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Induction of heparanase 2 (Hpa2) expression by stress is mediated by ATF3

Ibrahim Knani[1], **Preeti Singh**[1], **Miriam Gross-Cohen**[1], **Sharon Aviram**[1], **Neta Ilan**[1], **Ralph D Sanderson**[2], **Ami Aronheim**[1], **Israel Vlodavsky**[1,*]

[1]Technion Integrated Cancer Center, Rappaport Faculty of Medicine, Technion, Haifa, Israel

[2]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Abstract

Activity of heparanase, endoglycosidase that cleaves heparan sulfate side chains in heparan sulfate proteoglycans, is highly implicated in tumor progression and metastasis. Heparanase inhibitors are therefore being evaluated clinically as anti-cancer therapeutics. Heparanase 2 (Hpa2) is a close homolog of heparanase that lacks HS-degrading activity and functions as an endogenous inhibitor of heparanase. As a result, Hpa2 appears to attenuate tumor growth but mechanisms that regulate Hpa2 expression and determine the ratio between heparanase and Hpa2 are largely unknown. We have recently reported that the expression of Hpa2 is induced by endoplasmic reticulum (ER) and proteotoxic stresses, but the mechanism(s) underlying Hpa2 gene regulation was obscure. Here we expand the notion that Hpa2 is regulated by conditions of stress. We report that while ER and hypoxia, each alone, resulted in a 3–7 fold increase in Hpa2 expression, combining ER stress and hypoxia resulted in a noticeable, over 40-fold increase in Hpa2 expression. A prominent induction of Hpa2 expression was also quantified in cells exposed to heat shock, proteotoxic stress, lysosomal stress, and chemotherapy (cisplatin), strongly implying that Hpa2 is regulated by conditions of stress. Furthermore, analyses of the Hpa2 gene promoter led to the identification of activating-transcription-factor 3 (ATF3) as a transcription factor that mediates Hpa2 induction by stress, thus revealing, for the first time, a molecular mechanism that underlies Hpa2 gene regulation. Induction of Hpa2 and ATF3 by conditions of stress that often accompany the rapid expansion of tumors is likely translated to improved survival of cancer patients.

Keywords

Heparanase 2; ER stress; hypoxia; cisplatin; gene expression; ATF3

[*]Correspondence should be addressed: Israel Vlodavsky, Technion Integrated Cancer Center, Bruce Rappaport Faculty of Medicine; Technion, P. O. Box 9649, Haifa 31096, Israel, Vlodavsk@mail.huji.ac.il.

Declarations of interest

none

Introduction

Heparanase 2 (Hpa2) was cloned shortly after the cloning of heparanase, based on sequence homology [1]. Despite the close resemblance in nucleotide and amino acids sequences, Hpa2 seems molecularly and functionally very different from heparanase [2, 3]. Most importantly, Hpa2 do not show HS-degrading activity typical of heparanase, but its capacity to bind HS with high affinity is retained [4]. In fact, Hpa2 shows even higher affinity to HS than that of heparanase [4]. The consequence of this high-affinity interaction can lead to inhibition of heparanase activity, due to competition for HS binding [4]. This led to the notion that Hpa2 is endogenous inhibitor of heparanase, and that the ratio of heparanase:Hpa2 should be considered as an important parameter in human disorders [5]. In addition, we have recently reported that Hpa2 promotes the adhesion of normal and tumor-derived cells, and this was compromised by heparin [6]. Also, cell migration was attenuated by Hpa2, and this was reversed by heparin or anti-Hpa2 monoclonal antibodies [6, 7], suggesting that the high-affinity interaction of Hpa2 with HS on the cell surface is translated to molecular and cellular functions. Heparanase and Hpa2 are also very different in the role they play in cancer. Compelling evidence gathered in the last two decades strongly imply that heparanase functions to promote essentially all aspects of the tumorigenic process, namely tumor initiation, growth, metastasis, and chemo-resistance [8–17], pointing to heparanase as a drug target in cancer and encouraging the development of heparanase inhibitors [18, 19]. Unlike the extensive research effort devoted to exploring the role of heparanase in tumor progression and metastasis, very little attention was given to Hpa2 and its role in cancer. Evidence suggests, nonetheless, that Hpa2 functions to attenuate tumor growth. Unlike heparanase, Hpa2 is expressed to relatively high levels in normal epithelium of the bladder, breast, gastric, and ovarian tissues but its expression is decreased substantially in the corresponding carcinomas [3, 20, 21]. This expression pattern is characteristic of tumor suppressors. Furthermore, high levels of Hpa2 were associated with longer survival of head and neck, pancreatic and gastric cancer patients vs patients exhibiting low levels of Hpa2 [4, 22, 23]. Likewise, overexpression of Hpa2 in head and neck and pancreatic carcinoma cells resulted in a prominent decrease in tumor growth, mediated by lower vascular density [24], increased expression of Sox2 [25], and increased ER stress response that, once present constitutively, promote apoptotic cell death [22]. Notably, ER stress was found to induce the expression of Hpa2, thus establishing a feedback loop [22]. Here, we further examined the regulation of Hpa2 expression by conditions of stress. We report that while ER stress (thapsigargin, tunicamycin) and hypoxia, each alone, resulted in a 3–7 fold increase in Hpa2 expression, combining ER stress and hypoxia resulted in a noticeable, over 40-fold increase in Hpa2 expression. A prominent induction of Hpa2 expression was also quantified in cells exposed to heat shock, proteotoxic stress (MG132), lysosomal stress (LLOMe), and chemotherapy (cisplatin), strongly implying that Hpa2 is regulated by conditions of stress. We further subjected the Hpa2 promoter to site-directed mutagenesis and identified activating-transcription-factor 3 (ATF3) as a transcription factor that mediates Hpa2 induction by stress, thus revealing, for the first time, a molecular mechanism that underlies Hpa2 gene regulation.

Materials and methods

Cells, cell culture, immunoblotting and immunofluorescent staining

Human Panc-01 and AsPc-1 pancreatic carcinoma cells and SGC-7901 gastric carcinoma cells have been described previously [22, 23]. HT1080 and HOS sarcoma, HeLa cervical carcinoma, and SKOV-3 ovarian carcinoma cells were purchased from the ATCC. Cells were grown in Dulbecco's modified Eagle medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum and antibiotics. The human cell lines were authenticated in June 2020 by the short tandem repeat (STR) profile of 15 loci plus amelogenin for sex determination (X or XY) method, according to the manufacturer's (Promega) instructions [26]. Cells were passed in culture for no more than 2 months after being thawed from authentic stocks. ATF3-null HEK293 cells were generated by CRISPR methodology; mouse embryonic fibroblasts (MEF) isolated from ATF3-KO mice have been described previously [27, 28].

Preparation of cell lysates, immunoblotting and immunofluorescent staining were performed as previously described [22, 23, 29].

Antibodies and reagents

Anti-heparanase polyclonal antibody was purchased from Prospec (Rehovot, Israel). Anti-Hpa2 polyclonal (Ab 58) and monoclonal (20c5) antibodies have been described previously [4]. Anti-GRP78 (Bip), anti-CHOP and anti-ATF3 (ab254268) antibodies were purchased from Abcam (Cambridge, UK). Anti-AFT4 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz CA); Anti-HIF1-α antibody (610958) was purchased from BD Transduction Laboratories. Anti-ATF3 polyclonal antibody has been described previously [28]. Anti-actin antibody, thapsigargin, tunicamycin, MG132, Cisplatin, PERK inhibitor GSK2606414, and L-leucyl-L-leucine methyl ester (LLOMe) were purchased from Sigma. Tissue arrays of cervical and hepatocellular carcinomas were purchased from US Biomax (Rockville, MD).

Conditions of stress

Cells were plated in 6-well plates and were allowed to adhere for 24 hours. Plates were then switched to serum-free medium and were exposed to thapsigargin (1.5 uM), hypoxia (2% oxygen), or both for 24 hours. Cells were also subjected to tunicamycin, heat shock (42°C), MG132, or LLOMe for the indicated times and concentrations. Total RNA and protein extracts were then prepared and subjected to qPCR and immunoblotting.

Real time-PCR

Quantitative real-time-PCR (qPCR) analyses were performed using ABI PRISM 7000 Sequence Detection System employing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), essentially as described [22, 30, 31]. Expression of Hpa2 is presented as mean level (±SE) relative to control cells (Con), set arbitrarily to a value of 1, and calculated after normalization to actin. The primer sets utilized in this study are summarized in Suppl. Table 1.

EMSA

Electro-mobility shift assay (EMSA) was performed essentially as described [32]. Briefly, DNA probe was generated by annealing 5' IRDye®700 labeled forward oligonucleotides with unlabeled reverse oligonucleotides (Integrated DNA Technologies) (GCCAGCATGTAT**TGACGC**TCCCAAGACG-labelled and complementary CGTCTTGGGAGCGTCAATACATGCTGGC-unlabelled) to a final concentration of 5 μM in PNK buffer (New England Biolabs). One hundred femtomolar of labeled IRDye®700 probes were used in a 20-μl binding reaction containing 10 mM Tris, pH 7.5; 50 mM NaCl; 1 mM MgCl2; 4% glycerol; 0.5 mM DTT; 0.5 mM EDTA; 50 μg/ml poly(dI–dC); 200 μg/ml of BSA and the test sample. Following incubation (30 min, room temperature), the reaction mixture was subjected to electrophoresis on a native 4% polyacrylamide gel and band shift was visualized by Odyssey Infrared Imaging System. For competition assays, the indicated amount of cold competitor was added with the IRDye®700-labeled probe prior to the incubation.

Plasmids and gene constructs

A gene construct containing 1.4 kb of the HSPE2 gene promoter coupled to Gaussia luciferase (Gluc) was purchased from GeneCopoeia (Rockville, MD). Parts A (506bp), Part B (498bp) and Part C (482bp) of the promoter were generated by PCR using the primers sets summarized in Suppl. Table 2, and were cloned with appropriate restriction enzymes. Deletion constructs of the reporter gene were generated by site-directed mutagenesis, essentially as described previously [28]. The PCR products were assured by gel electrophoresis and DNA sequencing and were inserted upstream of the Gaussia luciferase (Gluc) reporter gene. ATF3 gene construct has been described previously [28]; ATF3-(1–100) gene constructs were kindly provided by Dr. Tsonwin Hai (Ohio State University, Columbus, Ohio) [33].

Statistics

Data are presented as means ± SE. Statistical significance was analyzed by the 2-tailed Student's *t*-test. Values of $p<0.05$ were considered significant. Data sets passed D'Agostino-Pearson normality (GraphPad Prism 5 utility software). All experiments were repeated at least 3 times with similar results.

Results

Hpa2 expression is induced by conditions of stress

Expression of Hpa2 varies considerably among tissues [34]. Unlike heparanase, Hpa2 is detected in normal epithelia but its expression is often decreased substantially in the respective carcinomas [3, 20, 21, 23], resulting in a high heparanase:Hpa2 ratio. We hypothesized that conditions of hypoxic stress that often characterize fast-growing tumors, induce Hpa2 expression when applied alone, and in combination with other forms of stress conditions, thus tilting the heparanase:Hpa2 ratio in favor of Hpa2. To examine this possibility we exposed cancer cells to hypoxia (2% O_2), ER stress (thapsigargin, 1.5 uM), or both. Notably, while thapsigargin and hypoxia, each alone, resulted in a, 3–7 fold increase

in Hpa2 expression, cells insulted by thapsigargin and hypoxia simultaneously revealed a noticeable, 40-folds increase in Hpa2 expression. This was evident by qPCR (Fig. 1A, Suppl. Fig. 1B), immunofluorescent staining (Fig. 1B), and immunoblotting (Fig. 1C) of pancreatic carcinoma (Panc-01, AsPC-1), sarcoma (HT1080) and cervical carcinoma (HeLa) cells. Similar pattern of Hpa2 induction was noted by the combination of tunicamycin and hypoxia (Fig. 1D). As expected, the expression of ER stress (Bip, CHOPS, ATF4) and hypoxia (HIF1α) markers was highly induced (Fig. 1C; Suppl. Fig. 1A). In contrast, the expression of heparanase was not altered under conditions of stress (Fig. 1A, lower panel; Fig. 1C, second panel), clearly signifying that the heparanase and Hpa2 genes are regulated differently. Induction of Hpa2 thus shifts the heparanase:Hpa2 ratio in favor of Hpa2. Notably, Hpa2 induction by conditions of ER stress and hypoxia was attenuated markedly in the presence of PERK inhibitor (Fig. 1E), suggesting that Hpa2 gene induction is dependent on the PERK arm of the ER stress pathway. Furthermore, Hpa2 expression was prominently induced in cells exposed to cisplatin (Fig. 2A–C), which results in ER stress and oxidative stress [35, 36], heat shock (Fig. 2D, E; Suppl. Fig. 1C), the proteasome inhibitor MG132 (Fig. 2F, G) that results in proteotoxic stress [37], and by LLOMe that elicits lysosomal stress (Suppl. Fig. 1D) [38]. Together, these results strongly imply that Hpa2 expression is induced by multiple stress conditions.

Identification of a promoter region that mediates Hpa2 induction by stress

To further reveal the molecular mechanism underlying Hpa2 induction by stress, we obtained a reporter gene construct in which 1468 bp of the Hpa2 gene promoter are placed upstream of a minimal promoter region followed by the Gaussia luciferase coding sequence (Suppl. Fig. 2A). We first validated that HEK 293 cells respond to ER stress by inducing Bip and CHOP to magnitude comparable to the tumor-derived cells (Suppl. Fig. 2B) and that Hpa2 expression is induced by thapsigargin and hypoxia in HEK 293 cells similar to tumor cells (Fig. 3A). Importantly, we found that the expression of luciferase in HEK 293 cells transfected with our reporter gene exhibited a comparable pattern and magnitude of induction following exposure to thapsigargin, hypoxia, or both (Fig. 3B). This indicates that the 1468 bp region upstream of the Hpa2 gene is sufficient to elicit Hpa2 induction by stress. To narrow down this region, we divided the 1468 bp region into three parts (Part A, Part B, Part C: Suppl. Fig. 2B), transfected each of them into HEK 293 cells, and the transfected cells were subjected to thapsigargin and hypoxia (Fig. 3C), heat shock (Fig. 3D), and MG132 (Fig. 3E). We found that only Part B responded to thapsigargin and hypoxia (Fig. 3C), exhibiting luciferase induction to magnitude comparable to the entire promoter region (Fig. 3B). In contrast, Part A or Part C regions of the promoter failed to respond to the stress by eliciting luciferase expression. Likewise, only Part B responded to heat shock (Fig. 3D), whereas each of the three parts of the promoter responded to proteotoxic stress (MG132), with part A being the most responsive (Fig. 3E). These results suggest that part B of the Hpa2 promoter contains the DNA elements that respond to ER stress/hypoxia and heat shock, whereas Hpa2 induction by proteotoxic stress is regulated by yet to be defined sequences.

Hpa2 induction by stress is mediated by ATF3

In order to identify transcription factors that are involved in Hpa2 induction by stress, we examined the expression pattern of activating transcription factor 4 (ATF4), an essential component of ER stress. Indeed, ATF4 expression was induced by thapsigargin (Fig. 1C), comparable in magnitude to the induction of Bip and CHOP (Fig. 1C), and by heat shock (Fig. 2E) and MG132 (Fig. 2G). In contrast, ATF4 expression was not induced by cisplatin (Fig. 2C) even though Hpa2 is induced by these conditions (Fig. 2A–C), suggesting that transcription factor/s other than ATF4 is involved. We hypothesized that an ATF4-related transcription factor, activating transcription factor 3 (ATF3), may regulate Hpa2 induction by stress because ATF3 is highly induced by conditions of stress [39]. Interestingly, we found that Hpa2 induction by all stresses used (Fig. 4A–C, upper panels) was paralleled with an increase in the expression of activating transcription factor 3 (ATF3). This was evident by qPCR (Fig. 4A–C, middle panels) and immunoblotting analyses (Fig. 4A–C, lower panels). Furthermore, double immunofluorescent staining of cells exposed to thapsigargin/hypoxia (Fig. 4D), cisplatin (Fig. 4E), and MG132 (Fig. 4F) showed that cells that are stained positive for nuclear ATF3 (green) are also stained positive for cytoplasmic Hpa2 (red). This co-staining pattern strengthens the notion that ATF3 and Hpa2 coincide. To examine the role of ATF3 in Hpa2 gene regulation, we exposed WT HEK293 cells and HEK293 cells that were edited by Crispr/cas9 and lacks ATF3 (HEK293 ATF3-null) to thapsigargin and hypoxia. Notably, we found that HEK293 cells that lack ATF3 lost their ability to induce the expression of Hpa2 following treatment with thapsigargin and hypoxia (Fig. 4D, lower panel, Fig. 5A) or treatment with cisplatin (Fig. 5B). In contrast, Hpa2 expression was induced in magnitude comparable to WT HEK293 in ATF3-null HEK293 cells treated with MG132. Similarly, Hpa2 induction by thapsigargin/hypoxia was lost in mouse embryonic fibroblasts (MEF) isolated from ATF3 knockout (KO) mice while WT MEF responded well to the stress condition (Fig. 5D). Moreover, transfecting ATF3-null cells with an expression plasmid encoding for ATF3 was sufficient to induce Hpa2 expression (Fig. 6A). In contrast, transfecting the ATF3-null cells with an expression plasmid encoding for ATF3 (ATF3 1–100) lacking the basic leucine zipper domain and hence does not bind to DNA, failed to induce Hpa2 expression (Fig. 6B).

We next identified two putative ATF3-binding sequences within Part B of the Hpa2 gene promoter (Suppl. Fig. 3A, red); Each ATF3-binding sequence was then deleted and the resulting luciferase reporter plasmids were transfected to HEK293 cells. Notably, while luciferase expression was noticeably increased by thapsigargin and hypoxia in cells carrying the entire reporter gene construct or Part B of the Hpa2 promoter (Fig. 6C, left and second left panels), the effect was practically abrogated upon deletion of ATF3-binding site 1 or 2 (Fig. 6C, right panels). Next, we labeled the cyclic AMP response element (CRE-ATF3-binding sites sequences) with IRDye (Suppl. Fig. 3, green) and used this probe (Probe 1, Suppl. Fig. 3) in a gel-mobility-shift assay. Cells were grown under normoxia or hypoxia conditions in the absence or presence of either thapsigargin or tunicamycin. Nuclear extracts were prepared and incubated with the labeled CRE probe. We found a noticeable increase in a mobility shift complex in cells exposed to hypoxia together with thapsigargin (Tg) or tunicamycin (Tun) as compared with cell lysates derived from cells cultured under normoxia conditions (Fig. 6D). Furthermore, ATF3 produced in-vitro using T7 in vitro translation

resulted in a specific mobility shift signal with the CRE probe, and this binding was significantly competed by a non-labeled probe (Fig. 6E). Together, these results strongly suggest that Hpa2 induction by stress conditions is mediated by ATF3.

To correlate our findings with clinically relevant human cancer, we found that Hpa2 is readily detected in normal cervical epithelium, but its expression is decreased substantially in the resulting cervical carcinoma (Fig. 7A). Interestingly, a similar staining pattern was noted for ATF3 (Fig. 7B). Importantly, analysis of Hpa2 expression obtained by the mining of data collections (GEO, EGA, and TCGA) and subjected to analysis by publically available computational tools (KM plotter service; https://kmplot.com/analysis) showed that high levels of Hpa2 and ATF3 are associated with prolonged survival of cervical (Fig. 7C, D) and hepatocellular (Suppl. Fig. 3B) carcinomas. These results suggest that an ATF3-Hpa2 axis occurs in human tumors and may lead to improved survival of patients exhibiting high levels of ATF3 and Hpa2.

Discussion

Activating transcription factor 3 (ATF3) belongs to the activator protein 1 (AP-1) family of transcription factors. It is also classified as a basic leucine zipper (bZip) transcription factor and a member of the ATF/cAMP response element-binding (CREB) proteins, which bind to the cyclic AMP response element (CRE) in many gene promoters with the consensus sequence TGACGTCA [39], but exceptions to this sequence restriction have been reported [39]. The basic region in the bZip domain mediates specific DNA binding, while the leucine zipper region is thought to facilitate the formation of homodimers or heterodimers with other bZip containing proteins. ATF3 has been demonstrated to be a transcriptional repressor by forming a homodimer mediated, in part, by recruitment of multiple histone deacetylase (HDAC) to TRE and CRE containing promoters [28]. In addition, ATF3 cooperates with other ATF/CERB family proteins or CCAAT/enhancer-binding protein (C/EBP) family members to form heterodimers, resulting in inhibitory or stimulatory effects, depending on cell type, experimental conditions, and gene promoter [39–41]. ATF3 is an immediate early gene, and its expression is highly induced in response to conditions of stress such as UV light, ER stress, chemotherapeutics, and DNA damaging agents, as well as many external stimuli (i.e., adipokines, chemokines, cytokines) [39, 40, 42]. Induction of ATF3 leads to repression or induction of numerous genes. Our results add Hpa2 to the growing list of genes under ATF3 regulation.

We have recently reported that the expression of Hpa2 is induced by ER and proteotoxic stresses [22, 23], but the mechanism(s) underlying Hpa2 gene regulation was obscure. Here we expand the notion that Hpa2 is regulated by conditions of stress, including ER, heat shock, hypoxia, lysosomal, chemotherapeutics, and proteotoxic stresses, suggesting that Hpa2 is a stress-inducible gene. Notably, we found that induction of Hpa2 was paralleled by a comparable induction of ATF3 (but not ATF4) (Fig. 4), suggesting that ATF3 is involved in Hpa2 induction. This notion was concluded and is supported by the following observations. Most importantly, we have found that ATF3-null cells (HEK293, MEF) failed to induce Hpa2 expression in response to ER stress, hypoxia, or chemotherapeutics (Fig. 5). In contrast, ATF3-null cells respond to MG132 (Fig. 5C), suggesting that induction of

Hpa2 by proteotoxic stress is ATF3-independent and involves different transcription factors. In addition, rescue of ATF3 expression in ATF3-null cells was sufficient to restore Hpa2 expression (Fig. 6A), whereas mutant ATF3 1–100 gene construct (which does not bind DNA) failed to induce Hpa2 (Fig. 6B), suggesting that interaction with the DNA is required. In line with this notion, we have identified two ATF3-binding motifs in the promoter of Hpa2 (Suppl. Fig. 3A) and demonstrated that deletion of either one is sufficient for the ablation of Hpa2 induction by stress (Fig. 6C). Moreover, by using the ATF3-binding motif as a probe, we were able to demonstrate that nuclear extracts of cells exposed to ER stress and hypoxia showed increased association with the probe (Fig. 6D). Furthermore, ATF3 protein produced in vitro was able to bind the CRE probe, and the binding appeared specific since a non-labeled probe competed with this binding (Fig. 6D, right). Altogether, these results are consistent with the notion that Hpa2 induction by stress is ATF3 dependent. Interestingly, we found that combining two stress conditions such as ER stress and hypoxia results in Hpa2 induction that is far greater than each stressor alone (Fig. 1; Fig. 4D). Similarly, inhibition of ER stress was sufficient to lower Hpa2 induction prominently (Fig. 1E), suggesting a synergistic effect of ER stress and hypoxia on Hpa2 induction. Notably, however, comparable ATF3 levels were detected in cells treated with thapsigargin (ER stress) or thapsigargin and hypoxia (Fig. 4A). This may indicate that ATF3 forms a heterodimer with hypoxia-inducible transcription factor and that this heterodimer (rather than ATF3 homodimer) is responsible for Hpa2 induction. Possibilities include JUN or JUND which, upon heterodimerization with ATF3, induce the transcription of target genes [43]. It should be noted that software analyses have identified potential binding sites for many transcription factors in the Hpa2 promoter, but identification and characterization of such putative factors await in-depth investigation beyond the scope of the current study.

ATF3 has been shown to play an important role in metabolic regulation, immune responses, and oncogenesis [39–42]. Studies indicate that the function of ATF3 varies considerably among various cancers. For example, high expression of ATF3 correlates with poor survival of lung [44], colorectal [45], and ovarian [46] cancer patients. In contrast, high levels of ATF3 were associated with prolonged survival of renal [47], liver [48], and male breast [49] cancer patients. Preclinical studies, employing overexpression or silencing of ATF3 support this duality and tie ATF3 with the enhancement or attenuation of tumor growth [39–42]. Notably, levels of ATF3 are prominently decreased in colon, renal, endometrial, and bladder tumors vs adjacent normal tissues [47, 50–52], expression pattern that resembles Hpa2 expression [3, 7, 21, 23] and is typical for tumor suppressors. Similarly, we found that Hpa2 levels are prominently decreased in cervical carcinoma (Fig. 7A), and that high levels of Hpa2 predict longer survival of cervical carcinoma patients (Fig. 7C). Interestingly, a similar staining pattern and prolonged patient survival times were found for ATF3-high cervical (Fig. 7B, D) and hepatocellular (Suppl. Fig. 3B) carcinomas. This is supported by preclinical studies, showing that ATF3 activates p53 in HPV-positive SiHa cervical carcinoma cells, resulting in cell cycle arrest and a prominent increase in apoptotic cell death [53]. Likewise, expression of ATF3 is induced by paclitaxel (common chemotherapy in cervical cancer) and over-expression of ATF3 potentiates paclitaxel-induced apoptosis of cervical carcinoma cells [54]. Thus, it is likely that stress conditions that often accompanied the rapidly expanding tumors lead to increased expression of ATF3 which, in turn, induces the expression of

Hpa2. Given its capacity to inhibit heparanase activity, induction of Hpa2 by conditions of stress likely tilt the heparanase:Hpa2 ratio in favor of Hpa2, resulting in reduced heparanase activity and favorable outcome of cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Expression of Hpa2 is markedly induced by stress
- Hpa2 induction by stress is mediated by ATF3
- High levels of Hpa2 and ATF3 are associated with longer survival of cancer patients

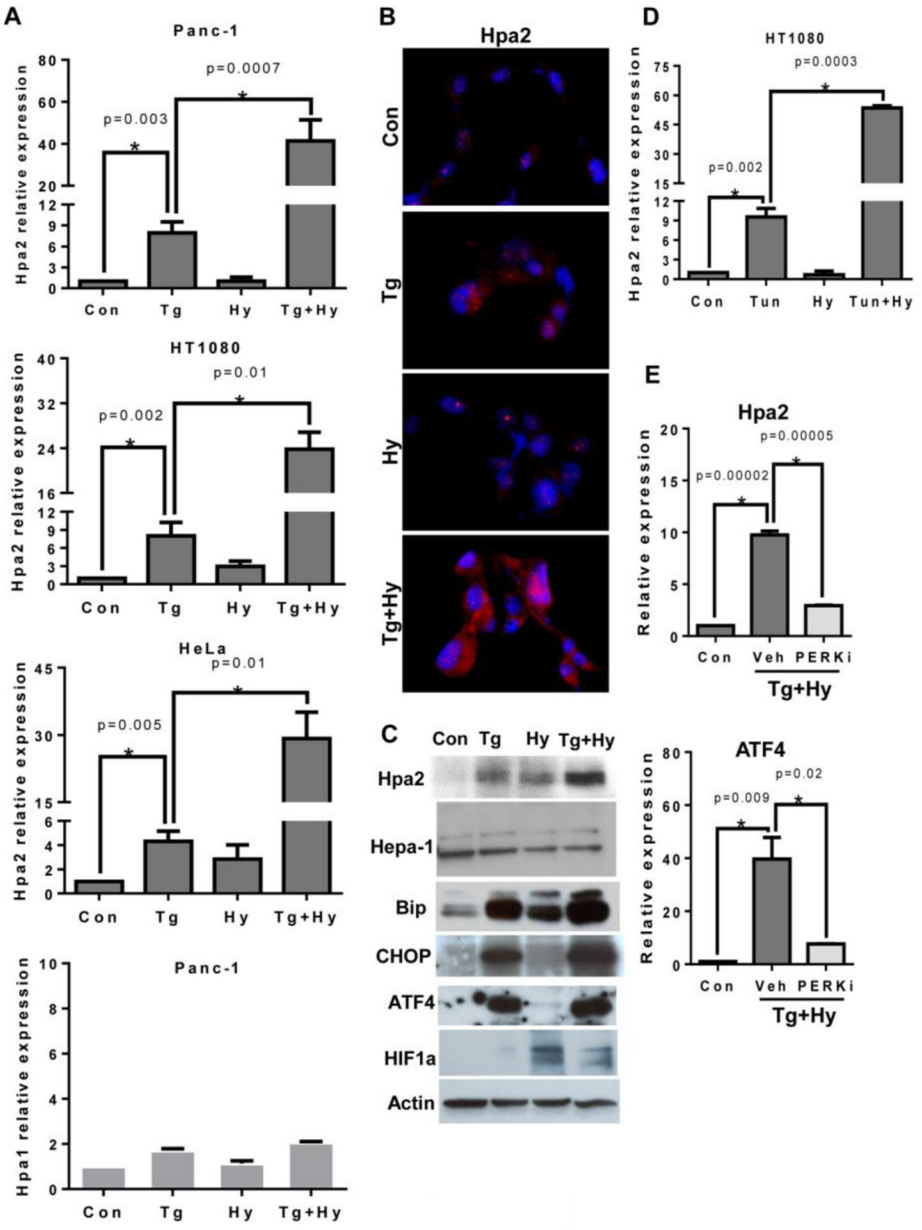

Figure 1.

Hpa2 expression is induced by conditions of stress. **A**. qPCR. The indicated cell lines were treated under serum-free conditions with vehicle control (DMSO; Con), thapsigargin (1.5 μM), hypoxia (2% O2), or both for 18 hours. Total RNA was then extracted and subjected to qPCR applying primers specific for Hpa2 (three upper panels) or heparanase (lower panel). Expression of Hpa2 is presented relative to control cells (Con), set arbitrarily to a value of 1, and calculated after normalization to actin. **B**. Corresponding Panc-01 cells were fixed with formalin and subjected to immunofluorescent staining applying anti-Hpa2 monoclonal antibody 20c5 (red). Shown are representative images together with nuclei counterstaining (DAPI, blue). **C**. Protein extracts from corresponding cultures were subjected to immunoblotting applying anti-Hpa2 (upper panel), anti-heparanase (second

panel), anti-Bip (third panel), anti-CHOP (fourth panel), anti-ATF4 (fifth panel), anti-HIF1α (sixth panel) and anti-actin (lower panel) antibodies. **D**. HT1080 cells were treated under serum-free conditions with vehicle control (DMSO; Con), tunicamycin (Tun; 2.5 μM), hypoxia (2% O2), or both for 18 hours. Total RNA was then extracted and subjected to qPCR applying primers specific for Hpa2. **E**. PERK inhibitor. Panc-01 cells were incubated with vehicle (DMSO) as control (Con) or were exposed to thapsigargin (1.5 μM) and hypoxia (2% O2) without (Veh) or with the PERK inhibitor GSK2606414 (5 μM). Total RNA was extracted after 18 hours and subjected to qPCR applying primers specific for Hpa2 (upper panel) and ATF4 (lower panel).

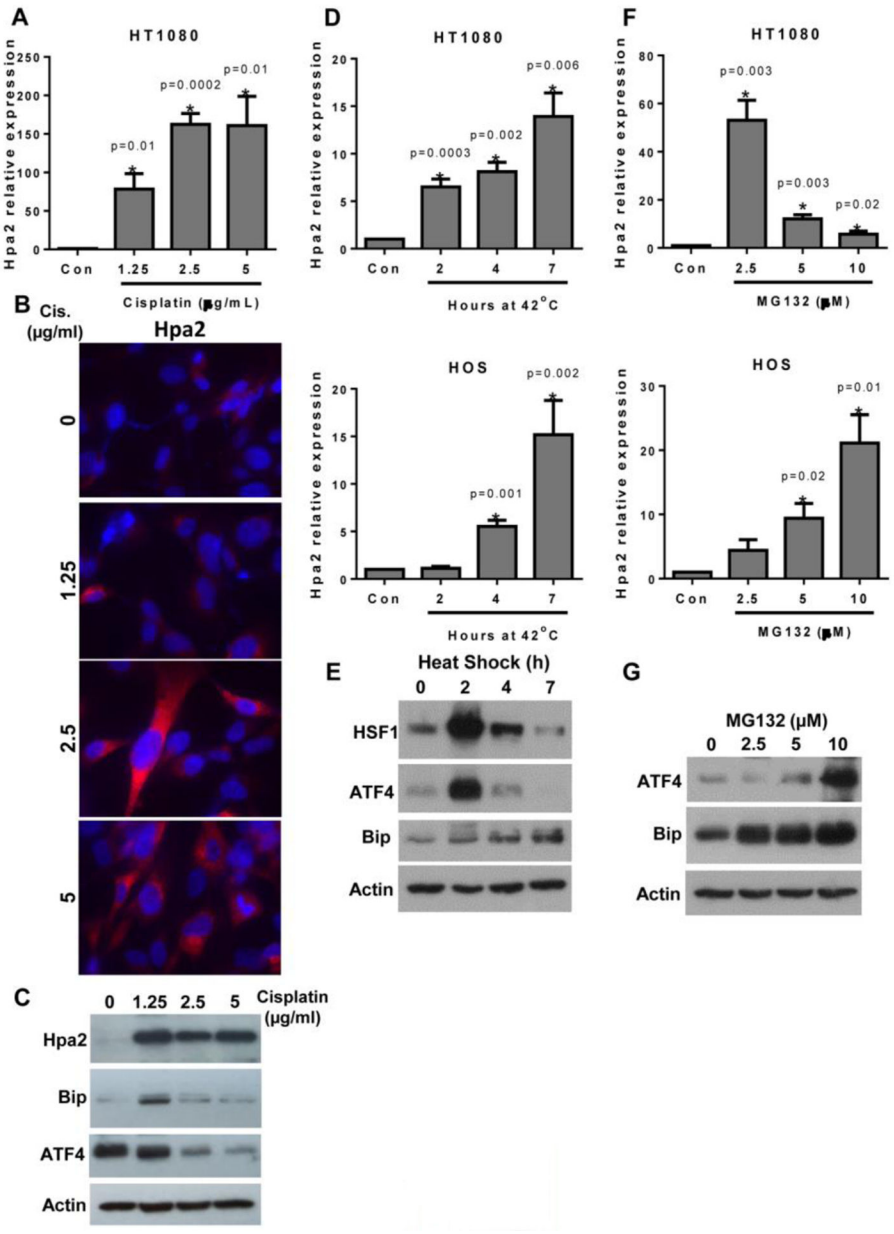

Figure 2.

Hpa2 expression is induced by cisplatin, heat shock, and proteotoxic stress. A. Cisplatin. HT1080 cells were treated under serum-free conditions with vehicle control (DMSO; Con) or the indicated concentration of cisplatin for 18 hours. Total RNA was then extracted and subjected to qPCR applying primers specific for Hpa2. **B**. Immunofluorescence. Corresponding cells were fixed with formalin and subjected to immunofluorescent staining applying anti-Hpa2 monoclonal antibody 20c5 (red). Shown are representative images together with nuclei counterstaining (DAPI, blue). **C**. Immunoblotting. Protein extracts from corresponding cultures were subjected to immunoblotting applying anti-Hpa2 (upper panel), anti-Bip (second panel), anti-ATF4 (third panel), or anti-actin (lower panel) antibodies. **D-E**. Heat shock. The indicated cell line was incubated at 37°C (Con) or 42°C for the

time indicated. Total RNA was then extracted and subjected to qPCR applying primers specific for Hpa2. (**D**). Protein extracts from corresponding cultures were subjected to immunoblotting applying anti-HSF-1 (upper panel), anti-ATF4 (second panel), anti-Bip (third panel), and anti-actin (lower panel) antibodies (**E**). **F-G**. Proteotoxic stress. The indicated cell line was treated under serum-free conditions with vehicle control (DMSO; Con) or the indicated concentration of MG132 for 18 hours. Total RNA was then extracted and subjected to qPCR applying primers specific for Hpa2. (**F**). Protein extracts from corresponding cultures were subjected to immunoblotting applying anti-ATF4 (upper panel), anti-Bip (second panel), and anti-actin (lower panel) antibodies (**G**).

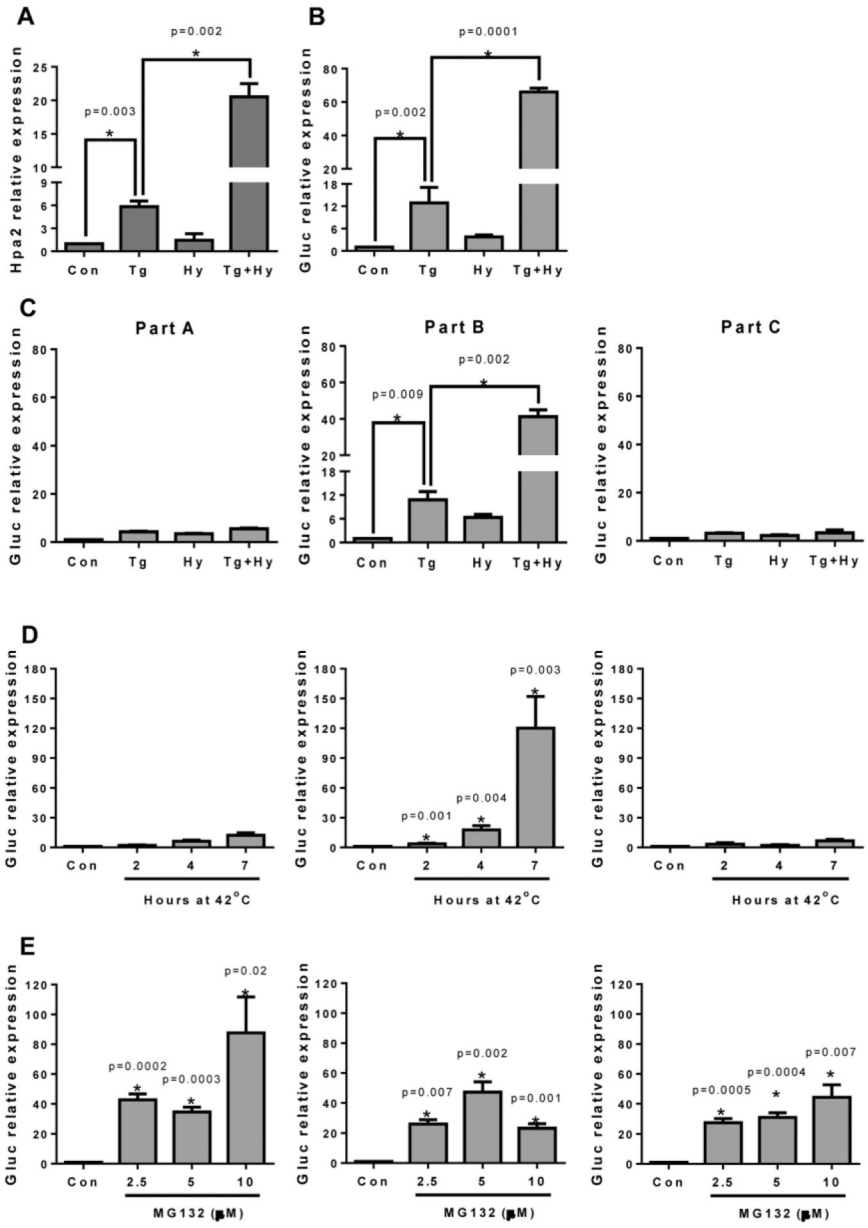

Figure 3.

Identification of a 498 bp promoter region that mediates Hpa2 induction by stress. HEK 293 cells were treated with vehicle (DMSO) as control (Con), thapsigargin (1.5 μM), hypoxia (2% O2), or both for 18 hours. Total RNA was then extracted and subjected to qPCR analysis applying primer set specific for Hpa2 (**A**). HEK 293 cells stably transfected with Hpa2 reporter gene were treated similarly with thapsigargin, hypoxia, or both for 18 hours. Total RNA was then extracted and subjected to qPCR applying primers specific for luciferase (**B**). Expression of luciferase is presented relative to control cells (Con), set arbitrarily to a value of 1, and calculated after normalization to actin. **C-E**. Identification of a promoter region that mediates Hpa2 induction by stress. HEK 293 cells were transfected with Part A [(−)1478- (−)972], Part B [(−)971-(−)474], or Part C [(−)473-(+)9] of the Hpa2

reporter gene and were subjected to thapsigargin, hypoxia or both (**C**), heat shock for the time indicated (**D**), or MG132 (20 μM) for 18 hours (**E**). Total RNA was then extracted and subjected to qPCR applying primer set specific for luciferase. Expression of luciferase is presented relative to control cells (Con), set arbitrarily to a value of 1, and calculated after normalization to actin. Note that luciferase induction by thapsigargin/hypoxia and heat shock stress is mediated by Part B of the Hpa2 promoter.

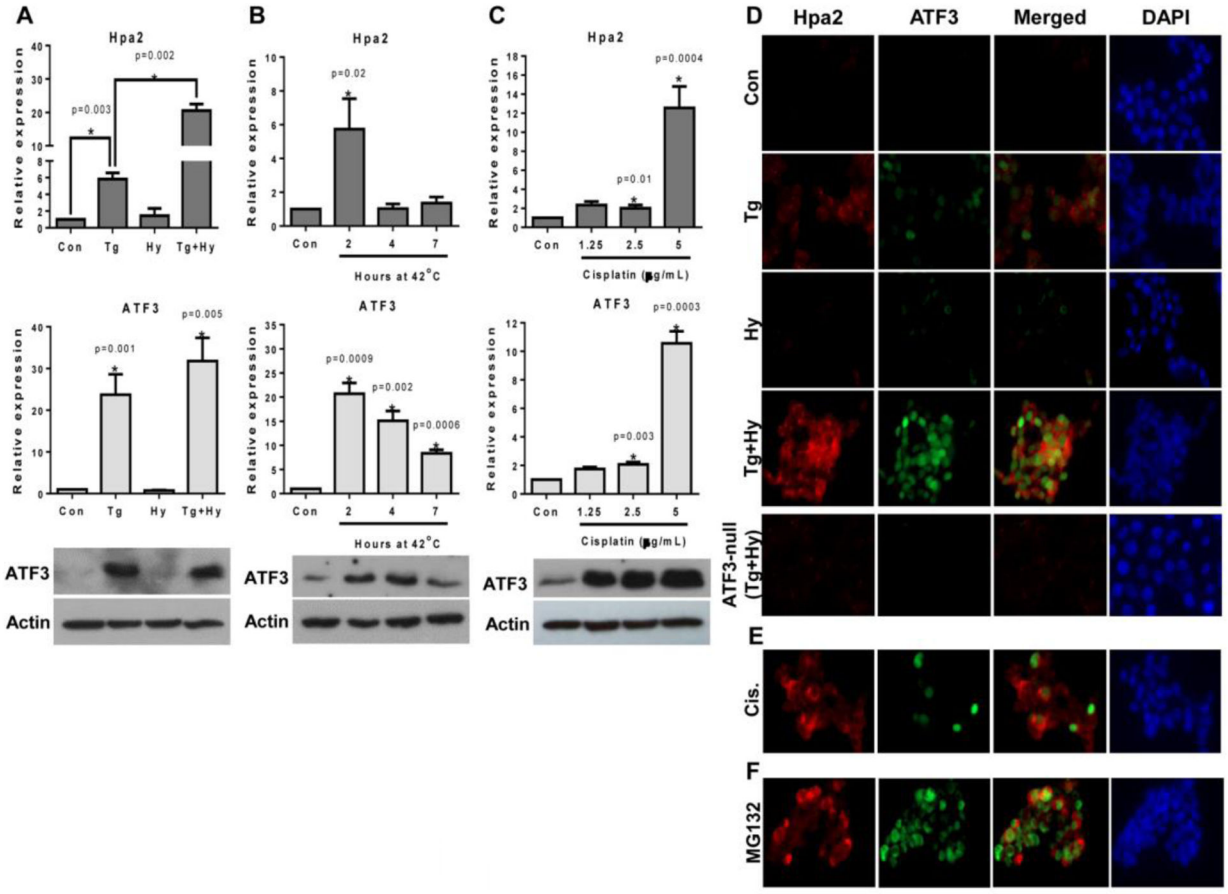

Figure 4.

ATF3 expression coincides with Hpa2. HEK293 cells were treated with vehicle (DMSO; Con), thapsigargin (Tg), hypoxia (Hyp) or both (**A**), heat shock (**B**), or cisplatin (**C**) for the time (B) and concentration (C) indicated. Total RNA was extracted and subjected to qPCR analyses applying primer sets specific for Hpa2 (**A**-**C**, upper panels) and ATF3 (**AC**, middle panels). Note that induction of ATF3 by the stress conditions parallels the induction of Hpa2. Protein extracts from corresponding cultures were subjected to immunoblotting applying anti-ATF3 and anti-actin antibodies (**A**-**C**, lower panels). **D**-**F**. Immunofluorescence staining. Cells were treated with vehicle (DMSO; Con), thapsigargin (Tg), hypoxia (Hyp), or both (**D**), cisplatin (**E**), or MG132 (**F**) for 18 hours. Cells were then fixed and subjected to immunofluorescent staining applying mouse anti-Hpa2 (red) and rabbit anti-ATF3 (green) antibodies. Shown are representative images together with nuclei counterstaining (DAPI, blue). Note that cells stained positive for (nuclear) ATF3 (green) are also positive for (cytoplasmic) Hpa2 (red).

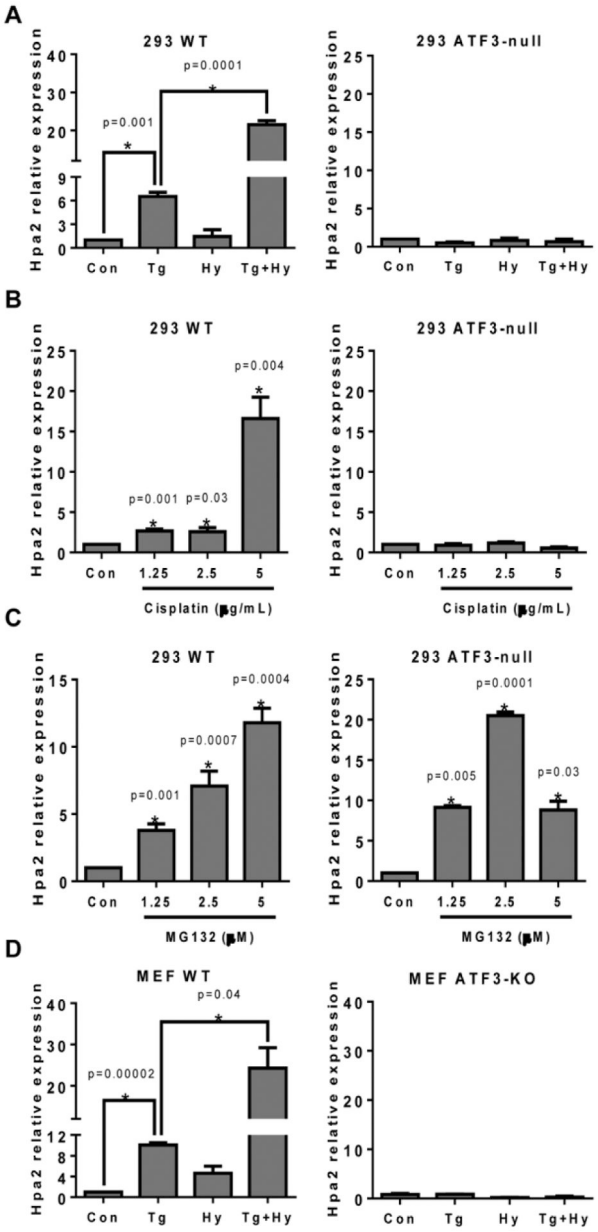

Figure 5.

Induction of Hpa2 expression by stress is ATF3-dependent. WT HEK293 (**A-C**, left panels) and HEK293 cells subjected to gene editing (Crispr/Cas9) and lacks ATF3 (**AC**, right panels) were subjected to vehicle (DMSO; Con), Tg, hypoxia, or both (**A**), the indicated concentration of cisplatin (**B**) or MG132 (**C**) for 18 hours and Hpa2 expression was quantified by qPCR. Note that cells deficient in ATF3 fail to induce Hpa2 expression following ER/hypoxia stress. Embryonic fibroblasts isolated from WT (**D**, left) and ATF3 KO mice (**D**, right) were treated with vehicle (DMSO; Con), thapsigargin (Tg), hypoxia (Hyp), or both for 18 hours and Hpa2 expression was quantified by qPCR. Note that cells deficient for ATF3 do not induce Hpa2 expression in response to thapsigargin and hypoxia or cisplatin, yet Hpa2 induction by MG132 is retained.

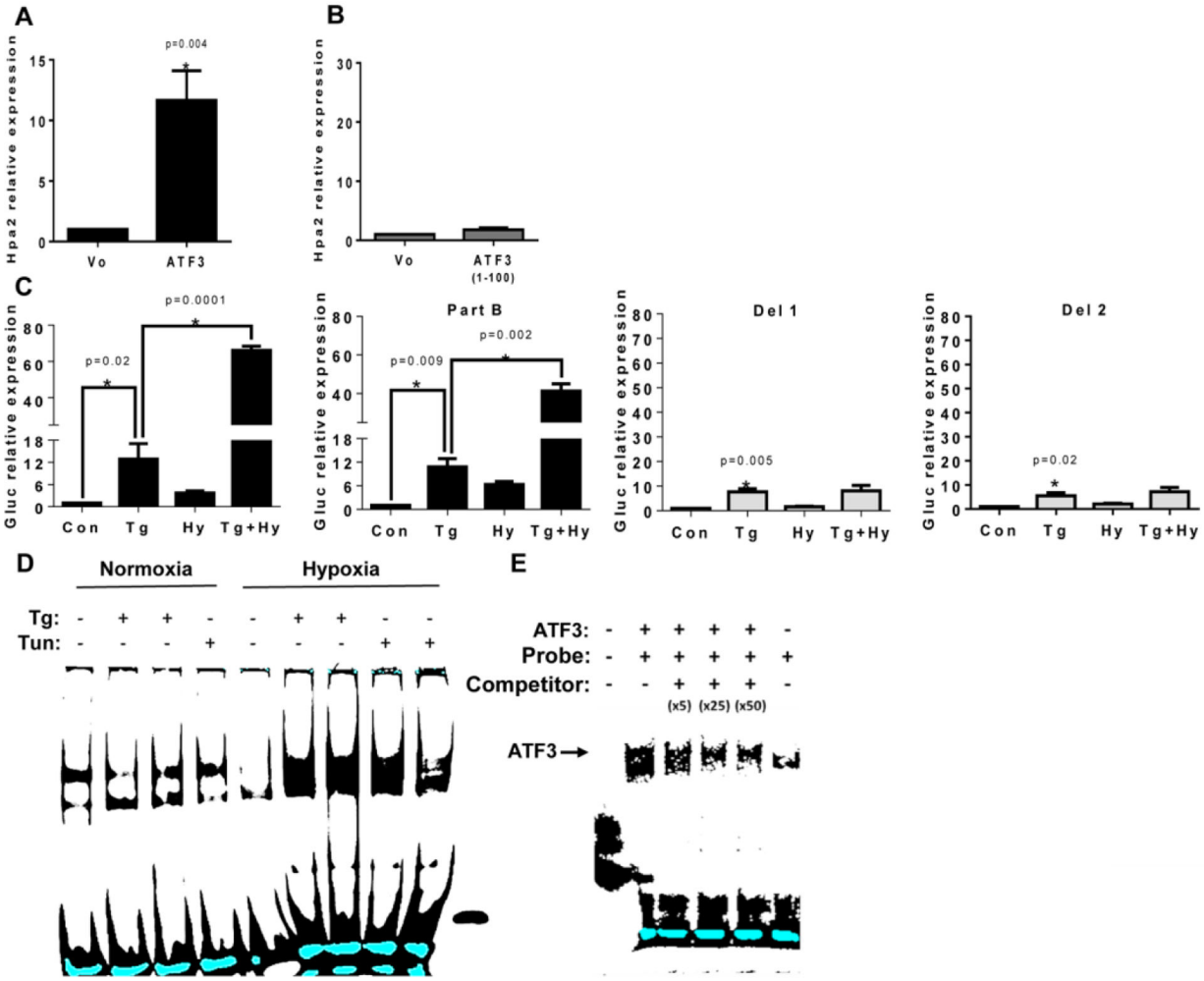

Figure 6.

Rescue of ATF3 and identification of ATF3-binding sequences. **A-B**. Rescue. ATF3-null HEK293 cells were transfected with control empty vector (Vo) or ATF3 gene constructs and expression of Hpa2 was quantified by qPCR (**A**). ATF3-null cells were similarly transfected with ATF3 gene construct that lacks the DNA-binding region of ATF3 (ATF3 1–100) and Hpa2 expression was quantified by qPCR (**B**). **C**. ATF3-binding sequences. HEK293 cells were transfected with Hpa2-reporter gene construct (C, left), Part B of the reporter gene (C, second left), or Part B of the reporter gene construct deleted for ATF3-binding site 1 (**C**, Del 1) or ATF-binding site 2 (**C**, Del 2). Cells were then treated with vehicle (DMSO; Con), thapsigargin (Tg), hypoxia (Hyp), or both and luciferase expression was quantified by qPCR after 24 h. Note that the strong effect of thapsigargin and hypoxia on Hpa2 induction is lost upon deletion of each of the ATF3 binding sites. **D-E**. Gel-shift assay. Nuclear extracts were prepared from HEK293 cells kept under normoxia or hypoxia in the absence (−) or presence (+) of thapsigargin (Tg) or tunicamycin (Tun) and incubated with the AFT3-binding site probe (**D**). Note increased binding of nuclear proteins from cells exposed to hypoxia and thapsigargin/tunicamycin to the ATF3 probe. **E**. ATF3 was

expressed by an in vitro translation method and was incubated with the labeled probe in the absence (−) or presence of increasing concentrations of unlabeled, competitor probe.

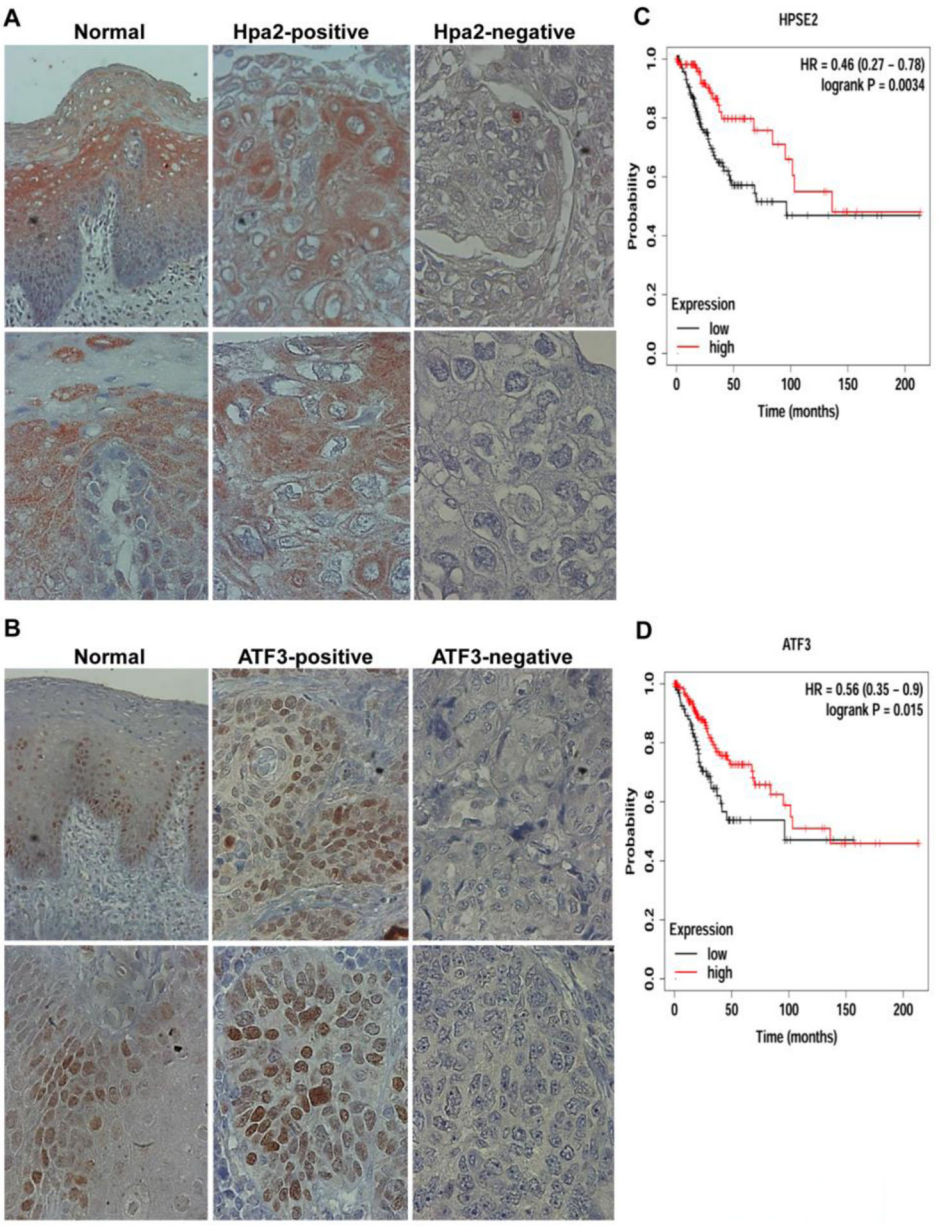

Figure 7.

Strong staining intensities of Hpa2 and ATF3 are associated with longer survival times of cervical cancer patients. **A, B**. Immunostaining. Five-micron sections of cervical carcinoma tumors were subjected to immunostaining applying anti-Hpa2 (**A**) and anti-ATF3 (**B**) antibodies. Shown are representative images at low(upper panels) and high (lower panels) magnifications. Note that Hpa2 and ATF3 are detected in the normal cervical epithelium (Normal) and are decreased in some of the carcinomas. **C, D**. Kaplan-Meier plotter. Survival of patients having cervical squamous cell carcinoma (n=304) was retrieved by the Kaplan-Meier plotter service according to the expression levels of Hpa2 (**C**) and ATF3 (**D**). Note

longer survival of patients exhibiting high levels of Hpa2 and ATF3 expression vs patients with low expression levels.