is conceivable that both UPR and genotoxic stress response may share, at least in part, some regulatory mechanisms. A likely candidate may be the critical UPR sensor, PERK. We have explored this hypothesis by testing initially if ERp29 interacts physically with PERK. Indeed, immunoprecipitation (IP) studies in A549 cells that are able to execute an canonical UPR and also express high endogenous PERK levels [23], confirmed that ERp29 binds to PERK (Fig. 5A), in line with a previous suggestion by Park et al. [24]. Interestingly, the amounts of ERp29 bound to PERK were only marginally increased upon the transfection with the increasing amounts of ERp29 encoding plasmids , suggesting that at least in resting conditions nearly all PERK molecules are occupied by ERp29 (Fig. 5A). Since in quiescent cells PERK (as well as the other UPR sensors) is usually silenced by the physical interaction with BiP [19] we have tested whether ERp29 may interfere with this mechanism. Overexpression of ERp29 demonstrated that this is not the case, ruling out the possibility that ERp29 may interfere directly with the binding of BiP to PERK (Fig. 5A,B). When, however, cells were exposed to DOX that stimulates the ERp29 expression (Fig. 1A,B, and C), the amount of BiP that was bound to PERK was higher in both A549 cells and in MEFs (Fig. 5C). Since BiP levels were not affected by ERp29 (Fig. 5B) we hypothesized that ERp29 might somehow affect the total levels of PERK. Indeed, as shown on Fig. 6A, ERp29 overexpression enhanced the total PERK levels, while suppression of ERp29 expression by siRNA or by a dominant negative form of the protein resulted in the reduction of PERK. In line with these data DOX treatment elevated the total PERK levels as evidenced by the increased amount of immunoprecipitated PERK following the exposure of MEFs, and to a lesser extent of A549 cells, to DOX (Fig. 5C). The existence of the reverse regulatory loop, namely modulation of ERp29 expression by PERK, is a less likely scenario since the overexpression of neither wild type nor mutant PERK had any effect on the ERp29 levels in A549 cells (Fig. 6B).

The ERp29-driven increase of PERK levels was detected on the protein level (Fig. 6A), while quantitative PCR data failed to demonstrate a similar trend implying the involvement of other, most probably post transcriptional mechanisms (data not shown).

In order to test whether ERp29 may modulate not only the levels of PERK but also its main activity, i.e. the phosphorylation of eIF2α, we have immunoblotted A549 cells for the phosphorylated form of eIF2α, 4 h and 8 h after the transfection with wild type and mutant ERp29 plasmids. Indeed, 8 h after the transfection the phosphorylation of eIF2α was positively affected by the wild type ERp29 whereas the transfection of the mutant ERp29 resulted in the decrease of p-eIF2α (Fig. 6C). Given the relative weakness of the wild type ERp29 effect, this observation can be interpreted as that ERp29 is needed but not sufficient for PERK activation.

While BiP expression was not affected by DOX (Fig. 1A,B) it increased the binding of BiP to PERK (Fig. 5C) in both A549 cells and MEFs. It could

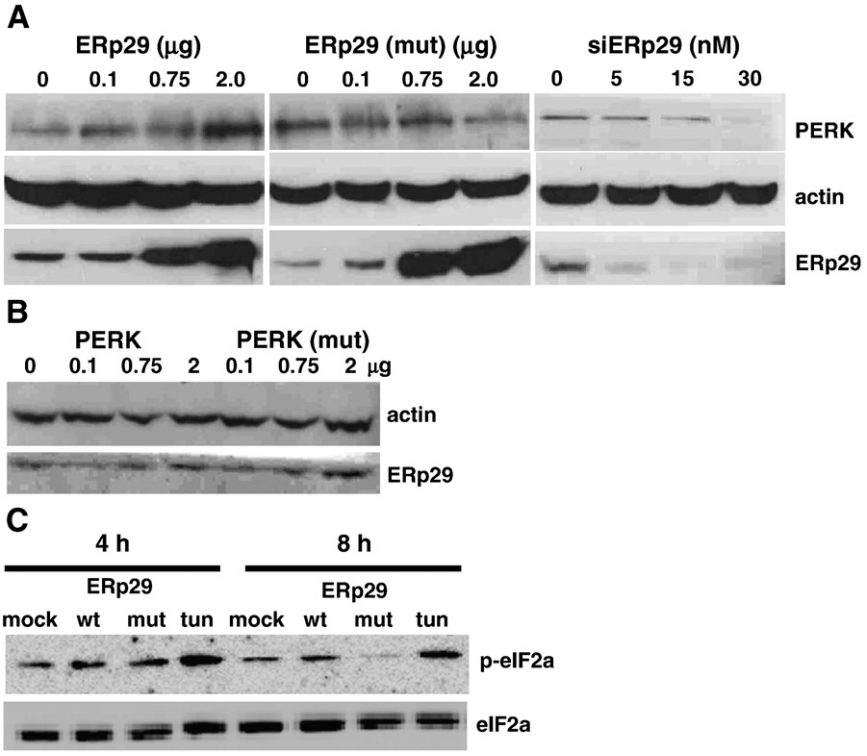

Fig. 6. ERp29 affects PERK levels. A. Immunoblot analysis of PERK and ERp29 following the transfection of A549 cells with pcDNA3.1-ERp29, pcDNA3.1-ERp29/C157 or siRNA for ERp29. B. Immunoblot analysis of ERp29 following the transfection of A549 cells with pcDNA1(mock), pcDNA1-PERK and pcDNA1-PERK/K618. Amounts of plasmid or siRNA are indicated above the immunoblots. C. Phosphorylation of eIF2α following the transfection of A549 cells with 2 μg of pcDNA3.1(mock), pcDNA3.1-ERp29 and pcDNA3.1-ERp29/D42A for 4 or 8 h. Actin or total eIF2α levels were used as a loading control. Tun, tunicamycin.

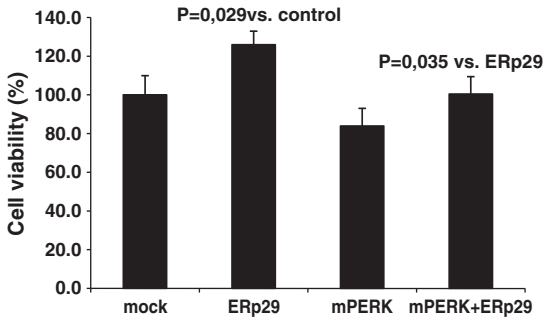

Fig. 7. ERp29 reduces the sensitivity of A549 cells to DOX in a PERK dependent manner. Clonogenic cell survival assay of A549 cells following the exposure of cells to DOX. Cell viability assays were performed in triplicates and average values +/− standard error of mean are shown on the graph. ERp29 overexpression increased the number of cells by about 20% vs. controls while mPERK restored sensitivity of the cells against DOX. P values (student's *t*-test) are shown.

be argued that ERp29 interferes with BiP binding to PERK. However, since plasmid-mediated overexpression of ERp29 was insufficient to increase total BiP levels, as well as the amount of BiP bound to PERK (Fig. 5A,B) we suggest that the regulation of BiP binding to PERK during DOX treatment is more complex and does not simply reflect the ERp29-dependent availability of PERK.

3.3. ERp29 regulates cell viability after DOX treatment

In an attempt to explore the physiological relevance of ERp29 stimulation by DOX we have examined the consequences of ERp29 overexpression on the viability of cells treated with DOX. ERp29 reduced the sensitivity of A549 cells to DOX as shown by clonogenic cell survival assay (Fig. 7) suggesting that ERp29 confers resistance to DOX. Furthermore, this effect was abolished by the overexpression of a mutant form of PERK, incapable of phosphorylating eIF2α [16] (Fig. 7) suggesting that the ERp29-dependent regulation of cell viability is mediated, at least in part, by PERK. These results are also consistent with the recently reported anti-apoptotic activity of ERp29 in cancer cells [25].

4. Discussion

The involvement of ER stress signaling in the cell survival during chemotherapy remains poorly explored. Present study suggests that the genotoxic stress response and UPR may have a common nodal point, namely one of the major sensors of UPR, PERK. Moreover, there are indications that PERK might be involved in the regulation of the therapeutic efficacy of DOX via the interaction with the putative secretion factor/escort chaperone ERp29, the assumption that is currently examined in more details in our laboratory.

Although ERp29 does not appear to affect PERK- and eventually UPR-activation *per se*, by increasing the amount of total PERK, it appears that ERp29 facilitates cell's response to genotoxic stress that ultimately results in the resistance against chemotherapy by DOX. This notion is also supported by the observation that while eIF2α phosphorylation, an immediate target of PERK activation, is not significantly stimulated by plasmid-mediated expression of ERp29 but a mutant form of this protein inhibited the phosphorylation of eIF2α. The latter is not consistent with an instructive but rather with a permissive role for ERp29 in the regulation of PERK activity.

The induction of ERp29 by DOX indicates that the above mentioned mechanism is physiologically relevant and is consistent with the operation of a cytoprotective cellular response that is triggered by chemotherapy and is modulated by ERp29. Interestingly, a recent study has shown that PERK regulates the p53/47 isoform of p53 leading to G2 arrest [26]. This finding in combination with our results implies the presence of a regulatory loop between PERK and p53 that apparently optimizes the cell survival: prosurvival effects triggered by DOX and mediated by ERp29 are compensated by the specific induction of p53/47-that causes G2 arrest.

The fact that ERp29-driven increase of PERK levels is not translated into the activation of UPR, rules out the possibility that ERp29 may directly regulate UPR via PERK. We have however noted that in the clonogenic assay the phosphorylation mutant form of PERK increased the sensitivity of A549 cells to DOX. Thus, the intact PERK activity is associated with the resistance against DOX, which is consistent with the elevation of PERK levels following the ERp29 overexpression. BiP overexpression has been associated with increased chemoresistance [27] and resistance to drug-induced apoptosis through the activation of PERK pathway [28] as well as the suppression of UPR [29,30]. Indeed, increased binding of BiP to PERK was observed during the treatment of cells with DOX, however, total BiP levels remained unaffected by DOX or by exogenous ERp29 expression. Thus, the resistance to DOX conferred by ERp29 is not likely to be related directly to BiP in particular or to UPR activation in general. In agreement with this notion, the modulation of chemoresistance by BiP may be related to alternative functions of this chaperone, other than the regulation of UPR, as proposed earlier [31].

5. Conclusions

In summary, in the present study we have identified ERp29 as a specific regulator of PERK during DOX treatment and showed that ERp29 expression is causally linked to resistance against DOX by a mechanism that requires PERK. These findings, not only expand our limited knowledge regarding ERp29, but also provide a direct link between the cellular response against genotoxic stress and the execution of UPR, identifying PERK as a nodal point between ER and genotoxic stress induction. The evaluation of ERp29 activity may be of importance for predicting the efficacy of the chemotherapeutic response while its specific modulation may increase the efficiency of anticancer therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2011.03.003.

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