# Stress-induced tyrosine phosphorylation of RtcB modulates IRE1 activity and signaling outputs.

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Keywords: Endoplasmic Reticulum, UPR, XBP1s

Running title: Tyrosine phosphorylation-dependent regulation of XBP1 splicing

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**Abstract**

Endoplasmic Reticulum (ER) stress is a hallmark of various diseases. Cells cope with ER stress through the activation of an adaptive signaling pathway named the Unfolded Protein Response (UPR) which is mediated by three ER-resident sensors. The most evolutionary conserved of the UPR sensors is IRE1α that elicits diverse downstream effects through its kinase and endoribonuclease (RNase) activities. IRE1α RNase activity can either catalyze the initial step of XBP1 mRNA unconventional splicing or degrade a number of RNAs through Regulated IRE1-Dependent Decay (RIDD). The degree of exertion of these two activities plays an instrumental role in cells' life and death decisions upon ER stress. Until now, the biochemical and biological outputs of IRE1α RNase activity have been well documented, however, the precise mechanisms controlling whether IRE1 signaling is adaptive or pro-death (terminal) remain unclear. This prompted us to further investigate those mechanisms and we hypothesized that XBP1 mRNA splicing and RIDD activity could be co-regulated within the context of the IRE1α RNase regulatory network. We showed that a key nexus in this pathway is the tRNA ligase RtcB which, together with IRE1α, is responsible for XBP1 mRNA splicing. We demonstrated that RtcB is tyrosine phosphorylated by c-Abl and dephosphorylated by PTP1B. Moreover, we identified RtcB Y306 as a key residue which, when phosphorylated, perturbs RtcB interaction with IRE1α, thereby attenuating XBP1 mRNA splicing and favoring RIDD. Our results demonstrate that the IRE1α RNase regulatory network is dynamically fine-tuned by tyrosine kinases and phosphatases upon various stresses and depending on the nature of the stress determines cell adaptive or death outputs.

# Introduction

The imbalance between the cellular demand to fold proteins and the Endoplasmic Reticulum (ER) capacity to achieve this function under certain circumstances can result in the accumulation of misfolded/unfolded proteins in this compartment, a situation known as ER stress [1]. Activation of the UPR sensors IRE1α (Inositol-Requiring Enzyme 1 alpha; referred to as IRE1 hereafter), ATF6α (Activating Transcription Factor 6 alpha) and PERK (Protein kinase RNA (PKR)-like ER kinase) has as principal purpose to alleviate the stress and is known as the adaptive UPR. However, when the stress cannot be resolved, the UPR instead triggers apoptosis, which is referred to as terminal UPR. Thus far, the mechanisms allowing the switch between adaptive and terminal UPR remain uncharacterized. Among the possible candidates, the IRE1 pathway being greatly conserved through evolution plays crucial roles in both physiological and pathological ER stress. Similar to the other sensors, IRE1 is an ER transmembrane protein activated after dissociation from BiP (Binding immunoglobin Protein) and/or direct binding to improperly folded proteins [2].

IRE1 is characterized by the presence of both kinase and endoribonuclease domains in its cytosolic region. After its dimerization/oligomerization, IRE1 trans-autophosphorylates, allowing the recruitment of TRAF2 and subsequent activation of the JNK pathway [3]. IRE1 dimerization and phosphorylation also yields a conformational change which activates its RNase domain and leads to unconventional splicing of the XBP1 mRNA and subsequent expression of a major UPR transcription factor XBP1s [4,5]. Importantly, the ligation following the IRE1-mediated cleavage of the 26 nucleotide intron in the XBP1 mRNA is catalyzed by the tRNA ligase RtcB [6–9]. Through its RNase domain IRE1 also accounts for the cleavage of mRNA [10,11], rRNA [12] and miRNA [13,14] sequences, a process named Regulated IRE1-Dependent Decay (RIDD) [10]. Interestingly, the degree of oligomerization of IRE1α may define its RNase activity towards XBP1 mRNA splicing or RIDD, ultimately impacting on cell fate [15–17]. Although the order of oligomerization required for XBP1 mRNA splicing vs. RIDD activity is still debated, there is a consensus on the cytoprotective effects of XBP1s and the cell death-inducing outputs of RIDD under acute ER stress [14–16]. RIDD was also described under basal conditions to maintain ER homeostasis [18]. In this model, while XBP1 mRNA splicing is induced in the adaptive UPR (aUPR) and inactivated during the terminal UPR (tUPR), RIDD displays an incremental activation pattern reaching unspecific RNA degradation during tUPR. Despite our knowledge on IRE1 signaling biological outputs, little is known on the integration of these two RNase signals and how their balance impacts on the life and death decisions of the cell. The existence of a multiprotein complex recruited in IRE1 foci that dynamically changes composition during the course of ER stress, named “UPRosome”, has been suggested as a possible modulation mechanism [19,20]. This is supported by the recent identification of IRE1 interactors (e.g. PP2A and TUBα1a) regulating XBP1 mRNA splicing differently from RIDD [21].

Herein, we hypothesize that XBP1 mRNA splicing and RIDD are part of an auto-regulatory IRE1 RNase network. RIDD targets could possibly impact on the splicing of XBP1 mRNA, thus fine-tuning the response to ER stress and impacting on subsequent cell fate decisions. We identified PTP1B mRNA as a RIDD target affecting XBP1s activity by dephosphorylating RtcB, the tRNA ligase that performs XBP1s ligation. The tyrosine kinase c-Abl is also part of this network as it phosphorylates RtcB, thus

revealing a post-translational regulatory mechanism of the IRE1 RNase signaling outputs. The study furthermore supports the true existence and dynamic nature of a UPRosome whose composition can determine the outcome of IRE1 signaling.

# Results

**PTP1B contributes to XBP1 mRNA splicing and is a RIDD target**

We first hypothesized that molecules involved in the IRE1-XBP1 pathway could at the same time be RIDD targets, and in this way, represent a balance mechanism between the two activities. To first identify XBP1s regulators in the context of ER stress and possible RIDD substrates we conducted two different high-throughput approaches. First, an siRNA library against ER protein-coding RNAs was used to transfect HEK293T cells expressing a XBP1s-luciferase reporter [22] (**Fig. S1A**). The cells were then subjected to tunicamycin-induced ER stress, and luciferase activity was tested with light emission to identify XBP1s positive and XBP1s negative regulators (**Fig. 1A**). The thereby identified 23 positive and 32 negative regulators (out of a siRNA library targeting >300 hits) included proteins involved in metabolic processes (e.g. CH25H, DHCR7), post-translational modifications (e.g. POMT2, DMPK), ERAD pathway (e.g. SYVN1, UBC6) and cellular growth (e.g. RRAS, CD74) (**Fig. 1B**). Second, to identify mRNA that could be cleaved by IRE1, we performed an *in vitro* IRE1 mRNA cleavage assay as described previously [23] (**Fig. S1B**) yielding a total of 1141 potential RIDD targets. XBP1s regulators that could also be subjected to RIDD-mediated degradation were determined by intersecting the hit lists from both screens, yielding 7 candidates, namely: ANXA6, ITPR3, PTPN1 (positive XBP1s regulators), RYR2, TMED10, KTN1 and ITPR2 (negative XBP1s regulators). The lists from this study were later combined with a list of XBP1s regulators coming from a genome-wide siRNA screen [24]. Although we did not observe any common XBP1s modulators with our own study, likely explained by a sensitivity issue due to the targeted aspect of the library used in the present work, we note the existence of 33 more hits in the intersection between XBP1 splicing and RIDD. The functional association network of the reported molecules shows their involvement in UPR regulation, RNA processing and cellular homeostasis (**Fig. 1D, E**). Interestingly, among the 7 hits found was PTPN1, that encodes for the Protein-Tyrosine Phosphatase 1B (PTP1B), a protein previously reported by us to potentiate XBP1 splicing in response to ER stress [25].

We repeated these experiments first by using RT-qPCR and confirmed that PTP1B$^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) exhibited reduced XBP1 mRNA splicing compared to their PTP1B$^{+/+}$ (wt) counterparts when exposed to tunicamycin (**Fig. 1F, Fig. S1C**). Regarding the UPR sensors ATF6 and PERK, the expression of both BiP and HERPUD was also attenuated in PTP1B$^{-/-}$ cells while that of CHOP was increased, most likely as a compensatory mechanism (**Fig. S1D-F**). In the same cellular setting, we also observed a basal activity of all three UPR sensors (**Fig. S1G**). We next sought to test whether PTP1B mRNA is indeed a RIDD substrate. To this end, we used U87 cells expressing a dominant negative form of IRE1α (IRE1 DN) or an empty vector (EV) [26] and conducted an Actinomycin D chase experiment under ER stress or basal conditions to see the effect at a post-transcriptional level. Under basal conditions, U87 DN cells presented higher expression levels of PTP1B compared to the control cells (**Fig. 1G**). Moreover, upon ER stress (DTT or Tunicamycin), PTP1B

mRNA degradation was higher in control cells than in DN cells (**Fig. 1H, Fig. S1H**). Collectively, these results show that PTP1B mRNA is a RIDD target and PTP1B promotes IRE1-dependent XBP1 mRNA splicing.

**RtcB is tyrosine-phosphorylated by c-Abl and dephosphorylated by PTP1B**

Based on the experiments reported above, we concluded that PTP1B may represent a key regulator of IRE1 activity and investigated how this protein could mechanistically alter IRE1 signaling. PTP1B is a tyrosine phosphatase, and thus we first searched the PhosphoSitePlus database [27] for the presence of phosphotyrosine residues in proteins described previously to regulate IRE1α/XBP1s signaling. This revealed that while there was no reported phosphotyrosine residue for IRE1 (a result confirmed experimentally in our laboratory), six phosphotyrosine residues were reported for RtcB and found in phosphoproteomics studies (**Table S1**, [27–29]). Interestingly, multiple protein sequence alignment of the human RtcB and its orthologues in different species from the metazoan/animal kingdom revealed not only a high conservation of the protein across evolution, but also the conservation of specific tyrosine residues (**Fig. S2A**).

To monitor RtcB tyrosine phosphorylation in cellular models, we transfected HEK293T cells with the pCMV3-mouseRTCB-Flag plasmid and left these untreated or treated them with the tyrosine-phosphatase inhibitor bpV(phen). Cell extracts were immunoprecipitated with anti-Flag antibodies, and the immune complexes were immunoblotted using anti-phosphotyrosine (pY) antibodies. BpV(phen) treatment augmented the pY signal in the whole cell lysates (**Fig. S2B**) and showed that RtcB-Flag exhibited increased tyrosine phosphorylation (**Fig. 2A, arrowhead**). An identical result was observed with the endogenous RtcB (**Fig. S2C**).

We then tested whether RtcB interacts with PTP1B. To this end, HEK293T cells were transfected with RtcB-Flag and either wt PTP1B or PTP1B-C215S (trapping mutant; [30,31]) plasmids. Cell extracts were immunoprecipitated using anti-PTP1B antibodies and the immune protein complexes were immunoblotted using anti-RtcB antibodies. RtcB-Flag exhibited a more stable association with the C215S PTP1B than what was observed for its wild-type form (**Fig. 2B**), suggesting that the pY residue(s) in RtcB might be trapped by the mutant PTP1B. To further document the functional relationship between PTP1B and the tyrosine phosphorylation of RtcB, mRtcB-Flag was transfected in PTP1B$^{-/-}$ and PTP1B$^{+/+}$ MEFs, and the RtcB-Flag phosphotyrosine status was evaluated as described above. This revealed that the pY signal of mRtcB-Flag was increased in PTP1B$^{-/-}$ MEFs when compared to PTP1B$^{+/+}$ MEFs (**Fig. 2C**), which directly demonstrates that PTP1B is involved in the dephosphorylation of RtcB.

We next sought to identify a tyrosine kinase that could be responsible for RtcB phosphorylation. A recent study showed that the tyrosine kinase c-Abl acts as a scaffold for IRE1 by assisting its oligomerization and thus enhancing terminal UPR but is not involved in IRE1 phosphorylation [32]. In line with these results, we observed reduced mRtcB-Flag tyrosine phosphorylation when cells were treated with the tyrosine kinase inhibitors dasatinib or imatinib both targeting c-Abl in a specific manner (**Fig. 2D**). To further document the possible phosphorylation of RtcB by c-Abl, we performed an *in vitro* kinase assay using recombinant human His-tagged c-Abl as kinase and recombinant human GST-RtcB as substrate. This experiment showed that c-Abl phosphorylated RtcB *in vitro* (**Fig. S2D, Fig. 2E**). We then mapped the tyrosine

phosphorylation sites on RtcB using mass spectrometry and identified three tyrosine residues Y306, Y316 and Y475 (**Fig. 2F**), which were also previously reported in independent proteomics screens (**Table S1**). Among them, the LVMEEAPESpY$^{475}$K phosphopeptide was found not only in the $TiO_2$ –purified but also in the crude sample indicating its probable greater abundance.

**Molecular dynamics simulations of tyrosine phosphorylated RtcB and possible outcomes**

We next evaluated the impact of tyrosine phosphorylation on RtcB structure using molecular dynamics (MD) simulations. The starting structure was a recent homology model of the human RtcB protein [33]. The systems that were prepared and subjected to MD simulations were distinguished by the presence (active) or absence (inactive) of bound GMP at the active site of RtcB, and by the different combinations of phosphotyrosines. After the simulations, certain conformational differences on the RtcB systems such as the movement of helices or specific amino acids were observed (**Fig. 3A, B, Fig. S3, 4**). By itself, the addition of a phosphoryl group to a tyrosine gives a negative charge to this modified amino acid, that in turn induces local changes to both the electrostatic surface and the conformation. Collectively, these alterations can have implications on protein structure, substrate specificity and interaction surface. To quantify these changes, we performed post-MD analyses including determination of the solvent accessibility surface area (SASA) and pKa values for the three tyrosine residues, as well as characterization of each tyrosine residue as “buried” within the protein structure or more solvent exposed (**Table S2, S3**). In addition, we quantified the dihedral angles for each of the three tyrosines in all the different RtcB systems as a measure of their rotation (**Fig. 3C**). We observed that when RtcB is fully phosphorylated, Y306 completely changes orientation to become more solvent exposed than buried in the body of the ligase (**Fig. 3D, Fig. 3A, B**). pY306 is characterized as a residue existing in both fully buried and solvent-exposed conformations (**Table S2**). Consistently, analysis of the final structures after the 200ns simulations revealed that, although the differences in the SASA values are not striking, pY306 has the highest value of SASA among the three phosphotyrosines (pY306/pY316/pY475), and a higher SASA value than Y306 in the non-pY system (**Table S3**). Next, we measured the docking score of GTP in the active site of RtcB in the differentially tyrosine phosphorylated systems. Phosphorylation of Y475 totally abrogated the binding of GTP in the active site (**Fig. 3E, F**). This is not surprising since Y475 is located at the active site of the protein and the bulky phosphoryl group creates a barrier that does not allow GTP to bind to the His 428 residue for the subsequent enzymatic catalysis.

**Cellular impact of RtcB tyrosine phosphorylation**

We then tested the effects of preventing phosphorylation of these tyrosine residues in a cellular environment. To this end, we generated RtcB-Flag Y-to-F mutants (**Fig. 4A**) using site-directed mutagenesis to keep the aromatic ring in a conformation that cannot be phosphorylated. Single (Y306F, Y316F, Y475F), double (Y306F/Y316F) and triple (Y306F/Y316F/Y475F) mutants were obtained. HEK293T cells were transfected with the wt and mutant plasmids, treated with bpV(phen) and lysates were then immunoprecipitated with anti-Flag antibodies. Anti-pY immunoblot revealed that while the Y306F and Y316F single mutants showed similar levels of tyrosine phosphorylation to wt, the Y475F mutant had a reduced degree of tyrosine phosphorylation indicating a potential preference for this residue by c-Abl (**Fig. 4B**).

The double Y306F/Y316F mutant showed reduced tyrosine phosphorylation compared to the corresponding single mutants (**Fig. 4B**). The triple mutant still displayed tyrosine phosphorylation suggesting that other Tyr residues could be phosphorylated most likely by other tyrosine kinases. The additional phosphotyrosines found in previous mass spectrometry-based analyses are shown in **Table S1**. The interaction of each RtcB mutant with the substrate trapping mutant PTP1B-C215S was also tested as described earlier for the wt form of RtcB in **Fig. 2B**. The double (Y306F/Y316F) and mostly the triple (Y306F/Y316F/Y475F) mutants showed a statistically significant reduction of their physical association with the PTP1B trapping mutant compared to the wt form of RtcB (**Fig. S5A, B**). This could be indicative of a lack of preference for PTP1B to bind to one of the tyrosine sites but rather that the tyrosine phosphatase is able to bind and dephosphorylate all three sites Y306, Y316 and Y475. Following this observation, we generated HeLa cell lines stably expressing either the wt or the above-described mutant forms of RtcB-Flag (including also the catalytically inactive mutant H428A) (**Fig. 4A**). Cycloheximide chase-experiments were conducted on these cells to compare the protein stability of each RtcB-Flag forms. These analyses revealed that all RtcB-Flag proteins display a similar half-life (**Fig. 4C-D, S6A-B**) which was comparable to that of endogenous RtcB (**Fig. 4C-D, S6C**).

**Phosphorylation of RtcB at Y306 attenuates XBP1 mRNA splicing and enhances RIDD activity**

Since we confirmed that RtcB could be phosphorylated in cells, we next tested the impact of these phosphorylation sites on XBP1 mRNA splicing. To this end, we treated the HeLa stable cell lines with 1µg/ml Tun for 0, 2, 4, 8, 16 and 24 hours to monitor the XBP1 mRNA splicing (**Fig. 5A, D**), and the results were normalized to the expression of RtcB-Flag (**Fig. 5C, F**), thereby indicating RtcB-Flag specific activity with respect to XBP1 mRNA splicing (**Fig. 5B, E**). We observed that cells expressing the Y306F RtcB-Flag showed increased XBP1 mRNA splicing activity (**Fig. 5A, B**). Furthermore, cells expressing the double mutant exhibited reduced XBP1 mRNA splicing compared to the wt RtcB-Flag but performed slightly better than cells expressing the catalytically inactive mutant H428A (**Fig. 5D, E**). The latter matched MD simulations results indicating that phosphorylation of RtcB on Y475 abrogates the binding of GTP in the active site of the protein, thereby halting its catalytic activity. Interestingly, cells expressing the triple mutant showed a slightly lower XBP1 mRNA splicing than those expressing the H428A mutant thereby suggesting that the complete absence of c-Abl-phosphorylated tyrosines negatively affects RtcB in its contribution to XBP1 mRNA splicing (**Fig. 5D, E**). Because of the observed effect of Y306F RtcB on XBP1 mRNA splicing, we wondered whether RIDD activity would change as well. To address this, we performed an Actinomycin D chase experiment under ER stress (induced by either Tun or DTT) and monitored the expression PER1 and SCARA3 mRNAs, two RIDD substrates. Upon ER stress Y306F RtcB-Flag-expressing cells showed higher levels of PER1 and SCARA3 mRNA compared to the wt cells (**Fig. 5G**) corresponding to a lower RIDD activity. The same was observed even when the endogenous RtcB protein was knocked-down by an siRNA targeting the 3'-UTR of the RTCB mRNA (**Fig. 5G, H**). Despite the changes in XBP1 mRNA splicing and RIDD activity, when the splicing of the three intron-containing tRNA molecules in human (corresponding to the Tyr, Arg and Ile tRNA) was compared between the Y306F and the wt RtcB-Flag-expressing cells, there was no change in tRNA splicing activity (**Fig. 5I**). As a control to this experiment the levels of the intronless tRNA molecules Pro and Val were also tested (**Fig. 5J**). These results indicated that phosphorylation of RtcB on

Y306 represents a major regulatory mechanism to control the balance between XBP1 mRNA splicing and RIDD activity downstream of IRE1 RNase. The specificity of this regulation becomes even more apparent by the fact that tRNA splicing is not influenced by the RtcB Y306F mutation.

**Non-phosphorylable RtcB Y306 (Y306F) rescues a defective XBP1 mRNA splicing and confers resistance to ER stress-induced cell death**

Based on the observations obtained from i) MD simulations that pY306 is almost totally exposed to the solvent and not buried in the protein in its fully-pY status, and ii) from the XBP1 splicing activity time-course of the respective HeLa line that Y306F RtcB-Flag has a much better XBP1 splicing activity, we next tested if the phosphorylation of Y306 impacted on the interaction of RtcB with IRE1. This interaction was previously reported and shown to contribute to proper XBP1 mRNA splicing [6]. HEK293T cells were co-transfected with IRE1 wt and either the wt or the mutant forms of RtcB-Flag plasmids. Twenty-four hours post-transfection, cell lysates were immunoprecipitated with anti-Flag antibodies and immunoblotted with anti-IRE1 antibodies. We confirmed the interaction between IRE1 and RtcB (**Fig. 6A**) and found that the Y306F RtcB-Flag mutant exhibited a stronger interaction with IRE1 than the wt form (**Fig. 6A**). This additional observation urged us to test whether the Y306F mutant would rescue the defective XBP1 splicing observed in PTP1B$^{-/-}$ MEFs (**Fig. 1F, Fig. S7A, B**). PTP1B$^{-/-}$ and PTP1B$^{+/+}$ MEFs were either left untransfected or transfected with wt RtcB-Flag or Y306F RtcB-Flag plasmids (**Fig. S7D**) and monitored for XBP1 mRNA splicing upon 0, 2, 4, 8, 16 and 24 hours of 50ng/ml Tun treatment (**Fig. 6B**). Both wt and Y306F RtcB-Flag rescued XBP1 mRNA splicing in PTP1B$^{-/-}$ MEFs. Importantly, the evaluation of the %XBP1s revealed an improved rescue of the Y306F RtcB-Flag consistent with the effect on the XBP1s activity observed in stable HeLa cell lines (**Fig. 6C, D**). The rescuing effect of Y306F RtcB-Flag was profound even under acute ER stress where 10μg/ml Tun was applied for 6 hours (**Fig. S7C**). The importance of the phosphorylation event on the Y306 site is reinforced by the successful docking of c-Abl with RtcB after MD simulation (**Fig. S7E**). The interacting residues on c-Abl (D325, D444) and RtcB (K279, R283, K357) create a surface of contact where Y306 of RtcB is well accessible to the active site of the kinase (**Fig. S7F**).

On the grounds of the pro-survival character of XBP1 mRNA splicing, we then wondered whether phosphorylation of Y306 in RtcB could have an effect on the cellular survival/death. When cells stably over-expressing the wt or the Y306F form of RtcB were treated for 24 hours with increasing concentrations of DTT to induce ER stress, the survival advantage of the latter cells became apparent (**Fig. 7A**). Y306F RtcB-Flag expressing cells presented even less necrosis when endogenous RtcB was knocked-down by using an siRNA targeting the 3'-UTR of the RTCB mRNA. This meant that the observed effect was solely due to the exogenously expressed Y306F RtcB mutant (**Fig. 7A**). Under these specific ER stress conditions, in contrast to necrosis/late apoptosis, early cell apoptosis was not evident (data not shown). Efficient silencing of endogenous RtcB was confirmed by both qPCR using primers spanning the 3'-UTR of the RtcB mRNA (**Fig. S7G**) and western blot analysis (**Fig. S7H**). These results indicate that RtcB Y306 is a key residue in the IRE1-XBP1/RIDD signaling since it is phosphorylated by c-Abl and dephosphorylated by PTP1B, and it regulates the interaction between RtcB and IRE1. Further to this, we realize that phosphorylation of RtcB on Y306 is able to direct a decision towards cell death in the presence of ER stress downstream of the IRE1 RNase signaling outputs. To further document the role

of the pY-dependent interaction between RtcB and IRE1 in the control of cell death, we relied on the recent discovery that DNA-damaging agents signaled through RIDD (*Dufey et al. in press*) and hypothesized that forcing the splicing of XBP1 mRNA through the expression of Y306F RtcB should sensitize the cells to cell death. To test this, cells expressing wt or Y306F RtcB were treated with Doxorubicin or Etoposide and in both cases Y306F RtcB expressing cells were more prone to apoptosis than cells expressing wt RtcB (**Figure 7B**), thereby indicating that the balance between XBP1 mRNA splicing and RIDD i) is tightly controlled by RtcB, ii) plays a key player in stress-dependent life and death decisions and iii) determine the nature of the biological output depending on the stress cells are exposed to.

## Discussion

The objective of this study was to better characterize how XBP1 mRNA splicing and RIDD activities can crosstalk and how their signals are integrated. We showed that RtcB, the tRNA ligase responsible for XBP1 mRNA splicing, is at the center of a kinase/phosphatase network and that the tyrosine phosphorylation-dependent regulation of RtcB interaction with IRE1α might represent a mechanism to shift IRE1α activity from XBP1 mRNA splicing to RIDD. We have identified c-Abl as a tyrosine kinase responsible for the phosphorylation of RtcB on the three sites Y306, Y316 and Y475, and PTP1B as a tyrosine phosphatase de-phosphorylating RtcB. Interestingly, the phosphorylation of Y475 on RtcB, a residue located in the active site, attenuated XBP1 mRNA splicing. Indeed, the phosphorylation of Y475 prevents GTP binding to His 428, thus halting the process of ligation. More surprisingly, phosphorylation of Y306 disrupted the interaction between RtcB and IRE1 thus reducing XBP1 mRNA splicing activity. This could in turn favor the formation of IRE1 oligomers thus leading to the observed increase in RIDD activity. In the same line of evidence, the mutant Y306F RtcB rescued the defective XBP1 splicing observed in PTP1B$^{-/-}$ cells indicating that phosphorylation on this specific residue impacts negatively on the XBP1s activity and constitutes a PTP1B-dependent mechanism in the UPR. Moreover, the fact that the mutant Y306F RtcB conferred resistance to ER stress-induced cell death poses the Y306 phosphorylation (preceding or following the phosphorylation on Y316 and Y475) as a key cellular event allowing life/death decisions to be determined (**Figure 7C**).

Our data verify the model posed as our initial hypothesis by demonstrating the existence of an auto-regulatory network emanating from the IRE1 RNase activity. In this regulatory network PTP1B was identified as a key actor by dephosphorylating RtcB, thereby facilitating the activation of the IRE1/XBP1s axis. In the meantime, PTP1B mRNA is a RIDD substrate such that when RIDD becomes dominant, PTP1B expression is prevented. Ultimately, one could propose a new model in which PTP1B would keep RtcB in a non-phosphorylated state thereby allowing the IRE1/RtcB complex to efficiently splice XBP1, hence exerting adaptive UPR. However, when the stress is un-resolvable, PTP1B expression would decrease through a RIDD-dependent mechanism. In parallel c-Abl is recruited to the ER membrane [32] where it phosphorylates RtcB on Y306, Y316 and Y475, thus further inhibiting IRE1/RtcB interaction initially lowered by the reduction of PTP1B expression, and in the meantime, enhancing RIDD by prompting IRE1 oligomerization, hence resulting in terminal UPR.

It was demonstrated in a recent report that c-Abl can dissociate from 14-3-3 proteins in the cytosol upon ER stress, and be recruited to IRE1 foci at the ER membrane augmenting terminal UPR [32]. Herein, we show that c-Abl can act not only as a scaffold for IRE1 but also through its catalytic activity with the phosphorylation of RtcB on tyrosine residues that in turn causes the reduction of its XBP1 mRNA splicing activity. From our results and other studies, we can also speculate that apart from c-Abl, other tyrosine kinases might be involved in the regulation the UPR. For instance, the Src tyrosine kinase has recently been reported to be activated by and bind to IRE1 under ER stress leading to ER chaperone-relocalization to the cell surface [34]. As such, the scaffolding role of IRE1 could represent a means to integrate multiple signaling pathways thus far unrelated to IRE1 signaling, and link them to a clear function in cell homeostasis.

In the present study, we identified RtcB as a new target of the tyrosine phosphatase PTP1B. Interestingly, besides our initial report that PTP1B is involved in the control of IRE1 activity [25], PTP1B was subsequently found to also target the PERK arm of the UPR by negatively regulating its activation through direct dephosphorylation [35]. As such, one might consider tyrosine kinase/tyrosine phosphatase signaling as a new key element in the regulation of the UPR to control life and death decisions. Our findings indicate that the exchange of a phosphatase for a kinase and vice versa is crucial for the XBP1 mRNA splicing catalysed by IRE1 and RtcB. In the molecular complex of IRE1 and RtcB, an additional member could potentially be the archease protein reported as a stimulatory co-factor of RtcB not only for tRNA but also for XBP1s ligation [7,36]. Our results demonstrate that the dynamic alteration of the constituents of a multi-protein complex at the ER membrane can drive its collective activities, which further supports the notion of a UPRosome in the control of cellular life and death decisions [19,20].

## Materials and Methods

*Materials* - Tunicamycin (Tun), dithiothreitol (DTT), Actinomycin D, cycloheximide (CHX) and sodium orthovanadate ($Na_3VO_4$) were obtained from Sigma-Aldrich. The phosphatase inhibitor bpV(phen) and the tysosine kinase inhibitors afatinib, crizotinib and sorafenib were from Santa Cruz Biotechnologies. The tyrosine kinase inhibitors dasatinib and imatinib (Imatinib Mesylate - STI571) were from Selleckchem. Protease and phosphatase inhibitor cocktail tablets were from Roche through Sigma-Aldrich. Pierce ECL Western Blotting Substrate was from Thermo-Fisher Scientific. The pCMV3-mRTCB-Flag plasmid encoding the wt form of mouse RtcB was purchased from Sino Biological. The various mutant forms of this plasmid were made with the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent using the appropriate primers each time (**Table S4**). The pcDNA3.1/Zeo-PTP1B wt and pcDNA3.1/Zeo-PTP1B C215S plasmids were used as previously described [37]. The pCDH-CMV-IRE1α-EF1-Puro-copGFP was cloned in the lab as previously described [23].

*Cell culture and transfection* - HEK293T and HeLa cells, and mouse embryonic fibroblasts (MEFs) wt or KO for PTP1B (obtained in [25]) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in a 5% CO2 incubator. For the generation of HeLa cell lines stably expressing each one of the RtcB-Flag proteins, cells were first transfected using Lipofectamine 2000 (Thermo-Fisher Scientific) with 1μg of the corresponding plasmid. After 24 hours, the medium of the cells was changed to medium containing 600μg/ml Hygromycin B (Thermo-Fisher Scientific) to start the selection process. The Hygromycin B-containing medium was replaced every three days for a total period of 21 days, when polyclonal populations of HeLa cells stably expressing each of the RtcB-Flag proteins were obtained. The stable cell lines were maintained in DMEM with 10% FBS containing 120μg/ml Hygromycin B. Transient transfections were achieved using either polyethylenimine (PEI) for the HEK293T cells or Lipofectamine 2000 for the HeLa cells and the MEFs together with the desired plasmid. For preparation of the PEI solution, branched polyethylenimine (average $M_w$~25,000) powder was purchased from Sigma-Aldrich.

*siRNA-based screening assay* - The library of approximately 300 small-interfering RNAs (siRNA) against genes encoding ER proteins was used in previous studies [38]. Each siRNA (25 nM) was transfected into HEK293T cells by transfection with the Lipofectamine RNAiMAX reagent (Thermo-Fisher Scientific). The cells were then transfected with an XBP1s-luciferase reporter in which the firefly luciferase gene is fused to the XBP1 gene [22]: under basal conditions where XBP1 mRNA remains unspliced, a stop codon at the gene fusion is in-frame thus not allowing the luciferase to be expressed. In contrast, under ER stress conditions the splicing of XBP1 mRNA shifts the ORF (open reading frame) resulting in the loss of the stop codon downstream of XBP1 and the subsequent expression of luciferase. ER stress was after applied to the cells and sequentially the substrate of the luciferase enzyme, luciferin. The cells were then tested for the emission of light that after appropriate quantification and a value threshold set-up led to the distinction of two major groups of genes: XBP1s positive and XBP1s negative regulators. High values of luminescence (>1 AU) signify that efficient XBP1 splicing took place so the luciferase expression was allowed, thus marking the silenced genes as negative XBP1s regulators. The opposite is true for values <1 AU that place the silenced genes in the category of positive regulators of XBP1 mRNA splicing.

*in vitro IRE1 mRNA cleavage assay* - As described previously [23], in this assay total RNA was extracted from U87 cells and incubated or not with recombinant IRE1α protein under appropriate physicochemical conditions. The resultant RNA sequences from the cleaved and uncleaved conditions underwent polyA mRNA isolation, the final different groups (non-polyA: cleaved or uncleaved, and polyA: cleaved or uncleaved) were reverse transcribed and the respective cDNA material was hybridized on a human transcriptome array revealing potential RIDD substrates.

*Immunoblot and Immunoprecipitation* - For preparation of whole cell extracts, cells were incubated in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0,5% sodium deoxycholate, 0.1% SDS, 2mM EDTA and 50mM NaF) supplemented with protease and phosphatase inhibitors, for 20 min on ice. Cells were scraped and centrifuged at 4 °C for 7 min at 17,000 x g to collect the supernatant containing the protein. The samples were applied to SDS-PAGE and analyzed by immunoblotting. Dilutions of primary antibodies used for immunoblotting were as follows: mouse monoclonal anti-FLAG M2 (Sigma-Aldrich), 1:2000; rabbit polyclonal anti-RtcB (Proteintech), 1:1000; for recognition of the human PTP1B, mouse monoclonal anti-PTP1B (3A7) (Santa Cruz Biotechnologies), 1:1000; for recognition of the mouse PTP1B, rabbit polyclonal anti-PTP1B (ab88481) (Abcam), 1:1000; rabbit polyclonal anti-IRE1α (B-12) (Santa Cruz Biotechnologies), 1:1000; mouse cocktail anti-pY-HRP (PY-7E1, PY20) (Invitrogen/Thermo-Fisher Scientific), 1:2000, goat polyclonal anti-Actin (I-19) (Santa Cruz Biotechnologies), 1:1000; mouse monoclonal anti-VCP (BD Transduction Laboratories), 1:1000; rabbit polyclonal anti-CNX/Calnexin (kindly provided by Dr. John Bergeron, McGill University, Montreal, QC, Canada), 1:1000. Polyclonal goat anti-mouse, goat anti-rabbit and rabbit anti-goat secondary antibodies conjugated to HRP (Dako-Agilent) were used at a dilution of 1:7000, 1:7000 and 1:3500, respectively. For the immunoprecipitation (IP), the cells were lysed in CHAPS buffer (30 mM Tris-HCl pH7.5, 150 mM NaCl and 1.5% CHAPS) supplemented with a cocktail of protease and phosphatase inhibitors. The cell lysis buffer was further supplemented with 1mM of the phosphatase inhibitor $Na_3VO_4$ when the immunoprecipitates were going to be tested for pTyr. The lysates were then incubated for 16 h at 4°C with the indicated IP antibody (1µg Ab/1000µg protein). After this, dynabeads protein G (Thermo-Fisher Scientific) were first washed with CHAPS lysis buffer and/or PBS, then mixed with the protein/Ab mixture, incubated at room temperature for 20 min or at 4°C for 40 min with gentle rotation and washed with CHAPS and/ or PBS. Finally, the beads were eluted with 1x Laemmli Sample Buffer (LSB), heated at 55-95°C for 5 min and loaded to SDS-PAGE. For the immunoblotting, the suitable primary and secondary antibodies were used.

*RT-PCR and RT-qPCR* - Total RNA was extracted from cells using Trizol (Thermo-Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized from the total RNA using the Maxima Reverse Transcriptase enzyme, random hexamer primers, dNTP mix and the Ribolock RNase inhibitor (Thermo-Fisher Scientific). PCR was performed on the template cDNA using Phusion High-Fidelity DNA Polymerase and dNTP mix (Thermo-Fisher Scientific). Quantitative PCR was alternatively performed for the cDNA using the SYBR® Premix Ex Taq™ (Tli RNase H Plus) (TAKARA-Clontech) using a QuantStudio5 system (Applied Biosystems). The primer sequences used for these experiments were synthesized by Eurogentec and are shown in **Table S4**.

*Cell death assay* - HeLa cells stably expressing RtcB-Flag proteins were left untransfected or transfected with 10nM of a duplex siRNA sequence (**Table S4**; IDT, Integrated DNA Technologies) targeting the 3'-UTR of the RTCB mRNA. Forty-eight hours later the cells were treated with 0, 1, 2.5, 5, 7.5 and 10 mM DTT or 0, 0.5, 1 and 2 μM Doxorubicin or 0, 5, 10 and 20 μM Etoposide for 24 hours. The cells were then collected in tubes, along with the corresponding supernatants and PBS washes, and centrifuged at 1700 rpm or 750 xg for 5 min. The cell pellets were transferred in a round-bottom 96-well plate where 1x Annexin V buffer and Annexin V were added. After a 15 min-incubation at room temperature and a PBS wash, 2% FCS in PBS and 7-AAD were added to the samples. The latter were incubated at room temperature for 5 more min and then analyzed by FACS (NovoCyte 3000).

*In vitro kinase assays* - To perform an *in vitro* kinase assay, we used 0.36-0.5μg of human recombinant His-tagged ABL (His-ABL1 or c-Abl: 126 kDa) from Carna Biosciences as the tyrosine kinase and 1μg of human recombinant GST-tagged RtcB (GST-C22orf28: 81.6 kDa) (P01) from Abnova as the substrate. These were incubated in 1x kinase buffer (25mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 1mM $MnCl_2$, 0.5mM DTT, 10μM ATP, 0.1 mM $Na_3VO_4$ and 5mM β-glycerophosphate) in a total volume of 30μl at 37°C for 30 min or at RT for 2 hours. Upon completion of the reaction, 5x LSB was added to the reaction samples which were heated to 100°C and applied in a 6% SDS-PAGE.

*Mass spectrometry analysis* - Samples for the mass spectrometry analysis came from a scaled-up *in vitro* kinase reaction containing 0.9μg of human recombinant His-c-Abl (His-ABL1 or c-Abl: 126 kDa) and 5μg of human recombinant GST-RtcB in the same conditions as described in the above section. The difference is that the samples were ran on a pre-cast 8% polyacrylamide gel (Eurogentec), which was afterwards stained with 10% CBB and decoloured to be sent for the analysis. At the proteomics platform, the gel was re-stained with Page Blue Protein Staining Solution (Thermo-Fisher Scientific). In-gel digestion was then performed with trypsin (cleavage site = K/R) or lysC (cleavage site = K) and $TiO_2$ purification (10% not purified as control) followed. For the MS analysis, the recovery volume was 15μl 0.1%TFA and the injection volume was 5μl. The instrument LTQ Velos and the PepMap 25cm or Orbitrap ELITE / C18 Accucore 50cm columns were used. 1 hour run MSA and HCD method was used. For the data processing, the software Proteome Discoverer 2.1 (Sequest HT / Percolator) was used with the thresholds of 1% FDR, ptmRS for site assignation.

*Parameterization, Molecular Dynamics simulations and post-MD analyses* - The starting structure for the molecular dynamics (MD) simulations was a homology model of the human RtcB protein [33]. For the modified versions of the protein regarding its tyrosine phosphorylation states, we edited the initial structure through YASARA (Yet Another Scientific Artificial Reality Application, v15.3.8) [39]. For the MD simulations using Gromacs 5.1 [40], preparation and generation of additional missing parameters to the AMBER ff14SB force field [41] was performed for each structure using the AmberTools17 package [42]. Since RtcB contains a $Mn^{2+}$ ion in its active site, we employed the python-based metal center parameter builder MCPB.py (version 3.0) [43] included in AmberTools17 for the parameterization of the metal site. In addition, separate pdb files were generated for the non-standard amino acid of phosphorylated tyrosine (named "TYP") and GMP bound to H428 (named "HIG"), using standard

protocols. The resulting topology and coordinate files from Amber were transformed to the respective Gromacs files in order to perform the necessary system preparations for the MD simulations. Each system was solvated with TIP3P water [44] under periodic boundary conditions with the minimum distance between any atom in the solute and the edge of the periodic box being 10.0 Å. $Na^+/Cl^-$ counterions were added as appropriate for the neutralization of the system. Energy minimization was conducted until the force was less than 1000 kJ $mol^{-1}$ $nm^{-1}$, followed by a 100ps NVT and a 100ps NPT equilibration, and a final 200ns classical MD simulation for each system, using the Gromacs 5.1 package [40]. The temperature was kept at 300 K by the velocity rescaling thermostat [45] with a coupling constant of 0.1 ps. In the NPT equilibration and MD simulation the pressure was kept at 1.0 bar using the Parrinello-Rahman barostat [46] with a coupling time of 2.0 ps. The leap-frog algorithm [47] was used with an integration time-step of 2 fs and the LINCS algorithm [48] was used to apply constraints on all bonds. UCSF Chimera [49] was used to visualize the processed systems. For quality control of our simulations we calculated the RMSF and RMSD of our systems. Post-MD analyses also included calculation of the surface solvent accessible area via Gromacs, pKa values and percentage of surface/buried area of the key tyrosines via the PROPKA 3.0 part of the PDB2PQR web server 2.0.0 [50]. To determine the relative positions of the tyrosines of interest and illustrate if and how much they are rotated, we set as a fixed point the $Mn^{2+}$ ion in the active site of RtcB and calculated the dihedral angles consisting of this metal ion and the CA, CB and O atoms of the tyrosine residue using UCSF Chimera. For the representation of the dihedral angles, polar charts were created using the matlablib 2.1.2 in python [51]. Furthermore, we calculated the docking score of the ligand GTP in the active site of the different RtcB systems (without GMP in the active site). For this purpose, among the programs of the Schrödinger Suites 2018-1 package, we used Protein Preparation Wizard [52] to prepare the protein structure, LigPrep to prepare the GTP ligand and Glide [53] for receptor grid generation and XP ligand docking. The grid in which the ligand was allowed to dock was determined by analyzing the interactions between GMP and its surrounding amino acids in MOE (Molecular Operating Environment, 2016.01; Chemical Computing Group) [54]. For the c-Abl – RtcB interaction, we used the active form of Abl (PDB-ID: 2GQG). The ATP molecule was aligned inside protein using Chimera, based on the crystal structure of ATP bound to the structurally similar kinase Src (PDB-ID: 5XP7). The proteins were docked using PatchDock [55], with Y306 (RtcB) and ATP (c-Abl) chosen as interacting units. Following visual inspection of obtained complexes, the best aligned complex was prepared for MD simulations as outlined above. In order for the complex to not immediately dissociate, a set of seven position restrained equilibrations, with gradually reduced restraints in each, were applied. Finally, the system was subjected to a 200 ns MD simulation without position restraints.

*Bioinformatics tools* - For the creation of Venn diagrams, we used the online tool http://bioinformatics.psb.ugent.be/webtools/Venn/. For the creation of gene networks and functional annotations, we used the online String database 11.0 (https://string-db.org/) [56]. Multiple sequence alignments were done using Clustal Omega 1.2.4 [57]. The PhosphoSitePlus database was used for the search of reported tyrosine phosphorylation sites on the proteins of interest [27].

*Statistical analyses* - Student's t-test, one-way ANOVA and two-way ANOVA were applied for the statistical analyses depending on the experimental setting through the GraphPad Prism 7.0a software.

## Acknowledgements

We thank Dr Anna Reymer, Antonio Carlesso, Dr Samuel Genheden and Johanna Hörberg for training on computational chemistry/biology (UGOT, Göteborg, Sweden), Dr Aeid Igbaria for critical reading of the manuscript. This work was funded by grants from Institut National du Cancer (INCa PLBIO), Fondation pour la Recherche Médicale (FRM, équipe labellisée 2018) to EC and RP; by EU H2020 MSCA ITN-675448 (TRAINERS) and MSCA RISE-734749 (INSPIRED) grants to EC and LAE. The Swedish Research Council (VR) and the Swedish National Infrastructure for Computing (SNIC) are gratefully acknowledged for funding and allocations of computing time at the C3SE and PDC supercomputing centers, respectively (LAE). AP is a Marie Curie early stage researcher funded by EU H2020 MSCA ITN-675448 (TRAINERS). AM was funded by the Fondation ARC pour la recherche contre le cancer.

## Conflict of interest

EC and LAE are founders of Cell Stress Discoveries Ltd. The authors declare no conflicting interests.

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# Figure Legends`

**Figure 1. siRNA screening and in vitro IRE1α cleavage assay identify PTP1B as both XBP1 splicing regulator and RIDD target.** (**A**) siRNA-based screening assay: HEK293T cells were transfected with siRNA sequences against genes encoding ER proteins. They were subsequently transfected with an XBP1s-luciferase reporter (Fig. S1A) and after 48h and the induction of ER stress, the cells were tested for the intensity of light signal after the addition of luciferin. (**B**) Luminescence quantification of the screening assay described in (A). (**C**) Venn diagram of the gene list resulting from the assay described in (A-B), a list of possible RIDD substrates coming from an in vitro IRE1α cleavage assay (Fig. S1B) and a genome-wide siRNA-based screening assay (described in [24]). (**D**) A schematic network of the genes in the intersections of the lists as described in (C). (**E**) Gene ontology (GO) annotation of the gene network shown in (D). (**F**) $PTP1B^{+/+}$ (wt) and $PTP1B^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were treated for 0 and 6 hours with 10μg/ml Tun. RNA was isolated and the resulting samples were analyzed with qPCR for spliced and total XBP1 mRNA levels. The bar graph presents the XBP1 mRNA splicing as the ratio of spliced XBP1 to total. For the latter, qPCR primers amplifying the region before the cleavage site or the region spanning the cleavage site were used (Fig. S1C). (**G-H**) PTP1B mRNA expression levels in U87 cells expressing or not a dominant negative form of IRE1α at 0h time-point (G) and during DTT treatment (1mM) with a 2-hour Actinomycin D (5μg/ml) pre-treatment (H). EV: empty vector, IRE1 DN: dominant negative (cytosolic-deficient) form of IRE1α. Data values are the mean ± SEM of $n \geq 3$ independent experiments (*$p < 0.05$, **$p < 0.01$). Error bars for (H) are not depicted.

**Figure 2. RtcB is a substrate of the tyrosine kinase c-Abl and the tyrosine phosphatase PTP1B.** (**A**) HEK293T cells were left untransfected (CTL) or transfected with 2μg of the pCMV3-mRTCB-Flag plasmid (RtcB-F) and treated 24 hours post-transfection with 15μM bpV(phen) for 2 hours. Immunoprecipitation was done in the cell lysates using Flag antibody and the immunoprecipitates were immunoblotted for phosphotyrosine. The membrane was re-probed with Flag and RtcB, sequentially. The corresponding input samples are also shown immunoblotted for Flag, RtcB, pY-HRP and VCP. (**B**) HEK cells were left untransfected (CTL) or transfected with 1μg of the wt RtcB-Flag and 1μg of either the wt PTP1B plasmid or the C215S mutant one. Immunoprecipitation was done in the cell lysates with the PTP1B antibody and the immunoprecipitates were immunoblotted for RtcB, while the membrane was re-probed with PTP1B. Inputs probed for RtcB, Flag, PTP1B and VCP are shown. (**C**) $PTP1B^{+/+}$ or $PTP1B^{-/-}$ MEFs were left untransfected (CTL) or transfected with 2μg wt RtcB-Flag and treated with 15μM bpV(phen) for 2 hours. Immunoprecipitation was done in the cell lysates using Flag Ab and the immunoprecipitates were immunoblotted for phosphotyrosine, while the membrane was re-probed with Flag. Input samples probed for Flag, RtcB, PTP1B, pY-HRP and VCP are shown. (**D**) HEK293T cells transfected with the wt RtcB-Flag were treated 24 hours post-transfection with 10μM of tyrosine kinase inhibitors afatinib, crizotininb, dasatinib, imatinib and sorafenib for 8 hours and 15μM bpV(phen) for 2 hours. Immunoprecipitation was done in the cell lysates using Flag antibody and the immunoprecipitates were first immunoblotted for phosphotyrosine and then re-probed with Flag Ab. Input samples probed for pY-HRP, Flag and VCP are shown. (**E**) A cartoon showing the tyrosine phosphorylation of RtcB by c-Abl. (**F**) Samples from a scaled-up in vitro kinase reaction containing recombinant human c-Abl and RtcB were analyzed with mass spectrometry. Fragment spectrums

corresponding to three different phospho-peptides containing tyrosine residues are depicted. The blots shown in (A-D) are representative of three or more independent experiments. In (A, C, D) the arrowhead denotes the RtcB-Flag protein and the band appearing below this at exactly 55kDa corresponds to the Ig heavy chains of the Flag Ab used for the IP.

**Figure 3. Molecular dynamics of RtcB tyrosine-phosphorylated systems and possible implications.** (**A**) Ribbon-like structures of the RtcB protein resulting from the MD simulations either non-phosphorylated (left) or phosphorylated at Y306, Y316 and Y475 (right). (**B**) Zoom-in at Y306, Y316 and Y475 after superposition of the two RtcB models shown in (A). (**C**) Scheme explaining the calculation of a dihedral angle for a tyrosine relative to the $Mn^{2+}$ in the active site of RtcB. The example shows Y306 in the unphosphorylated RtcB system. (**D**) The dihedral angles in degrees (°) of each of the three tyrosines 306, 316 and 475 relative to the metal ion in the protein active site in all RtcB systems after 200 ns MD simulation, depicted in polar charts. (**E**) Scheme showing the docked GTP ligandin the active site of the unphosphorylated RtcB protein. (**F**) Glide XP docking scores of GTP in the active site of the different RtcB systems.

**Figure 4. Generation of cellular models with altered RtcB tyrosine phosphorylation status.** (**A**) Scheme showing the RtcB-Flag protein encoded by the pCMV3-mRtcB-Flag plasmid and the mutant forms that were created in this study. (**B**) HEK293T cells were left untransfected (CTL) or transfected with 2μg of the different RtcB-Flag plasmids. 24 hours after transfection the cells were treated with 15μM bpV(phen) for 2 hours. IP was performed in the cell lysates using Flag antibody and the immunoprecipitates were immunoblotted for phosphotyrosine. The membrane was re-probed with Flag. The corresponding input samples are also shown immunoblotted for Flag and VCP. The arrowhead denotes the RtcB-Flag protein and the band appearing below this at 55kDa corresponds to the Ig heavy chains of the Flag Ab used for the IP. The blot shown is a representative one out of three independent experiments. (**C**) HeLa stable lines expressing wt or mutant RtcB-Flag were treated with 10μg/ml CHX for 0, 1, 2, 4 and 8 hours to block translation and monitor the current protein levels. Here, RtcB-Flag expression levels were monitored in the resulting protein lysates. The Parental HeLa cells were included to additionally monitor the levels of the endogenous RtcB. VCP was used as a loading control. The blots shown here are representative of three independent experiments. (**D**) Half-life ($t_{1/2}$) calculated for the different forms of the RtcB-Flag protein or the endogenous RtcB in the case of the Parental HeLa cells.

**Figure 5. Phosphorylation of RtcB on Y306 negatively affects XBP1 mRNA splicing but not RIDD activity.** (**A, D**) HeLa lines stably expressing wt or mutant RtcB-Flag were treated with 1μg/ml Tun for 0, 2, 4, 8, 16 and 24 hours. cDNA coming from these were analyzed by qPCR for XBP1s and XBP1 total mRNA levels with their ratio being the XBP1 mRNA splicing as depicted in the y axis of the graphs. The Parental HeLa cells are also included for comparison. (**B, E**) The peak of XBP1s activity at 8 hours (A, D) was then normalized to the protein levels (C, F) to obtain the RtcB specific activity. (**C, F**) Stable HeLa lines were lysed and analyzed for RtcB-Flag expression levels by immunoblotting with anti-Flag. Actin was used as a loading control. (**G**) HeLa lines stably expressing wt or Y306F RtcB-Flag were left untransfected or transfected with an siRNA targeting the 3'-UTR of the RTCB mRNA. 48 hours post-transfection the

cells were pre-treated for 2 hours with 5μg/ml Actinomycin D and then treated either with 5μg/ml Tun for 4 hours or 1mM DTT for 2 hours. The graph shows the mRNA levels of PER1 (upper part) and SCARA3 (lower part) after normalization to the untreated samples (0h time-point). (**H**) RT-qPCR analysis of the untreated samples from (G) for the endogenous RTCB mRNA using primers spanning its 3'-UTR. (**I**) cDNA from HeLa stable lines expressing wt or Y306F mutant RtcB-Flag was analyzed with qPCR for the splicing of the three intron-containing tRNA molecules in human Tyr, Arg and Ile. The graph shows the percentage of each spliced tRNA molecule to their total levels (unspliced + spliced). The presented values have been normalized to the levels of % tRNA splicing in Parental HeLa cells. (**J**) The levels of the intronless tRNA molecules Pro and Val were measured by RT-qPCR in the samples of (I). Values have been normalized to the levels measured in Parental HeLa cells. Data values for all the presented graphs are the mean ± SEM of n≥3 independent experiments (*p < 0.05, **p < 0.01). Error bars for (A, D) are not depicted.

**Figure 6. The Y306F RtcB mutant rescues a defective XBP1 mRNA splicing.** (**A**) HEK cells were co-transfected with 1μg of wt IRE1α and 1μg of wt or mutant RtcB-Flag plasmid. 24 hours later, cell lysis and IP for Flag was performed. The immunoprecipitates were blotted for IRE1α and the input samples for IRE1α, Flag and Actin. The arrowhead denotes the RtcB-Flag protein and the band appearing below this at 55kDa corresponds to the Ig heavy chains of the Flag Ab used for the IP. (**B**) $PTP1B^{+/+}$ (wt) and $PTP1B^{-/-}$ (KO) MEFs untransfected (CTL) or transfected with 2μg of wt or Y306F RtcB-Flag plasmid were treated 24 hours post-transfection with 50ng/ml Tun for 0, 2, 4, 8, 16 and 24 hours. Their cDNA was analyzed by PCR using primers recognizing the XBP1 mRNA. (**C**) Quantification of the gels in (B). (**D**) The slope of each curve in (C) was calculated and for each condition the slope of the wt cells was subtracted from the one of the KO cells, called as Δslope. Data values for are the mean ± SEM of n≥3 independent experiments (ns: non-significant, *p < 0.05, ***p < 0.001, ****p < 0.0001).

**Figure 7. RtcB Y306 phosphorylation is a key event in modulating stress-induced cell life and death decisions. A**) HeLa lines stably expressing wt or Y306F RtcB-Flag were transfected or not with siRNA sequences against the endogenous RTCB mRNA. 48 hours post-transfection they were treated with 0, 1, 2.5, 5, 7.5 and 10 mM DTT for 24 hours. The resulting samples were then analyzed by FACS for cell necrosis and apoptosis through 7-AAD and Annexin V staining, respectively. Data values are the mean ± SEM of n≥3 independent experiments (ns: non-significant, ***p < 0.001, ****p < 0.0001). **B**) HeLa lines stably expressing wt or Y306F RtcB-Flag were left untransfected or transfected with siRNA sequences against the endogenous RTCB mRNA. 48 hours post-transfection they were treated with 0, 0.5, 1 and 2 μM Doxorubicin, or 0, 5, 10 and 20 μM Etoposide for 24 hours. The resulting samples were then analyzed by FACS for cell necrosis and apoptosis through 7-AAD and Annexin V staining, respectively. Data from 2 μM Doxorubicin and 5 μM Etoposide treatments are shown. Data values are the mean ± SEM of n = 4 independent experiments in Two-Way ANOVA and post-Tukey multiple comparisons test (*p < 0.05, ***p < 0.001). **C**) Model representation of IRE1 activation and signaling towards XBP1s or RIDD. A decrease in PTP1B expression driven by RIDD is thought to reduce the formation of the IRE1/RtcB complex thereby pushing towards unleashed RIDD and terminal UPR.

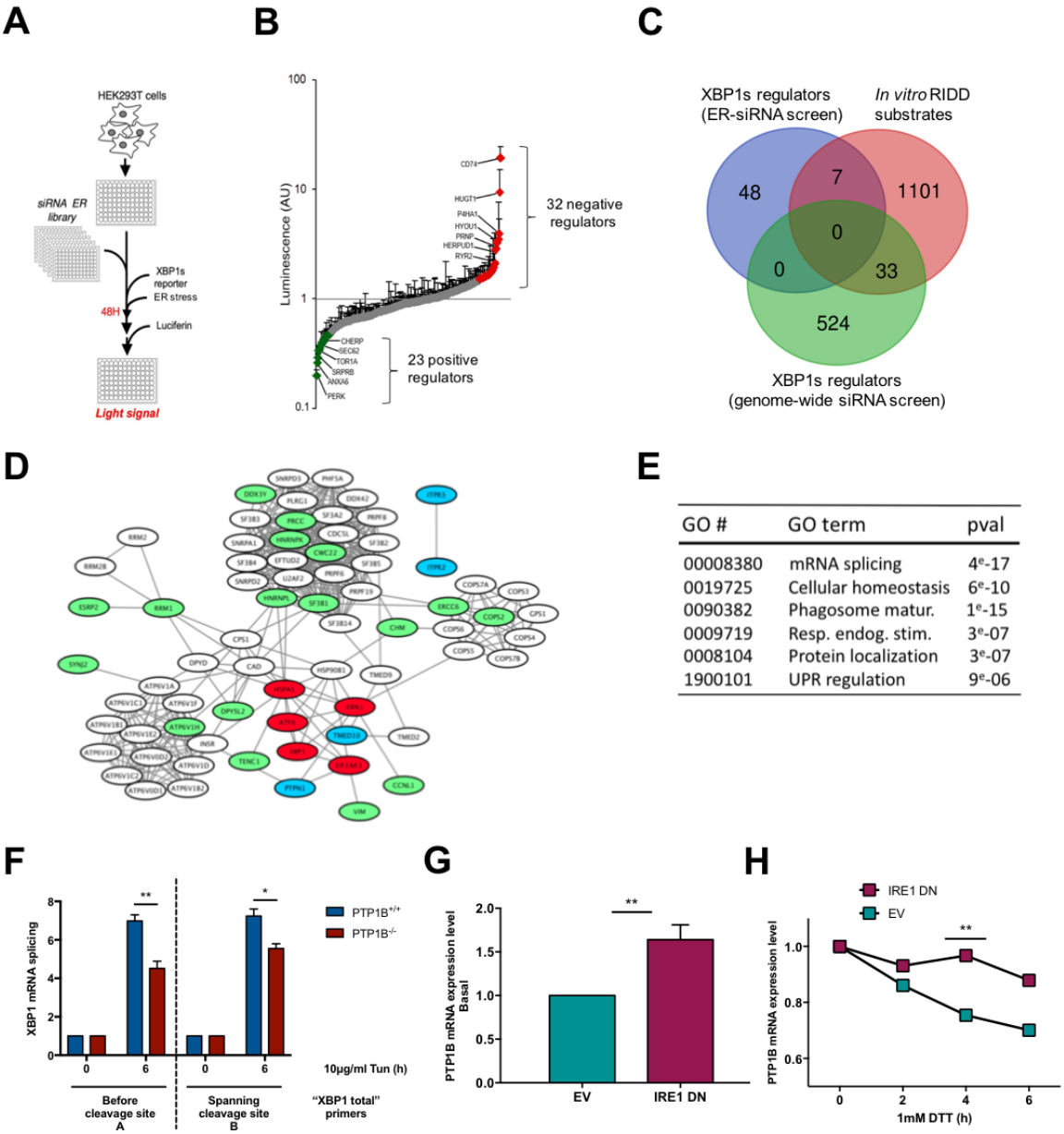
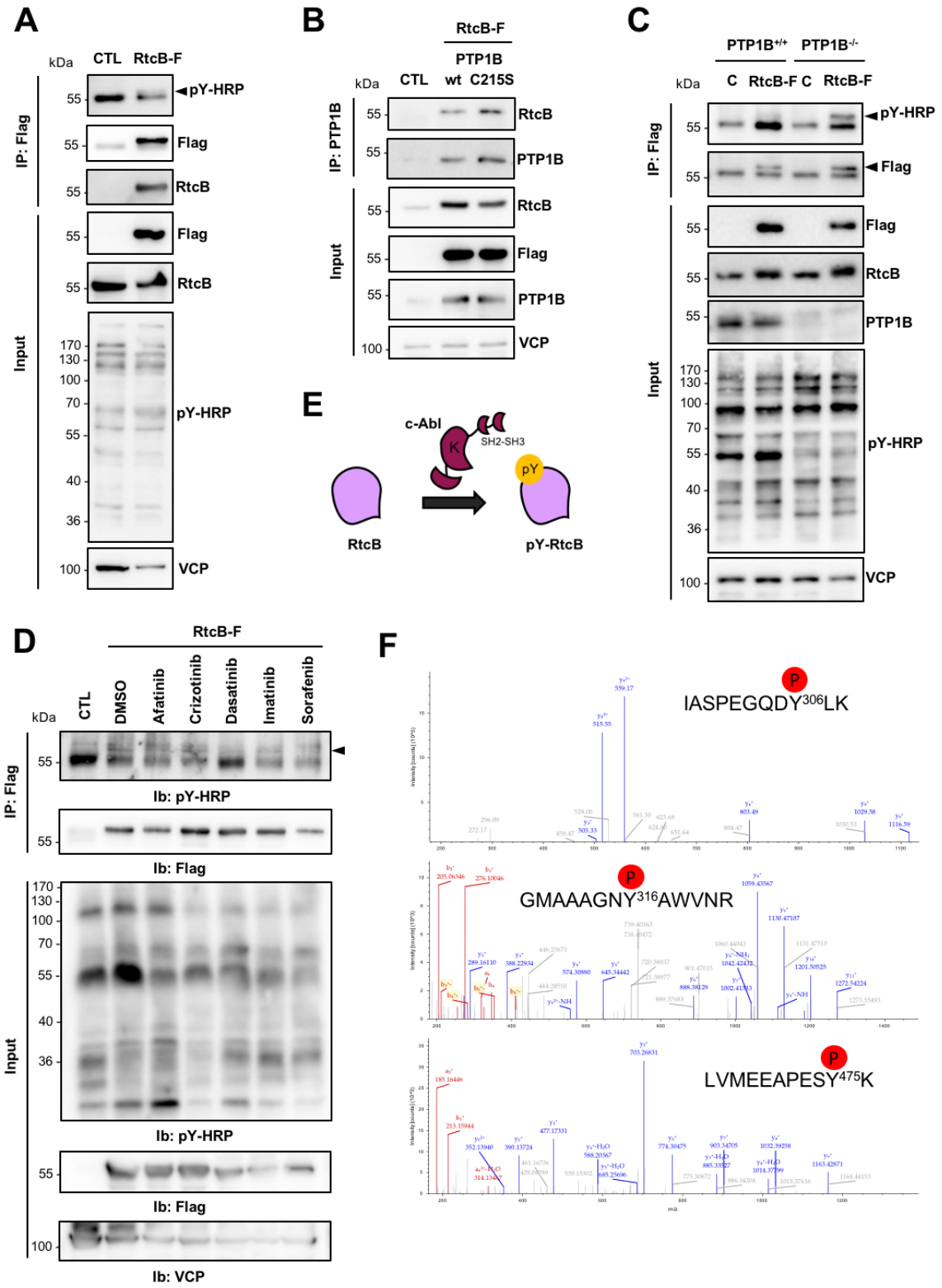

A
HEK293T cells
siRNA ER library
XBP1s reporter
ER stress
48H
Luciferin
Light signal
B
Luminescence (AU)
32 negative regulators
23 positive regulators
C
XBP1s regulators (ER-siRNA screen)
In vitro RIDD substrates
48
7
1101
0
0
33
524
XBP1s regulators (genome-wide siRNA screen)
D
E
GO # | GO term | pval
00008380 | mRNA splicing | 4e-17
0019725 | Cellular homeostasis | 6e-10
0090382 | Phagosome matur. | 1e-15
0009719 | Resp. endog. stim. | 3e-07
0008104 | Protein localization | 3e-07
1900101 | UPR regulation | 9e-06
F
XBP1 mRNA splicing
PTP1B+/+
PTP1B-/-
10µg/ml Tun (h)
Before cleavage site A
Spanning cleavage site B
"XBP1 total" primers
G
PTP1B mRNA expression level Basal
EV
IRE1 DN
H
IRE1 DN
EV
PTP1B mRNA expression level
1mM DTT (h)

**FIGURE 1**


A
kDa
CTL
RtcB-F
IP: Flag
pY-HRP
Flag
RtcB
Input
Flag
RtcB
pY-HRP
VCP
B
RtcB-F
PTP1B
kDa
CTL
wt
C215S
IP: PTP1B
RtcB
PTP1B
Input
RtcB
Flag
PTP1B
VCP
C
PTP1B+/+
PTP1B-/-
kDa
C
RtcB-F
C
RtcB-F
IP: Flag
pY-HRP
Flag
Input
Flag
RtcB
PTP1B
pY-HRP
VCP
E
c-Abl
SH2-SH3
K
pY
RtcB
pY-RtcB
D
RtcB-F
kDa
CTL
DMSO
Afatinib
Crizotinib
Dasatinib
Imatinib
Sorafenib
IP: Flag
Ib: pY-HRP
Ib: Flag
Input
Ib: pY-HRP
Ib: Flag
Ib: VCP
F
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GMAAAGNY316AWVNR
LVMEEAPESY475K
P

**FIGURE 2**

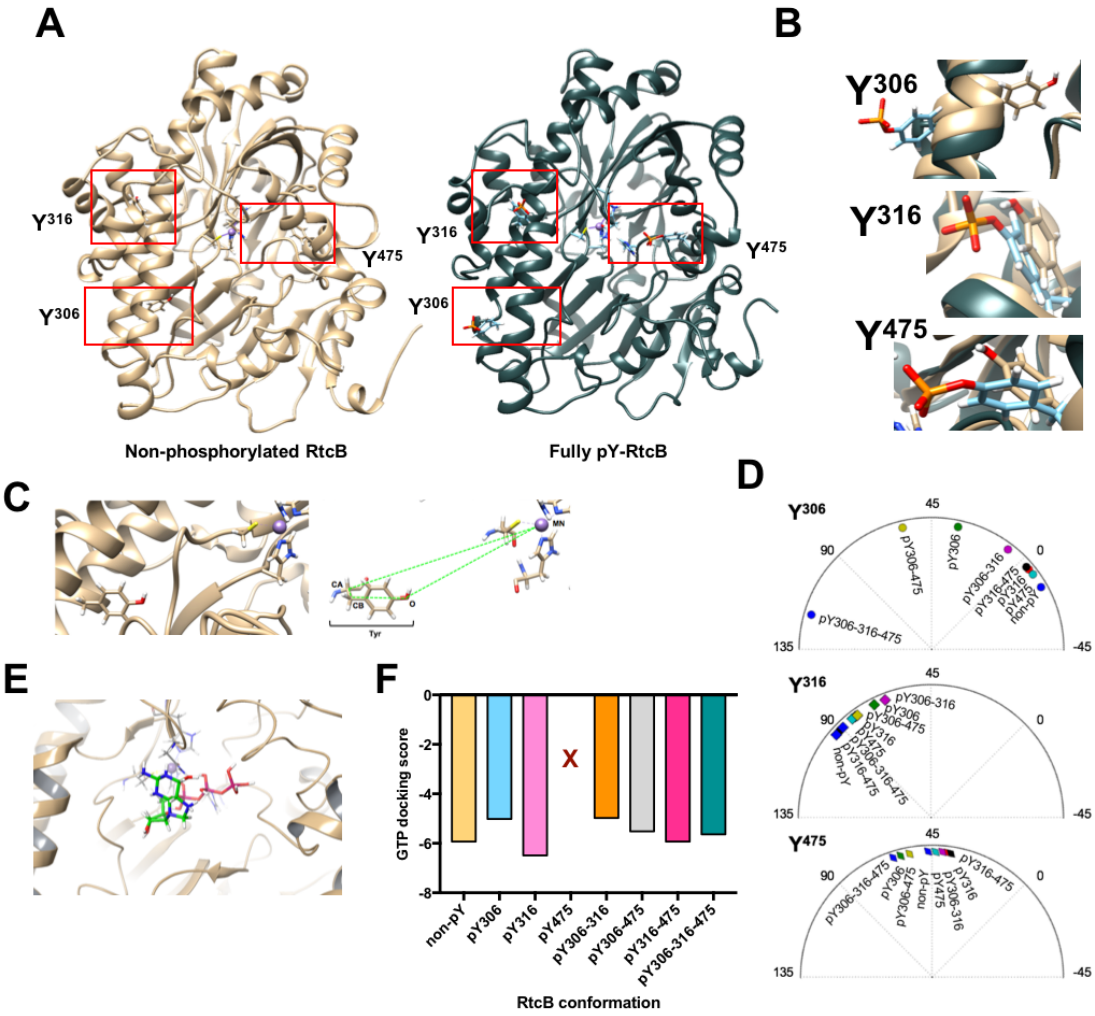

A
Y316
Y475
Y306
Y316
Y475
Y306
Non-phosphorylated RtcB
Fully pY-RtcB
B
Y306
Y316
Y475
C
D
Y306
45
90
0
135
-45
pY306-475
pY306
pY306-316
pY306-316-475
Y316
pY306-316
pY306
pY306-475
pY316
pY475
pY306-316-475
non-pY
pY316-475
Y475
pY316-475
pY306-316
pY316
pY475
non-pY
pY306-475
pY306
pY306-316-475
E
F
GTP docking score
0
-2
-4
-6
-8
X
non-pY
pY306
pY316
pY475
pY306-316
pY306-475
pY316-475
pY306-316-475
RtcB conformation

**FIGURE 3**

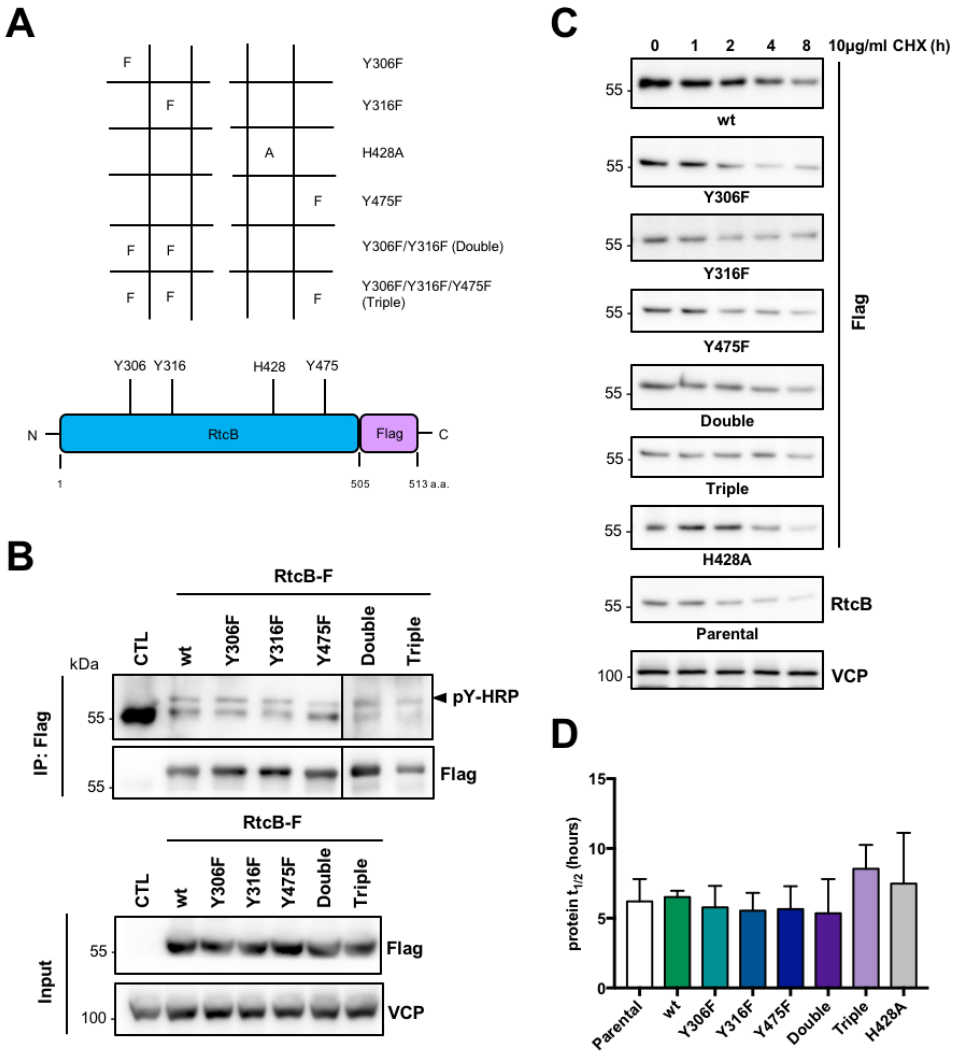

A
Y306F
Y316F
H428A
Y475F
Y306F/Y316F (Double)
Y306F/Y316F/Y475F (Triple)
Y306 Y316 H428 Y475
N
RtcB
Flag
C
1
505
513 a.a.
B
RtcB-F
CTL
wt
Y306F
Y316F
Y475F
Double
Triple
kDa
IP: Flag
55
pY-HRP
Flag
Input
100
VCP
C
0 1 2 4 8 10μg/ml CHX (h)
wt
Y306F
Y316F
Y475F
Double
Triple
H428A
Parental
RtcB
VCP
D
protein t1/2 (hours)
15
10
5
0
Parental
wt
Y306F
Y316F
Y475F
Double
Triple
H428A

**FIGURE 4**

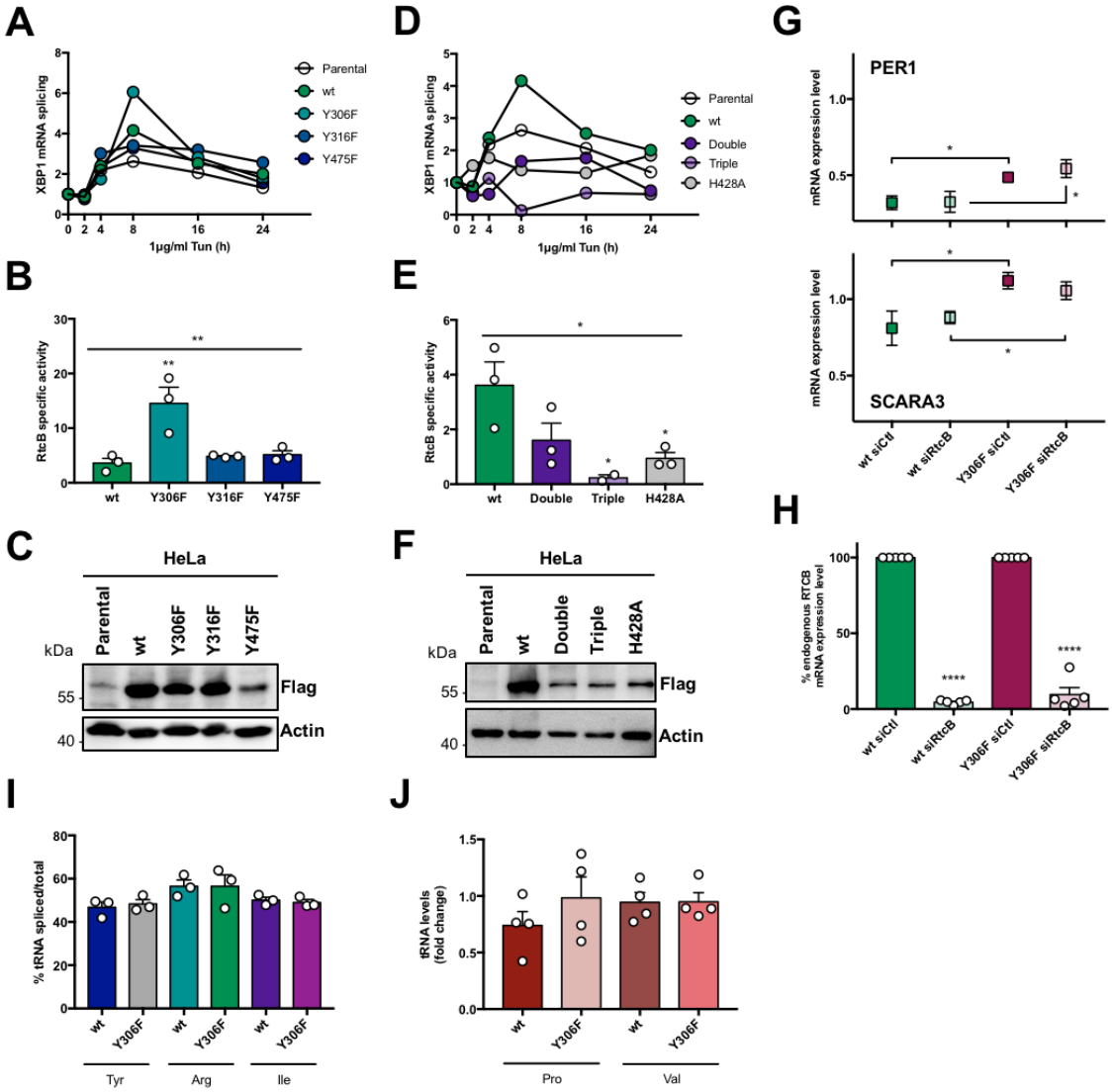

A
XBP1 mRNA splicing
1µg/ml Tun (h)
Parental
wt
Y306F
Y316F
Y475F
D
XBP1 mRNA splicing
1µg/ml Tun (h)
Parental
wt
Double
Triple
H428A
G
PER1
mRNA expression level
SCARA3
wt siCtl
wt siRtcB
Y306F siCtl
Y306F siRtcB
B
RtcB specific activity
wt
Y306F
Y316F
Y475F
E
RtcB specific activity
wt
Double
Triple
H428A
H
% endogenous RTCB mRNA expression level
wt siCtl
wt siRtcB
Y306F siCtl
Y306F siRtcB
C
HeLa
Parental
wt
Y306F
Y316F
Y475F
kDa
55
40
Flag
Actin
F
HeLa
Parental
wt
Double
Triple
H428A
kDa
55
40
Flag
Actin
I
% tRNA spliced/total
wt
Y306F
Tyr
Arg
Ile
J
tRNA levels (fold change)
wt
Y306F
Pro
Val

**FIGURE 5**

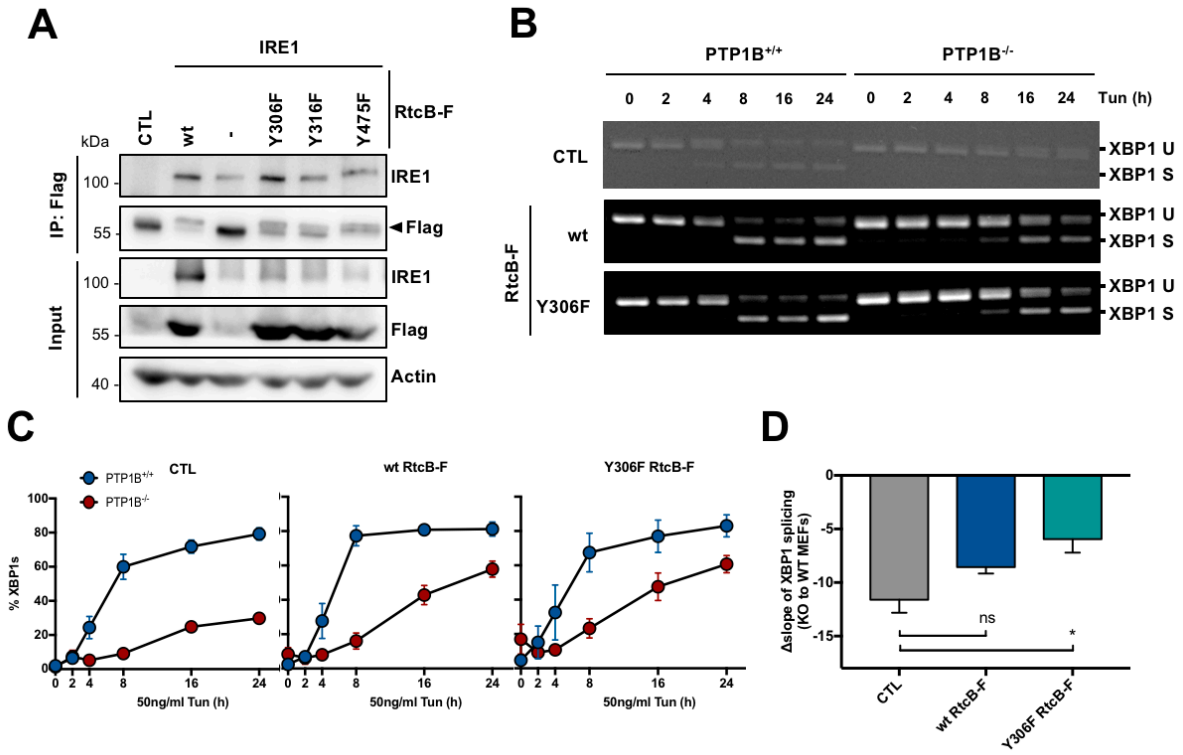
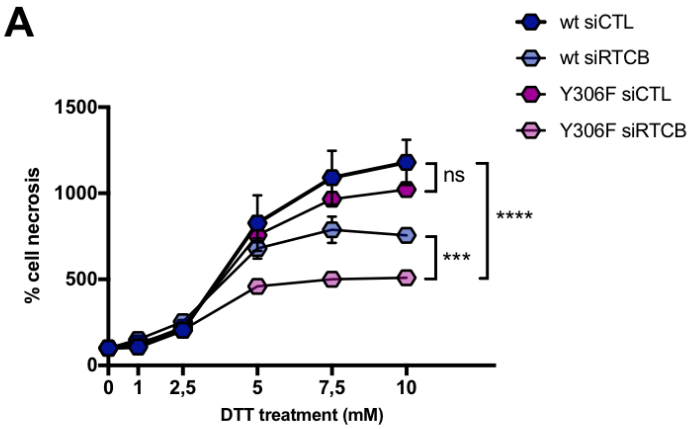
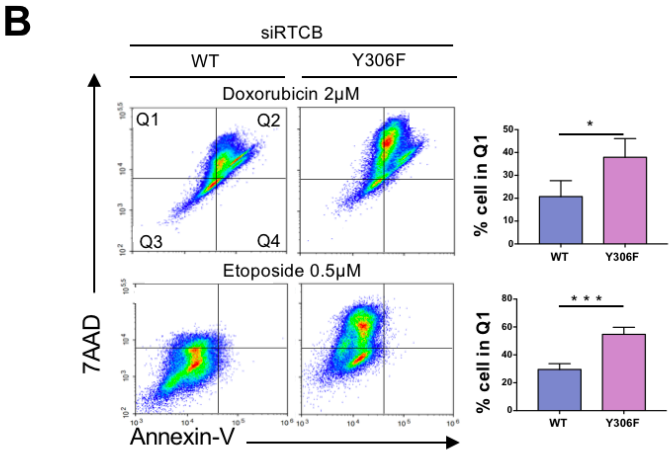

A
IRE1
RtcB-F
CTL
wt
-
Y306F
Y316F
Y475F
kDa
IP: Flag
100
55
IRE1
Flag
Input
100
55
40
IRE1
Flag
Actin
B
PTP1B+/+
PTP1B-/-
0 2 4 8 16 24 0 2 4 8 16 24 Tun (h)
CTL
RtcB-F
wt
Y306F
XBP1 U
XBP1 S
C
PTP1B+/+
PTP1B-/-
CTL
wt RtcB-F
Y306F RtcB-F
% XBP1s
50ng/ml Tun (h)
D
Δslope of XBP1 splicing (KO to WT MEFs)
ns
*
CTL
wt RtcB-F
Y306F RtcB-F

**FIGURE 6**


A
wt siCTL
wt siRTCB
Y306F siCTL
Y306F siRTCB
% cell necrosis
1500
1000
500
0
0
1
2,5
5
7,5
10
DTT treatment (mM)
ns
****
***


B
siRTCB
WT
Y306F
Doxorubicin 2µM
Q1
Q2
Q3
Q4
Etoposide 0.5µM
7AAD
Annexin-V
% cell in Q1
WT
Y306F
*
* * *

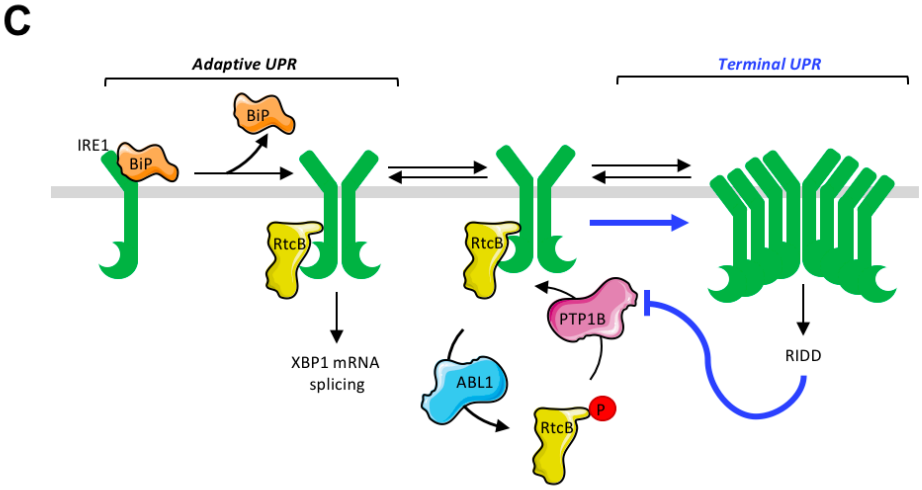

C
Adaptive UPR
Terminal UPR
IRE1
BiP
BiP
RtcB
RtcB
PTP1B
XBP1 mRNA
splicing
ABL1
RtcB
P
RIDD

**FIGURE 7**