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HSPB1 is essential for inducing resistance to proteotoxic stress in beta-cells

Vinicius M. Gomes[1], Rosangela A. M. Wailemann[1], Gabriel S. Arini[1], Talita C. de Oliveira[1], Daria Raquel Queiroz[1], Ancély F. dos Santos[1], Letícia F. Terra[1], Stephan Lortz[2] and Leticia Labriola[1*]

[1] Chemistry Institute, University of São Paulo, São Paulo, Brazil.
[2] Institute of Clinical Biochemistry, Hannover Medical School (MHH), Carl-Neuberg-Straße, 1, 30625, Hannover, Germany
* Correspondence: LL: labriola@iq.usp.br

Abstract: During type 1 diabetes mellitus (T1DM) development, beta-cells undergo intense endoplasmic reticulum (ER) stress that could result in apoptosis through the failure of adaptation to the unfolded protein response (UPR). Islet transplantation is considered an attractive alternative among beta-cell replacement therapies for T1DM. To avoid the loss of beta-cells that will jeopardize the transplant´s outcome, several strategies are being studied. We have previously shown that prolactin induces protection against pro-inflammatory cytokines and redox imbalance-induced beta-cell death by increasing heat shock protein B1 (HSPB1) levels. Since the role of HSPB1 in beta cells has not been deeply studied, we investigated the mechanisms involved in unbalanced protein homeostasis caused by intense ER stress and overload of the proteasomal protein degradation pathway. We tested whether HSPB1-mediated cytoprotective effects involved UPR modulation and improvement of protein degradation via the ubiquitin-proteasome system. We demonstrated that increased levels of HSPB1: attenuated levels of pro-apoptotic proteins like CHOP and BIM, increased protein ubiquitination and the speed of proteasomal protein degradation. Our data showed that HSPB1 induced resistance to proteotoxic stress and thus enhanced cell survival via an increase in beta-cell proteolytic capacity. These results could contribute to generate strategies aiming at optimization of beta-cell replacement therapies.

Keywords: heat shock proteins; Diabetes mellitus; beta-cells; endoplasmic reticulum stress; proteostasis; HSPB1; cytoprotection; apoptosis.

1. Introduction

Pancreatic islet transplantation is an attractive alternative for the treatment of T1DM recommended mainly for patients suffering from frequent episodes of severe unnoticed hypoglycemia [1–3]. Even when several modifications have been adopted to maintain longer survival and functionality of the graft [3,4], the loss of beta-cell mass by autoimmunity, rejection and instant blood mediated inflammatory reaction (IBMIR) [5] is still an important factor deserving more attention to increase the success of pancreatic islet transplantation [3,4].

Beneficial effects of prolactin (PRL) or placental lactogen in the process of isolation and culture of these structures during the period prior to transplantation have recently being reported [6–8]. Results from our group have shown that PRL was able to induce the up-regulation of HSPB1, a protein from the family of small Heat Shock Proteins (sHSP), which turned out to be a key mediator of PRL-mediated inhibition of beta-cell apoptosis [9,10].

Understanding the mechanisms of beta-cell protection by HSPB1 has crucial importance for future cell-based strategies, since PRL concentration used to promote beta-cell cyto-protection is not compatible with a clinical application because of the side effects displayed by the hormone in other tissues. It is well known that pro-inflammatory cytokines like interleukin 1 β (IL-1-β), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) can promote beta-cell death by inducing oxidative and endoplasmic reticulum (ER) stress as well as a reduced capacity to completely mount a functional autophagic response for protein degradation [11,12]. Our results have demonstrated that increased levels of HSPB1 can inhibit beta-cell apoptosis induced by pro-inflammatory cytokines, menadione-induced oxidative stress as well as thapsigargin-induced ER-stress [9,10].

ER stress has been implicated in the pathogenesis of both type 1 and 2 diabetes [13–16]. Incorrectly folded proteins are retained in the ER to undergo refolding or degradation. Dysregulation of these processes causes ER stress-dependent activation of the Unfolded Protein Response (UPR), which is involved in minimizing stress and restoring homeostasis. Upon prolonged or stronger stress situations UPR can also lead to apoptosis [17,18]. One of the fates of unfolded proteins in the ER is the Endoplasmic Reticulum Associated Protein Degradation (ERAD) which promotes the degradation of ubiquitinated proteins by the proteasome system [19]. UPR activation impacts ERAD pathway leading to ER stress attenuation and thus cell survival [20]. Upon intense induction, and if all the intracellular mechanisms are not able to sustain proper protein quality control, ER stress can finally trigger cell death by inducing apoptosis through the up-regulation of CCAAT-enhancer-binding *protein* Homologous *Protein* (CHOP) that counteract the effect of anti-apoptotic proteins. There is substantial evidence for the presence of ER stress in beta-cells suggesting defective UPR and failure to resolve ER stress in the context of T1DM and T2DM [12,21]. Indeed, CHOP has been proposed as the main link between ER stress induction and beta-cell apoptosis in T1DM [16,22]. Under stress conditions the induction of CHOP and further activation of BH3-only proteins like Bcl-2 Interacting Mediator of cell death (BIM) can cause mitochondrial outer membrane permeabilization and cell death [23,24]. The participation of BIM as a cell death inducing factor involved in pancreatic beta-cell death has also been reported [12].

In view of the scarce amount of studies properly addressing the role of HPSB1 in beta-cells, we set out to unveil the molecular mechanisms involved in HSPB1-induced resistance to proteotoxic stress in insulin producing cells.

2. Materials and Methods

Mouse islets isolation and culture

Islet isolation from male BALB/c mice (8–12 weeks old) was performed according to Ricordi and co-authors with modifications [25]. Before terminal surgery, healthy animals (5 per cage) were kept in a normal dark/light cycle and *ad libitum* access to water and food in an environmental enriched ambient in the animal facility of the Chemistry Institute (University of São Paulo). Briefly, animals were submitted to terminal surgery for pan-

creas retrieval after euthanasia by intraperitoneal administration of a lethal dose of anesthetics (xylazine hydrochloride [Kensol, Koenig; 10 mg/kg] and ketamine hydrochloride [Vetanarcol, Koenig; 100 mg/kg]). The Internal Animal Care and Use Committee of the Chemistry Institute at the University of São Paulo approved all protocols on 23/08/2017 (process n°74/2017) following all norms established by the National Council for the Control of Animal Experimentation. Islets were handpicked and kept in RPMI medium supplemented with 10% (v/v) FCS, 2 mmol/L glutamine, 100 units/mL ampicillin and 100 μg/mL streptomycin at 37 °C in a 5% (v/v) CO2 atmosphere.

Cell culture of Min6 cells

Mouse insulinoma-derived Min6 cells [26] and all HSPB1-modified cells [10,11] and their respective controls were maintained in RPMI medium (Life Technologies, CA, USA) supplemented with 10% (v/v) FCS, 100 units/mL ampicillin, 100 μg/mL streptomycin and 10 mmol/L HEPES (Sigma-Aldrich, St. Louis, Mo, USA). Cell lines were periodically submitted to PCR and Hoechst staining to analyze mycoplasma contamination and were only used in case of negative results.

HSPB1 silencing and overexpression in mouse islets

HSPB1 silencing: Dispersed islets were infected with (M.O.I: 4.6) recombinant lentiviral particles containing a mixture of five validated shRNAs for murine hspb1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or the corresponding controls as previously described [27,28].
Transient HSPB1 overexpression: pEGFPhsp27 wt FL, a gift from Andrea Doseff (#17444; Addgene, Cambridge, MA, USA) [29] or the empty vector pEGFP (#19056; Addgene, Cambridge, MA, USA) were used to transfect mouse islets as previously described [9]. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen). Lipid-DNA complexes (0.2 μl Lipofectamine: 500 ng plasmid) were formed in Opti-MEM (Invitrogen) at room temperature for 20 min and added to primary cultures of mouse islets in antibiotic-free medium for overnight transfection. Islet cells were maintained in culture for a 24 h recovery period before experiments were carried out. Transfection efficiency was validated by Western blot (WB) and by monitoring eGFP fluorescence by epifluorescence microscopy as previously described [9].

Cell treatments

Cells were seeded at different concentrations depending on further experimentation and allowed to attach for a period of 24 h. Cells were then serum-starved for 24 h in the respective medium supplemented with 0.1% (v/v) FCS. Treatments with a cocktail of pro-inflammatory cytokines (IL-1β, 0.8 ng/mL; TNF-α, 8 ng/mL; IFN-γ, 4 ng/mL), tunicamycin (mouse islets:7.5 or Min6 cells: 15 μg/mL), or thapsigargin (75 nM) (both from Sigma-Aldrich) were carried on for 16 h (for cell viability) or 30 min, 1 h, 3 h, 6 h and 9 h

(for protein extraction). The proteasome was inhibited by pre-incubating the cells for 1 h with MG132 (0.1 or 1 µM, Calbiochem). PRL (300 ng/mL; Peprotech, Mexico DF, Mexico) incubations were started 30 min before the addition of either cytokines or ER stress inducers and kept through the whole treatment.

Cell viability evaluated by microscopy

Cells were stained with 15µg/mL propidium iodide (PI, Sigma Aldrich) and 15µg/mL Hoechst 33342 (HO, Sigma-Aldrich) for 10 min. The percentage of viable and dead cells was determined as already described [9]. A minimum of 500 cells was counted for each experimental condition.

Western blots

Total cell lysates were prepared from cells plated at a density of $3x10^4$ cells/cm^2.
Equal amounts of proteins from each extract were solubilized in sample buffer (60 mmol/L Tris-HCl [pH: 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue) and subjected to SDS-PAGE (10-16%). Proteins were transferred to PVDF membranes, which were blocked and then incubated with the antibodies listed in Suppl. table 1. Detection was performed by enhanced chemiluminescence (Millipore, Billerica, MA, USA) using horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). As a loading control, the membranes were stripped and re-probed with mouse monoclonal anti-α-Tubulin (B512) (Sigma-Aldrich, St Louis, USA). For the analysis of protein phosphorylation levels the membranes were stripped and re-probed respectively with the corresponding anti-total protein. Quantitative densitometry was carried out using ImageJ software (National Institutes of Health). The volume density of the chemiluminescent bands was calculated as an integrated optical density × mm^2 after background correction from at least two different images from each independent experiment (n≥3).

Study of the rate of protein degradation by the ubiquitin-proteasome system

The rate of protein degradation by the ubiquitin-proteasome system was measured by checking the levels of the GFP protein whose sequence was modified (G76V) so that it can be easily ubiquitinated and consequently degraded by the proteasome system , according to the methodology described by Dantuma and co-workers [30,31]. Briefly, Lipid-DNA complexes were formed in Opti-MEM (Invitrogen) in a proportion of 0.2 µL Lipofectamine for 500 ng plasmid, at room temperature for 20 minutes and then added to the cells in antibiotic-free medium for 16 h. The medium was then replaced by RPMI containing 10% FCS. Min6 oxHSPB1 and EV cells were subjected to the UbG76V-GFP (GFP-Ub) vector transfection process performed using Lipofectamine RNAiMAX (Invitrogen). Cells were serum starved and incubated for 3, 6 and 9 h with pro-inflammatory cytokines, ER stressors or vehicle. Total protein extracts were obtained and GFP and α-tubulin levels were detected by western blot. Since the speed of GFP degradation reflects the ubiqui-

tin-proteasome protein degradation pathway activity [30,31], the rate of GFP degradation was measured by calculating the area under the normalized curve, which shows the amount of protein normalized to the α-tubulin content present throughout the treatment time.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 software. All results were analyzed for Gaussian distribution and passed the normality test. In cell viability tests, the statistical differences between the means of the experimental groups were tested through one-way ANOVA analysis followed by the Tukey post-test for multiple comparisons. The statistical differences comparisons between protein levels of the different cell lines submitted to treatments at different times were tested by two-way ANOVA analysis followed by Bonferroni's post-test for multiple comparisons. For all tests, a value of $p < 0.05$ was considered to be statistically significant.

3. Results

HSPB1 is required for beta-cell cytoprotection against ER stress-induced apoptosis.
Since we have shown that HSPB1 was involved in prolactin-promoted cytoprotection in the context of T1DM and ER stress-induced cell death by thapsigargin [9], we investigated the role of this chaperone in ER stress-induced cell death. Min6 cells and primary cultures of mouse islets were challenged with a combination of pro-inflammatory cytokines or endoplasmic reticulum stressors: tunicamycin or thapsigargin for 16 h in the presence or absence of PRL. Results displayed in Figure 1A, C-E showed that prolactin was able to inhibit apoptosis by approximately 50% in all cell lines expressing HSPB1 (Min6, Min6 scC, and Min6 EV). This effect was abrogated in the absence of the chaperone (Min6 shHSPB1). Additionally, overexpression of HSPB1 (Min6 oxHSPB1) was enough to protect beta-cells against induced death independently of hormonal treatment. A more pronounced HSPB1-dependent cytoprotective effect was observed in primary cultures of mouse islets overexpressing the chaperone (Islets oxHSPB1) (Figure 1B, F-H). These results demonstrated that HSPB1 is an essential player in the PRL-induced beta-cell cytoprotection against ER stressors' cytotoxic properties. Moreover, the sole overexpression of this protein was sufficient to provide a significant pro-survival effect independent of PRL treatment.

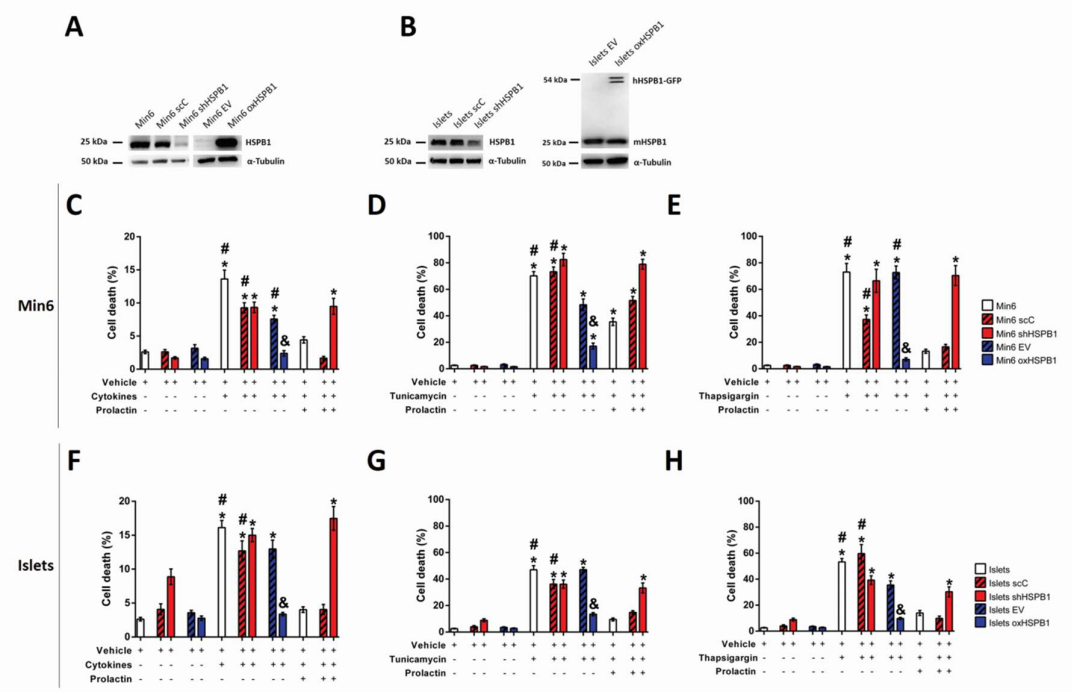

Figure 1. *HSPB1 is a key mediator of prolactin-mediated cytoprotection against ER stress in Min6 cells and mouse islets.* HSPB1 expression was confirmed by western blot in **(A)** Min6 cells and **(B)** mouse islets. HSPB1 silenced Min6 cells and mouse islets (Min6 shHSPB1, Islets shHSPB1), HSPB1 overexpressing (Min6 oxHSPB1, Islets oxHSPB1) and their respective controls (Min6 scC, Min6 EV; Islets scC, Islets EV) were exposed to a combination of cytokines **(C, F)** (TNF-α 8 ng/mL, INF-γ 16 ng/mL, IL-1-β 1.6 ng/mL), **(D, G)** tunicamycin (15 or 7.5 µg/mL, respectively) or **(E, H)** thapsigargin (75 nM) in the presence or absence of PRL (300 ng/ml) for 16 h. Cell death was evaluated by PI / Hoescht staining using fluorescent microscopy . Results are presented as means ± SEM (Each data point represents means ± SEM from three replicates of at least three independent experiments); * $p<0.05$ vs. control (vehicle); # $p < 0.05$ vs PRL. &: $p< 0.05$ vs EV.

HSPB1 modulates UPR in mouse islets andMin6 cells.

To investigate the molecular mechanisms leading to the cytoprotective effect of HSPB1 protein levels of several components of the UPR pathways were analyzed.

Since we have already shown that caspase-3 activity reached a plateau upon 9 h of cytokines treatment [9], protein levels and phosphorylation states were analyzed in kinetic experiments (0-9 h). The quantitative analyses between the cells were performed by comparing protein expression levels 6 or 9 h after ER stress stimuli.

HSPB1 overexpressing cells displayed significant stress attenuation. The first evidence of this effect was the lower level of Binding Immunoglobulin *Protein* (BIP) observed in oxHSPB1 islets, as well as in Min6 oxHSPB1cells after 6h of pro-inflammatory cytokines or tunicamycin exposure when compared with the silenced cells (islets shHSPB1 and Min6 oxHSPB1 respectively) (Figure 2 and Suppl. Figure 1).

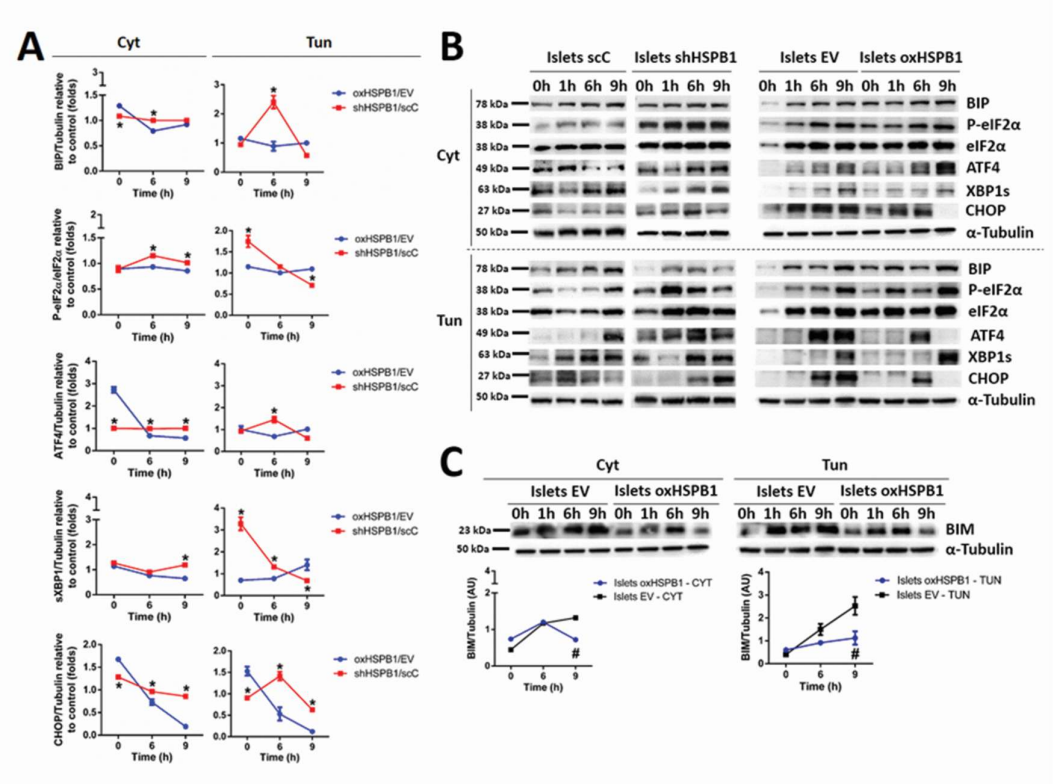

Figure 2. *HSPB1 overexpression attenuates ER stress induced by pro-inflammatory cytokines or tunicamycin in mouse islets*. HSPB1 silenced mouse islets (shHSPB1), HSPB1 overexpressing (oxHSPB1) and their respective controls (scC, EV) were exposed to a combination of cytokines (TNF-α 8 ng/mL, INF-γ 16 ng/mL, IL-1-β 1.6ng/mL) or tunicamycin (7.5 µg/mL) for 6 and 9 h. Protein expression of BIP, ATF4, XBP1s, CHOP, as well as phosphorylation state of eIF2α were analyzed by western blotting. **(A)** Graphical representation of BIP, eIF2α, ATF4, XBP1s and CHOP protein levels presented as arbitrary densitometry unit (AU). After normalization to the corresponding α-tubulin content, the data of the silenced HSPB1 cells (Min6 shHSPB1, Islets shHSPB1) or overexpressed (Min6 oxHSPB1, Islets oxHSPB1) were plotted as the ratio between the values obtained in silenced or overexpressing cells and the one in their respective controls (scC or EV). **(B)** Immunoblots of islets are shown as representative results. **(C)** Graphical representation of normalized protein levels and immunoblots of BIM protein levels in islets presented as arbitrary densitometric units (AU). Each data point represents mean ± SEM from three replicates and at least three independent experiments were carried out for each cell type submitted to the different cell incubation conditions; *: $p<0.05$ vs. oxHSPB1/EV. #: $p<0.05$ vs. EV.

Regarding the *Protein* kinase RNA like endoplasmic reticulum kinase-Eukaryotic Initiation Factor 2 alpha-*Activating transcription factor 4* (PERK-eIF2α-ATF4) UPR´s branch, we have detected increased PERK phosphorylation levels upon 9h of islets oxHSPB1 exposure to either of the ER stressors when compared with silenced cells (Figure 2). This effect was also observed in Min6 oxHSPB1 cells treated with pro-inflammatory cytokines

(Suppl. Figure 2). It is important to note that this last model showed an earlier response since the increase was already significant after 6h of treatment (Suppl. Figure 2). Phosphorylated eIF2α (P-eIF2α) levels were decreased in both cell models overexpressing HSPB1 already after 6h of cytokines exposure and remained at lower levels until 9h of treatment (Figure 2, Suppl. Figure 1). Tunicamycin-exposed cells, either oxHSPB1islets or Min6 oxHSPB1 cells, displayed a significant decrease in P-eIF2α only after 9h of ER stress induction. Finally, unlike Min6 oxHSPB1, isletsoxHSPB1 also presented reduced levels of ATF4 after 6h of exposure to any of the ER stressors. This same profile was maintained until the last time point analysed (Figure 2, Suppl. Figure 1). Overall these results led us to conclude that higher abundance of HSPB1 promoted the attenuation of the UPR pathway initiated by PERK activation in both primary mouse islets and Min6 cells.

Phosphorylation levels of Inositol-Requiring Enzyme 1 alpha (pIRE1α) as well as spliced X-box Binding Protein 1 (XBP1s) were differently modulated in HSPB1 overexpressing cells depending on the ER stressor and on the cell model used. While cytokines exposure promoted an increase in pIRE1α, tunicamycin treatment led to lower phosphorylation levels of the protein in both cell models bearing higher chaperone levels (Suppl. Figure 1, 2). Of note is the slightly different kinetic displayed by mouse islets and Min6 cells. While in primary mouse islets the effect appears to be transient, in the latter model the effect was more sustained (Suppl. Figure 1, 2). When we studied the abundance of IRE1α's downstream effector, XBP1s, significant lower levels were detected in islets oxHSPB1 after 9h of cytokines exposure. Only a trend of decrease was detected in Min6 oxHSPB1 (Figure 2 and Suppl. Figure 1). Regarding tunicamycin treatment, both cell types presented significant diminished levels of the spliced form of the protein upon this strong and specific ER stress induction (Suppl. Figure 1, 2). A delayed response was observed in Min6 oxHSPB1cells when compared with the earlier decrease displayed by primary mouse islets (Suppl. Figure 1, 2). Since activation of both PERK as well as IRE1α signaling pathways can lead to upregulation of the CHOP 25,26 which, will then lead to the increase of BH3 proteins finally involved in apoptosis activation [32,33], we analyzed the levels of some of these proteins in our HSPB1 overexpressing and silenced cell models. A decrease of around four folds was observed in pro-apoptotic CHOP protein levels in mouse islets exposed to pro-inflammatory cytokines (oxSHPB1/EV: 0.18±0.03 (9h); shHSPB1/scC: 0.85±0.01 (9h)) or tunicamycin (oxSHPB1/EV: 0.12±0.03(9h); shHSPB1/scC: 0.62±0.01(9h)) (Figure 2). A greater reduction in the levels of the same protein was observed in Min6 oxHSPB1cells (Suppl. Figure 1). It is important to highlight that all the response promoted by HSPB1 overexpression was completely lost in HSPB1 silenced cells (shHSPB1/scC) (Figure 2A and B, Suppl. Figure 1). Of note are also the significant lower levels of CHOP's target BIM (EV: 1.32±0.05 (9h); oxHSPB1: 0.71±0.03(9h)) observed in oxHSPB1mouse islets (Figure 2C).

Additionally, decreased levels of cleaved ATF6, another factor promoting CHOP upregulation, were detected in MIN6 oxHSPB1 cells after 9h of cytokines or tunicamycin exposure (Suppl. Figure 1). All together, these results further confirmed our hypothesis that HSPB1 overexpression in insulin producing cells is able to attenuate ER stress-induced UPR activation.

In conclusion, despite some discrepancies in the response of the different cell models, these data highlight the importance of increased HSPB1 expression in order to promote ER stress attenuation compatible with the restoration of cell homeostasis. The observed UPR modulation included reduction of pro-apoptotic factors like CHOP and BIM interrupting the main link between UPR and apoptosis.

HSPB1-mediated beta-cell survival requires a functional ubiquitin-proteasome protein degradation system.

One of the fates of unfolded proteins in the ER is the ERAD pathway, which is mediated by the proteasome system [34,35].

In order to confirm whether this degradation process was an essential mechanism involved in the cytoprotective effect induced by HSPB1 up-regulation, the cells were incubated in the presence or absence of the proteasome inhibitor MG132 and then treated with pro-inflammatory cytokines or ER stressors. Exposure to the proteasome inhibitor alone promoted significant cell death (Suppl. Figure 3). Interestingly, this effect in Min6shHSPB1 cells was the highest among all the cells tested. Independently of HSPB1 overexpression, cell death rate was further increased when Min6 or islet cells were treated with cytokines or tunicamycin in the presence of the proteasome inhibitor (Figure 3). These results suggest that proteasome activity is not only important to maintain cell viability but also that this system is essential for the cytoprotection mediated by HSPB1 overexpression.

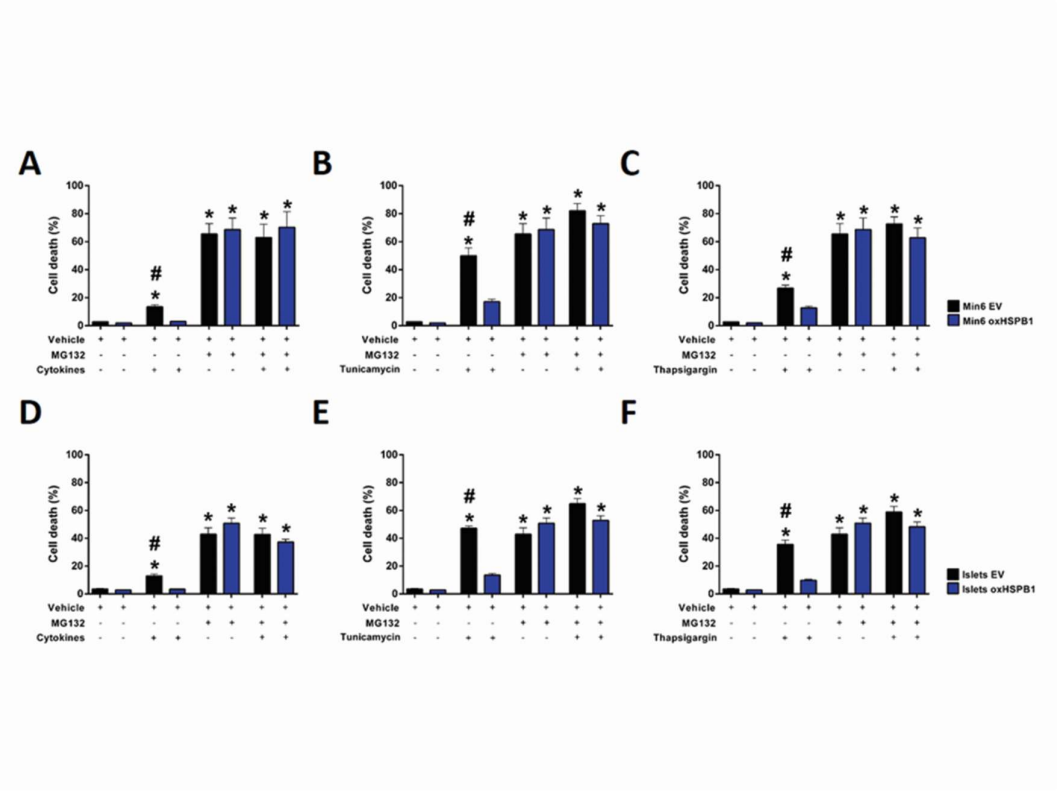

Figure 3. *Proteasomal activity is essential for the cytoprotective effect of HSPB1 against cytokine-, tunicamycin- or thapsigargin-induced cell death*. **(A-C)** HSPB1overexpressing Min6 cells and

(D-F) mouse islets (Min6 oxHSPB1, Islets oxHSPB1) and their respective controls (Min6 EV; Islets EV), were submitted to serum starvation (0.1% FCS) and then exposed to a combination of **(A, D)** cytokines (TNF-α 8 ng/mL, INF-γ 16 ng/mL, IL-1β 1.6 ng/mL), **(B, E)** tunicamycin (15 or 7.5 µg/mL respectively) or (C, F) thapsigargin (75 nM) in the presence or absence of MG132 (2 µM) for 16 h. Cell death was evaluated by PI/Hoescht staining using fluorescent microscopy. Each data point represents means ± SEM from three replicates and at least three independent experiments were carried out for each cell type submitted to the different cell incubation conditions; *p<0.05 vs. control (vehicle); # p < 0.05 vs oxHSPB1 respective treatment.

We then assessed whether protein ubiquitination was involved in HSPB1-mediated beta-cell survival. A significant increase in protein ubiquitination was detected in mouse islets ox HSPB1 incubated with cytokines or tunicamycin after 6 and 9 h (Figure 4). The same effect was displayed by Min6 oxHSPB1cells compared with silenced cells (Suppl. Figure 4).

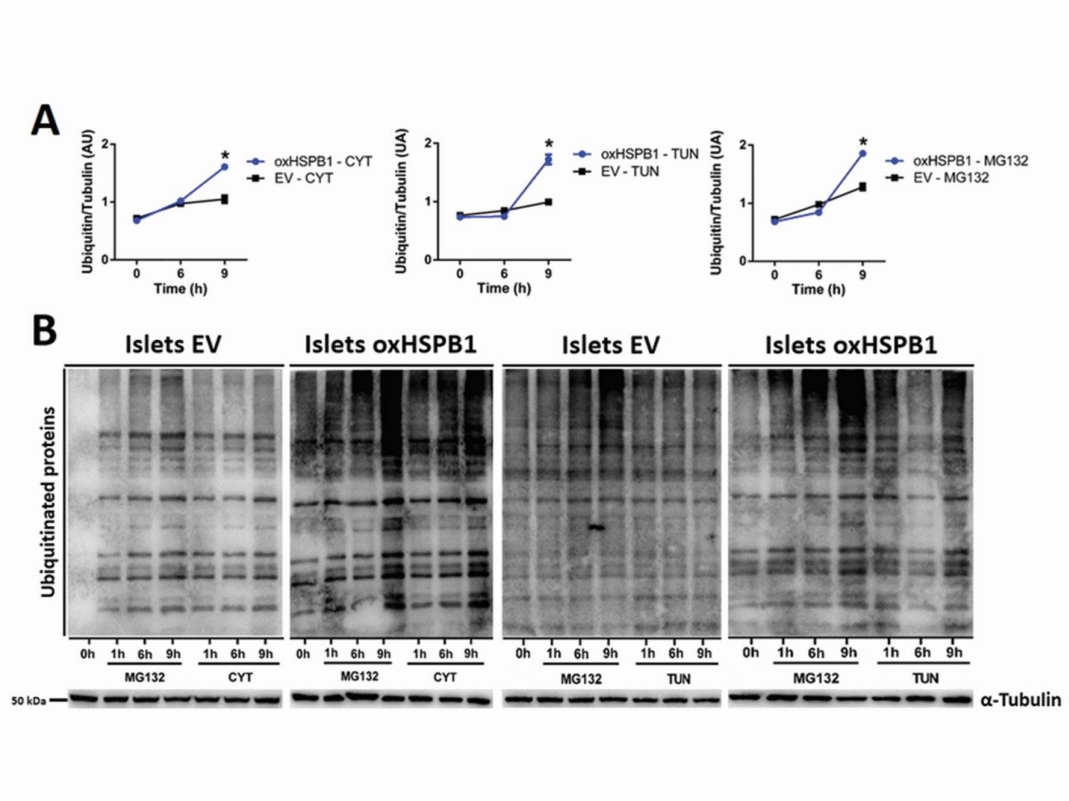

Figure 4. *HSPB1 overexpression leads to increased global protein ubiquitination.* Mouse islets overexpressing HSPB1 (oxHSPB1), and the respective controls (EV) were serum starved and then exposed to a combination of cytokines (TNFα 8 ng/mL, INFγ 16 ng/mL, IL-1β 1.6 ng/mL), tunicamycin (7.5 µg/mL) or MG132 (2 µM) for 6 and 9 h. **(A)** Graphical representation of ubiquitinated proteins results presented as arbitrary densitometry units (AU) after normalization by their respective α-tubulin contents. **(B)** Representative images of immunoblots showing the levels of ubiquitinated proteins of islets treated with cytokines and tunicamycin. Each data point represents means ± SEM from three replicates and at

least three independent experiments were carried out for each cell type submitted to the different cell incubation conditions; *: $p<0.05$ vs. EV.

As a positive control, a significant increase of ubiquitinated proteins was also observed when proteasomal activity was pharmacologically inhibited (Figure 4, Suppl. Figure 4). These data confirmed that HSPB1 overexpression was responsible for the higher protein ubiquitination as part of the cell response involved in HSPB1-mediated beta-cell survival.

HSPB1 accelerates the rate of proteasomal protein degradation.

In view of the above reported results, the following step was to evaluate the impact of this chaperone on the rate of protein degradation via the proteasome system using a reporter strategy. This included the transient transfection of cells with an expression vector containing a modified version of GFP that is rapidly polyubiquitinated and thus targeted to degradation [30,31]. By monitoring the levels of this modified GFP over time, we were able to assess changes in protein degradation rates. Min6 oxSHPB1 cells showed significant lower GFP levels than those observed in Min6 EV after 9 h of cytokines (EV: 1.15±0.07; oxHSPB1: 0.85±0.04) or tunicamycin (EV: 1.36±0.08; oxHSPB1: 0.95±0.05) treatment (Figure 5A, B). We also assess this parameter by analyzing the areas under the curves that integrate GFP levels during the whole period of treatment. We obtained smaller values for Min6 oxHSPB1 compared with Min6 EV cells after ER stress induction either by cytokines (oxHSPB1: 8.58±0.28; EV: 9.34±0.15) or tunicamycin (oxHSPB1: 9.47±0.12; EV: 10.26±0.14) (Figure 5C). These results suggested that HSPB1 overexpression was able to increase the rate of proteasomal protein degradation in cells exposed to ER stressors further contributing to an adaptive response leading to manageable ER stress and thus avoiding the fatal fate observed in wild type and further enhanced in silenced cells.

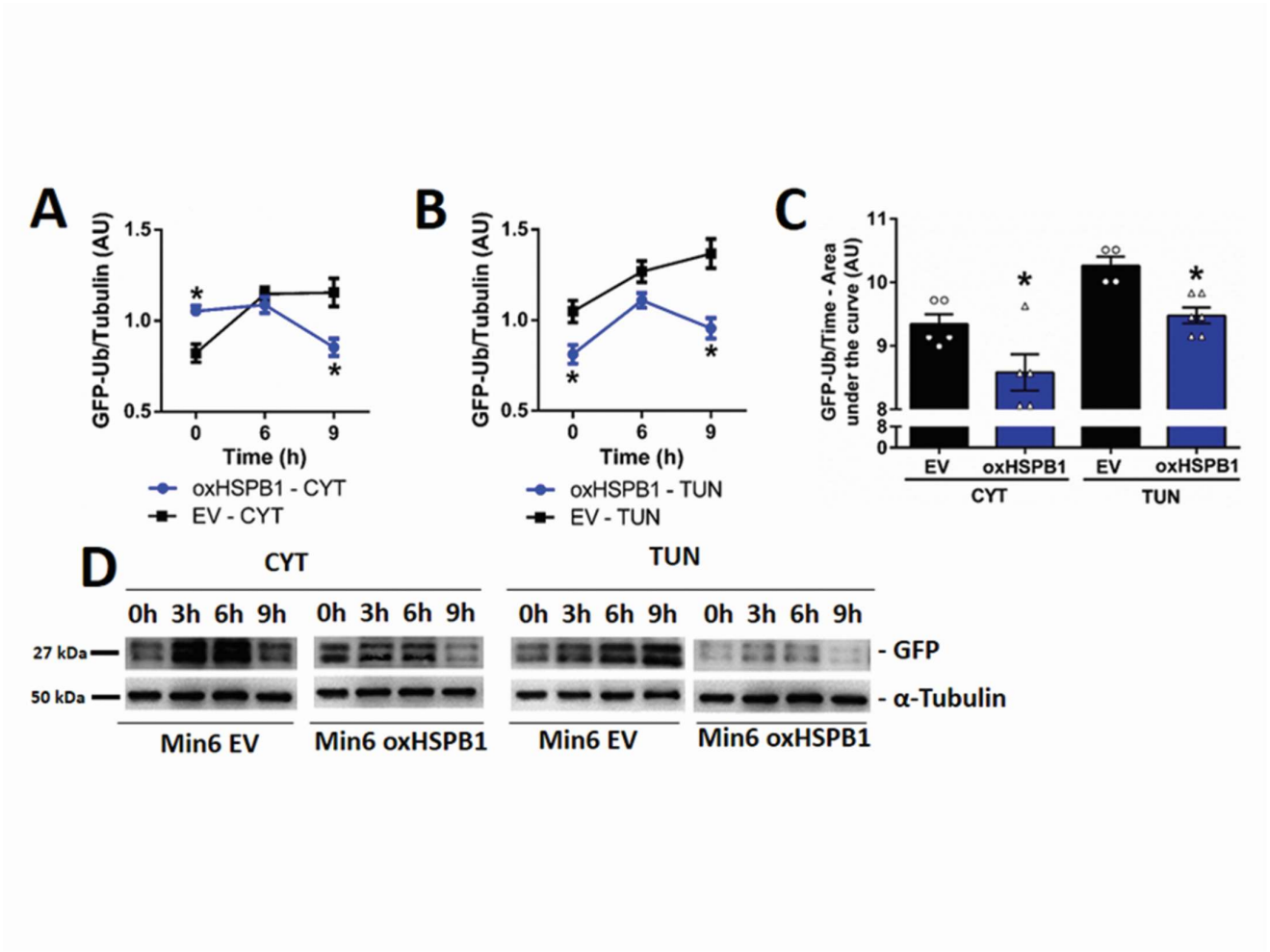

Figure 5. HSPB1 overexpression accelerates the rate of protein degradation by decreasing the levels of ubiquitinated proteins in Min6 cells submitted to ER stress. Min6 OX and Min6 EV cells were transfected with the GFP-Ub vector and were then exposed to a combination of pro-inflammatory cytokines (TNF-α 8 ng/mL, INF-γ 16 ng/mL, IL-1β 1.6 ng/mL) or tunicamycin (15 μg/mL). GFP levels were analyzed in total protein extracts obtained after 6 and 9h of treatment and analyzed by western blot assays. Quantification of the GFP levels presented as arbitrary densitometric units (AU) after normalization to the corresponding α-tubulin content in cells treated with **(A)** cytokines or **(B)** tunicamycin. **(C)** Histogram representation of the area under the GFP level curves of the different cell types submitted to ER stressors for 9 h. **(D)** Representative images of the immunoblots showing GFP expression in Min6 cells treated with cytokines or tunicamycin. Each data point represents means ± SEM from three replicates and at least three independent experiments were carried out for each cell type submitted to the different cell incubation conditions; *: $p < 0.05$ vs. EV.

3. Discussion

In the present study we were able not only to validate that HSPB1 is a key mediator of the cytoprotective effects mediated by prolactin in type 1 diabetes context [9,10] but also to demonstrate that HSPB1 up-regulation independently of PRL treatment is sufficient to

protect beta-cells and murine islets against ER stress-induced cell death. Moreover, this protein was capable to attenuate the UPR response by preventing the raise of the pro-apoptotic proteins CHOP and BIM. We have also shown that one of the HSPB1 cyto-protective mechanisms relies in the modulation of the proteasomal degradation pathway by enhancing not only the overall protein ubiquitination but also their degradation rate.

Increased expression of heat shock proteins is known to mediate the attenuation of various types of stress, including UPR activation and ER stress [36], and thus promoting cell survival [37]. It was recently demonstrated that prolactin and lactogen hormones protect INS1 cells, primary islets from Akita mice and human islets against cell death induced by tunicamycin and thapsigargin, by modulating UPR and attenuating pro-apoptotic proteins [8]. This effect was completely abrogated by STAT inhibition. These results further reinforce ours since we have previously shown, using human and mouse islets, that already after 2 h of PRL treatment HSPB1 protein expression was increased. This step was essential for cytoprotection and depended on JAK-STATs activation [9,38,39]. In the present study, we have further demonstrated that just HSPB1 overexpression is enough to improve cell viability via ER stress attenuation and beta-cell death inhibition.

Reduction of UPR pathways mediating cell death like PERK/eIF2α/ATF4, IRE1/XBP1s/CHOP and IRE1α/ASK1/JNK observed in the present study could be pointed as one of the reasons for increased beta-cell viability promoted by HSPB1 overexpression. It has been reported already that ATF4 regulates the expression of the pro-apoptotic factor CHOP, which is the main link between UPR and beta-cells apoptosis [40,41]. This one in turn, upregulates BIM, another factor directly linked to beta-cell death [42]. The attenuation of IRE1α phosphorylation may also be linked to a lower activation of the IRE1/XBP1s/CHOP pathway. It is known that in situations of prolonged neuronal stress, XBP1s can increase the expression of the pro-apoptotic factor CHOP 40. A recent report using mouse beta-cells showed that overexpression of XBP1s was associated with increased apoptosis and impairment of insulin expression as well as glucose-stimulated insulin secretion [43]. Sharma and collaborators have unveiled the crosstalk between ATF6 activation pathway and XBP1 targets in mouse islets submitted to ER stressors [44]. It is important to note that in their report ER stress was not induced with pro-inflammatory cytokines but with thapsigargin or tunicamycin. In their case, despite certain differences in the kinetics of the process, this crosstalk was independent on the ER stressor used [44]. Our results showed that apoptosis inhibition observed in two different models of insulin producing cells overexpressing HSPB1 could be associated with a decrease of cleaved ATF6 as well as XBP1s levels starting from 6 h and lasting at least 9 h. This effect was also cell type and stressor dependent. It is important to take into account that Min 6 cells are cells derived from an insulinoma [26] and, among the landmark of cancer cells a greater resistance to avoid cell death pathways is one of them.

The activation of the IRE1α-ASK1-JNK signaling pathway has also been postulated as a cell death trigger. JNK pro-apoptotic protein kinase that leads to the inhibition of anti-apoptotic BCL-2 proteins by phosphorylating them and thus inducing cell death in HEK 293T, lung cancer (A549) cells [45,46], hepatocytes [47] and beta-cells [48]. Like HSPB1, exendin-4 was reported to alleviate ER stress induced by exposure of rat insu-

linoma cells to high glucose and palmitate. This attenuation was mediated by a decrease of PERK, eIF2α, IRE1α and JNK phosphorylation, as well as reduced ATF6 levels [47].

HSPB1 overexpression promoted, in general, an attenuation of this pathway in murine pancreatic islets. This effect can be associated with the decrease of unfolded proteins in the ER through the activation of the ERAD that translocate non-functional proteins to the cytosol, which in turn will be ubiquitinated and directed to proteasomal degradation [19]. Synthesis and folding of insulin places a significant demand on beta-cells and failure to adapt to ER stress contributes to loss of function and beta-cell death [49–53]. Indeed, the increase in protein ubiquitination and speed of degradation observed in insulin-producing cells overexpressing HSPB1 in this study further corroborate this hypothesis. Moreover, the interactoma data obtained by our research group showed that only when PRL treatment promoted the up-regulation of HSPB1, this chaperone was able to interact with enzymes involved in the ubiquitination of proteins as well as with several catalytic subunits of the proteasome in Min6 cells incubated with pro-inflammatory cytokines [10]. Thus, one can state that by decreasing the levels of unfolded proteins in the ER the activation of the UPR may be significantly attenuated by HSPB1. Indeed, it has been implicated that UPR coupled with ERAD promotes better cell survival by mitigating ER stress [54–56]. In this scenario, heat shock protein 22 (HSP22) has been involved in protein degradation as an adapter protein between the unfolded substrate and the ubiquitination complex in rat cardiac myocytes [57]. There is also evidence that in cancer cell models HSPB1 is able to improve proteasome stability, allowing an increase in the complex ability to degrade proteins during ER stress by UPR [58]. Degradation of pro-apoptotic proteins may also be one of the pathways for improving cell survival. HSPB1 has been reported to participate in the degradation process of the pro-apoptotic factor BIM, promoting greater survival of pheochromocytoma-derived PC12 tumor cells subjected to ER stress [59]. In the context of T1DM, the up-regulation of BIM has already been reported as being part of the signals involved in beta-cell apoptosis induction [60,61]. HSPB1 was shown to increase the degradation of ubiquitinated proteins in response to stress stimuli triggered by TNF-α when interacting with the 26S portion of the proteasome in human leukemia (U937), murine embryogenic fibroblast and rat colon carcinoma cells [62].

Our previous studies of HSPB1´s interactoma in beta-cells have shown that HSPB1 was interacting mainly with several catalytic subunits of the proteasome [10]. In the present study, we have demonstrated that HSPB1-induced beta-cell survival depends on proteasomal activity. The high sensitivity of beta-cells to ER stress induced apoptosis can be related to the synthesis and secretion of insulin [63], requiring an efficient cellular protein translation, quality control machinery. Disturbances can lead to a rapid activation of cell death pathways [64].

Altogether, our data contributed to unveil a cytoprotective molecular mechanism where HSPB1 overexpression promotes UPR modulation resulting mainly in ER stress attenuation by increasing protein ubiquitination and proteasomal protein degradation. Overall, this process led to decreased levels of the key players linking ER stress and apoptosis such as CHOP and BIM and thus contributed beta-cell protection (Figure 6).

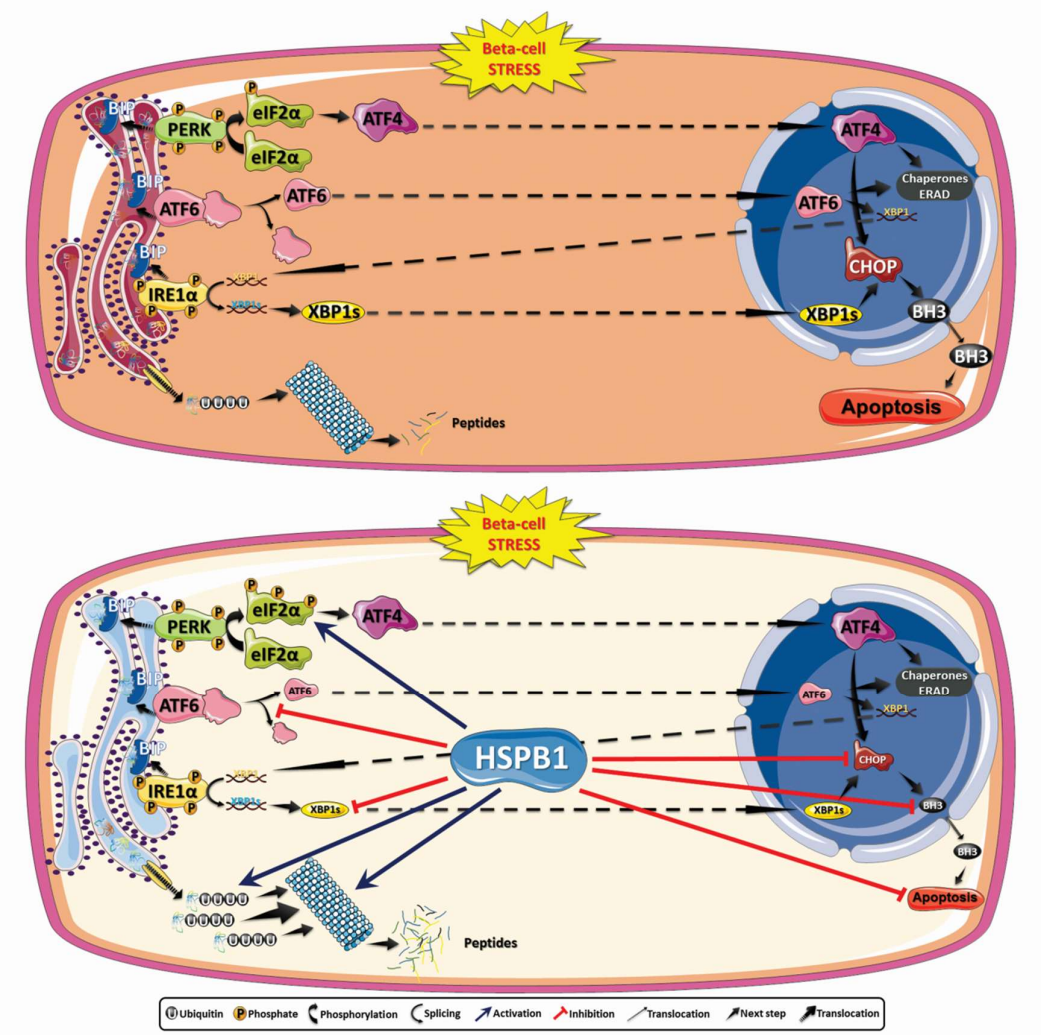

Figure 6. *HSPB1 inhibits ER stress-induced cell death by modulating UPR, protein ubiquitination and protein degradation rate via proteasome*. The UPR is activated by stress to restore cellular homeostasis. **(A)** Under conditions of intense and prolonged stress, UPR activates mechanisms of cell death by increasing the expression of pro-apoptotic proteins such as CHOP and proteins of the BH3-only family. **(B)** HSPB1 overexpression in mouse pancreatic islets promotes increased PERK phosphorylation, and attenuation of eIF2α phosphorylation, ATF4 and cleaved ATF6 levels, IRE1α pathway as well as pro-apoptotic mediators CHOP and BIM expression. This process, associated with an increase in protein degradation by the ubiquitin-proteasome system, allows keeping under control cellular proteostasis and thus avoids the activation of pro-apoptotic signaling pathways.

Collectively, our results provide deeper knowledge of HSPB1 action in beta-cells submitted to ER stress. They underscore the importance of further studies for the development of strategies to mitigate beta-cell death independently of the immune system modulation by upregulating HSPB1-activated endogenous protection pathways and thus pave the way for new therapeutic alternatives improving the outcome of islet transplantation by increasing beta-cell viability.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Supplementary Table 1**: List of primary antibodies used for protein detection by Western blot; **Supplementary Figure 1**: Increased HSPB1 expression modulates the UPR in Min6 cells under ER stress induced by pro-inflammatory cytokines or tunicamycin; **Supplementary Figure 2**: Increased HSPB1 expression modulates PERK and IRE1α phosphorylation in mouse islets under ER stress induced by pro-inflammatory cytokines or tunicamycin; **Supplementary Figure 3**: The presence of HSPB1 promotes a longer survival of murine pancreatic beta-cell with inhibited proteasome; **Supplementary Figure 4**: HSPB1 levels modulates global protein ubiquitination in Min6 cells.

Author Contributions: The authors have contributed to this study and manuscript by performing the following activities: Conceptualization, V.M.G and L.L.; methodology, R.A.M.W., A.F.S., L.F.T and T.C.O.; validation, D.R.Q.A and G.S.A.; formal analysis, V.M.G. and L.L.; investigation, V.M.G., S.L and L.L..; resources, S.L. and L.L.; data curation, L.L; writing—original draft preparation, V.M.G and L.L.; writing—review and editing, S.L. and L.L; visualization, A.F.S..; supervision, L.L..; project administration, L.L..; funding acquisition, L.L. All authors have read and agreed to the published version of the manuscript.

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