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mTOR inhibition increases cell viability via autophagy induction during endoplasmic reticulum stress – An experimental and modeling study

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ABSTRACT

Unfolded or misfolded proteins in the endoplasmic reticulum (ER) trigger an adaptive ER stress response known as unfolded protein response (UPR). Depending on the severity of ER stress, either autophagy-controlled survival or apoptotic cell death can be induced. The molecular mechanisms by which UPR controls multiple fate decisions have started to emerge. One such molecular mechanism involves a master regulator of cell growth, mammalian target of rapamycin (mTOR), which paradoxically is shown to have pro-apoptotic role by mutually interacting with ER stress response. How the interconnections between UPR and mTOR influence the dynamics of autophagy and apoptosis activation is still unclear. Here we make an attempt to explore this problem by using experiments and mathematical modeling. The effect of perturbed mTOR activity in ER stressed cells was studied on autophagy and cell viability by using agents causing mTOR pathway inhibition (such as rapamycin or metyrapone). We observed that mTOR inhibition led to an increase in cell viability and was accompanied by an increase in autophagic activity. It was also shown that autophagy was activated under conditions of severe ER stress but that in the latter phase of stress it was inhibited at the time of apoptosis activation. Our mathematical model shows that both the activation threshold and temporal dynamics of autophagy and apoptosis inducers are sensitive to variation in mTOR activity. These results confirm that autophagy has cytoprotective role and is activated in mutually exclusive manner with respect to ER stress levels.

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1. Introduction

The maintenance of homeostasis of a multicellular organism against both internal and external stimuli (such as nutrient availability, growth factors etc.) depends on the ability of the cells to generate appropriate response (cell growth, division or cell death) to the input signals [1,2]. Cellular response depends on the molecular network comprising of crosstalk between diverse signaling pathways [3]. The inter-connections between pathways influence the dynamics and robustness of the regulatory system with respect to multiple signals [3,4].

Endoplasmic reticulum (ER) is a eukaryotic cellular component that has a crucial role in cellular homeostasis by sensing and generating signals to drive cellular responses [1,5]. ER has major

functions in synthesizing and packaging both secreted and membrane proteins, lipid biosynthesis, carbohydrate metabolism and several signaling processes [6–8]. The redox homeostasis of ER lumen is an important integrator of various stimuli. The proper redox state of both the luminal thiol/disulfide and pyridine nucleotide systems, as well as the Ca²⁺ rich luminal environment are essential for the ER function [9,10]. Any imbalance of luminal ER homeostasis results in ER stress [5,11–13].

Prototypic experimental inducers of ER stress, namely thapsigargin (TG) and tunicamycin (TM) are shown to perturb ER homeostasis [11,14]. TG disrupts the calcium storage of ER by blocking calcium reuptake into the ER lumen, thus by depleting calcium from the organelle [11,15–17]. TM inhibits N-linked glycosylation of secretory and membrane proteins in the ER [11,18]. Both ER stressors are shown to induce autophagy but higher concentration of TG is shown to disrupt the fusion of autophagosomes and lysosomes [19]. Both agents are also powerful inducers of apoptosis [20,21].

High luminal NADPH/NADP⁺ ratio is essential for the proper function of the ER [9,22]. The luminal NADPH concentration is

Abbreviations: ER, endoplasmic reticulum; mTOR, mammalian target of rapamycin; UPR, unfolded protein response

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maintained high by the coordinated action of the ER glucose-6-phosphate transporter (G6PT) and the luminal hexose-6-phosphate dehydrogenase (H6PD), while NADPH can be consumed by different luminal reductases (such as carbonyl reductases) [9,23,24]. Decreasing NADPH level by adding both metyrapone and menadione sensitizes the ER to oxidative injuries and can result in increased autophagosome formation [22]. We have shown that high concentration of metyrapone alone is sufficient to induce the autophagic process [25].

A recent study has shown that cells under ER stress activate autophagosome formation [26], which results in macroautophagy (autophagy) [26,27]. Since autophagy has an essential role in promoting cellular-survival during starvation by “self-eating” of parts of the cytoplasm and intracellular organelles, it was suggested that autophagy also has a crucial protective role under ER stress [3,26,28–30]. On the other hand, severe ER stress results in apoptotic-dependent cell death *via* sustained c-Jun terminal kinase (JNK) activity [29,12].

The accumulation of unfolded or misfolded proteins in ER during different stress situations is shown to trigger an adaptive response known as unfolded protein response (UPR) [7,14,31]. The three branches of UPR are activated by ER stress through ER membrane-associated proteins IRE1 (inositol requiring 1), PERK (PKR-like ER kinase) and ATF6 (activating transcription factor 6) [6,14]. All three sensors are bound by the chaperone Grp78/BIP under stress free situation to keep them inactive [7,14]. Under ER stress, the activation of IRE1 and ATF6 promotes transcription of UPR target genes (such as chaperones) and activation of PERK-controlled pathway leads to the general inhibition of protein translation [6,14]. UPR is shown to activate both autophagy and apoptosis depending on the ER stress levels [29,32].

Intriguingly, recent evidences suggest the existence of a crosstalk between UPR and mammalian target of rapamycin (mTOR) signaling pathways in the control of cell survival and cell death decisions [6,14,33–35]. mTOR is the master regulator that integrates inputs from the external and internal signals, such as growth factors, amino acids, glucose and energy status to control growth and metabolism [34,36]. It also inhibits autophagy under nutrient rich condition [34]. In contrast, the down-regulation of mTOR activity by rapamycin is shown to enhance cell viability under ER stress [29,37]. Similarly, pre-treatment with rapamycin also protects cells against apoptotic cell death [29,38]. The pro-apoptotic role of mTOR is coupled to the downstream activation of both PERK and IRE1 branches of UPR under severe ER stress [37,39,40]. Further evidences suggest that UPR also has a function upstream of mTOR to control its activation under ER stress. Thus, mTOR and UPR regulate each other to form a positive feedback loop [35].

In addition to crosstalk between mTOR and UPR, the existence of a crosstalk is also suggested between the cell survival and death pathways at the level of effector molecules, such as caspases and autophagy-inductor Beclin1 regulated by Bcl2 [41–44]. Recently, we proposed a minimal mathematical model of Bcl2 – Beclin1 – caspase regulatory motif to show how sequential activation of cell survival and cell death mechanisms can be achieved robustly by the crosstalk between the activators of cell fate decisions [45]. We suggested that the feedback loops between autophagy and apoptosis inducers make the system bistable to ensure mutually exclusive decisions and transition from cell survival to irreversible cell death [45]. How the feedback loops between autophagy and apoptosis inducers are coupled to the mutual regulation of mTOR and UPR is still unclear.

In this study, we examine with help of experiments and mathematical modeling the interplay of mTOR and UPR in the control of cell survival and cell death decision. Thapsigargin and metyrapone were used as ER stress inducers. In the presence of thapsigargin, we

studied the effect of perturbing the mTOR activity on autophagy and cell viability by either using rapamycin or metyrapone. Autophagy was monitored upon conversion of LC3I to LC3II, mTOR pathway activity was evidenced by measuring p70S6 phosphorylation, activation of apoptosis was detected by the decrease of procaspase-3 level and by the cleavage of PARP, while p62 levels were measured as an indicator of autophagy inhibition. 3-methyladenine and bafilomycin A were used as inhibitors of autophagy. Cell viability was assayed by two alternative methods.

We observed that the inhibition of mTOR pathway leads to increase in cell viability under ER stress and is accompanied by increase in autophagy activity. We also showed that autophagy is activated under severe ER stress condition but gets inhibited with increase in duration of stress. These results demonstrate that ER stressors can initiate apoptosis or autophagy, which depends on the chemical nature of the stressor, the extent and the duration of the stress. However, autophagy has cytoprotective role under ER stress. Our mathematical model shows that the activation thresholds and temporal dynamics are sensitive to mTOR activity.

2. Materials and methods

2.1. Materials

Metyrapone (Sigma–Aldrich, M2696), thapsigargin (Sigma–Aldrich, T9033), rapamycin (Sigma–Aldrich, R0395), tunicamycin (Sigma–Aldrich, T7765) and 3-methyladenine (Sigma–Aldrich, M9281), bafilomycin A (Sigma–Aldrich, M17931) were purchased. All other chemicals were of reagent grade.

2.2. Cell culture and maintenance

As model systems, human liver carcinoma (HepG2) and human embryonic kidney (HEK293) cell lines were used. Both cell lines were maintained in DMEM (Life Technologies, 41965039) medium supplemented with 10% fetal bovine serum (Life Technologies, 10500064) and 1% antibiotics/antimycotics (Life Technologies, 15240062). Culture dishes and cell treatment plates were kept in a humidified incubator at 37 °C in 95% air and 5% CO₂.

2.3. SDS–PAGE and Western blot analysis

Cells were harvested and lysed with 20 mM Tris, 135 mM NaCl, 10% glycerol, 1% NP40, pH 6.8. Protein content of cell lysates was measured using Pierce BCA Protein Assay (Thermo Scientific, 23225). During each procedure equal amounts of protein were used. SDS–PAGE was done by using Hoefer miniVE (Amersham). Proteins were transferred onto Millipore 0.45 μm PVDF membrane. Immunoblotting was performed using TBS Tween (0.1%), containing 5% non-fat dry milk for blocking membrane and for antibody solutions. Loading was controlled by developing membranes for actin or dyed with Ponceau S in each experiment. The following antibodies were applied: antiGADD153 (SantaCruz, sc-575) antiLC3B (SantaCruz, sc-16755), antiactin (SantaCruz, sc-1616), anticaspase-3 (SantaCruz, sc-7272), anti-p70S6 (SantaCruz, sc-11759), anti-p70S6 (SantaCruz, sc-230), antiPARP (Cell Signaling, 9542S), anti-4EBP1 (Cell Signaling, 236B4), anti-4EBP1 (Cell Signaling, 53H11), anti-p62 (Cell Signaling, 5114S) and GAPDH (Santa Cruz, 6C5), HRP conjugated secondary antibodies (SantaCruz, sc-2020 and Cell Signaling, 7074S, 7076S).

2.4. Cell viability assays

Cell viability was detected using a trypan blue exclusion assay. Cells were incubated with isotonic solution of trypan blue 0.6% (Sigma–Aldrich, T6146) and permeabilized cells were counted pro-

portionally to total cell numbers. Cell viability was also evaluated using CellTiter-Blue assay (Promega, G8080). Cells were grown and treated on 96-well plates, and were incubated with resazurin for 2 h at 37 °C. Absorbance was measured at 620 nm, and expressed in arbitrary unit, being proportional to cell toxicity. For each of these experiments at least three parallel measurements were carried out.

2.5. Mathematical modeling

The regulatory network given in Fig. 5A was translated into a set of nonlinear ordinary differential equations (ODEs) and analyzed using the techniques of dynamical system theory [46–48]. Dynamical simulations, phase plane and bifurcation analysis were carried out using the program XPPAUT, which is freely available from <http://www.math.pitt.edu/~bard/xpp/xpp.html> [47,48]. ODE describes the time-rate of change of level or activity of a component (such as crosstalk element, autophagy and apoptosis inducers). The initial conditions and parameter values used for simulations are given in Supplementary material. All variables are dimensionless, rate constants (k 's) have a dimension of min^{-1} , while Michaelis Menten constants are dimensionless. The total level of mTOR, autophagy and apoptosis inducers were assumed to be constant (one unit). We provide the XPP code (in the Supplementary material) that can be used to generate all the figures in the manuscript.

3. Results

3.1. Apoptotic events are preceded by autophagy-dependent survival under ER stress

We firstly studied the cellular response to ER stress by checking cell viability in HepG2 cells treated with different concentrations of drug thapsigargin (TG) or tunicamycin (TM) for 24 h (Fig. 1A and Fig. S1A). Cell viability did not decrease significantly at lower concentration of TG (0.001–0.01 μM), whereas at a high concentration of TG (25 μM TG), cell viability dropped dramatically, below 5% (Fig. 1A). At intermediate level of ER stressor (0.1–1 μM) drastic decrease of cell viability was not observed (data not shown). To further characterize cell phenotypes under drug treatment, we analyzed the well-known markers of autophagy and apoptosis by immunoblotting. The lower concentration of TG induced transformation of cytoplasmic LC3I to membrane bounded LC3II, indicating an increase in autophagosome formation. On the other hand, higher concentration of TG not only induced transformation of LC3I to LC3II but also equally showed more intense p62 band (Fig. 1B), suggesting that autophagosome formation was disrupted. Meanwhile, procaspase-3 level decreased and cleaved PARP level increased at high level of ER stressor indicating the activation of apoptotic events, consistently with the observation of drastic drop in cell viability at high TG concentration (Fig. 1A). Similar results were observed when HEK cells were treated with TM or TG

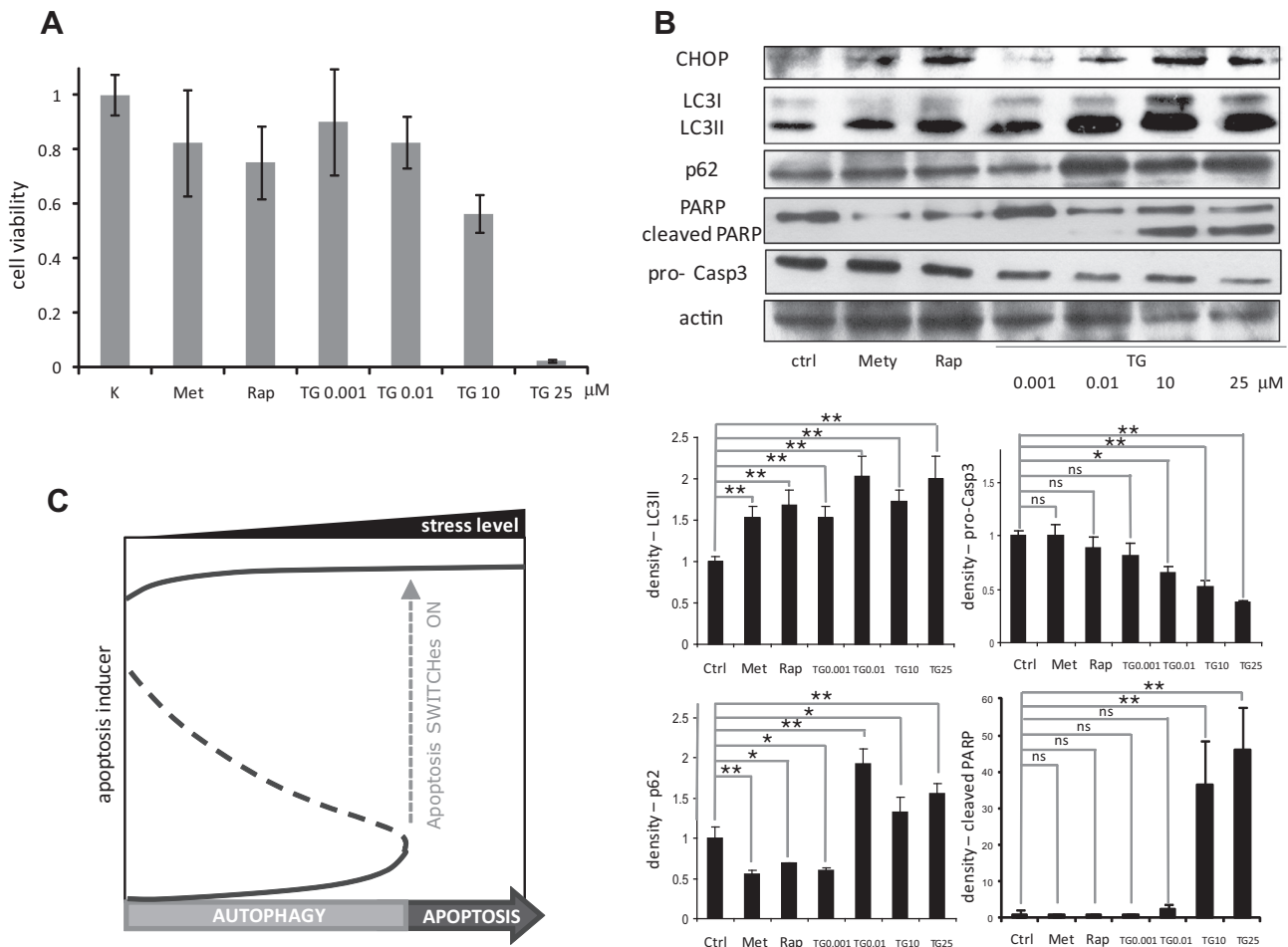


Fig. 1. Choosing between autophagy and apoptosis with respect to different cellular stressors. HepG2 cells were treated with rapamycin (100 nM) or metyrapone (100 μM) for 2 h or with increasing concentration of TG for 24 h. (A) Cell viability observed upon the treatments. (B) Western blot analysis of GADD153/CHOP, LC3, p62, PARP, procaspase-3 and actin. Densitometry data represent the intensity of LC3II, p62, procaspase-3, and cleaved PARP bands in arbitrary units normalized for actin (asterisks indicate statistically significant difference: * $p < 0.05$; ** $p < 0.01$). (C) Presumed signal-response curve of apoptosis inducer.

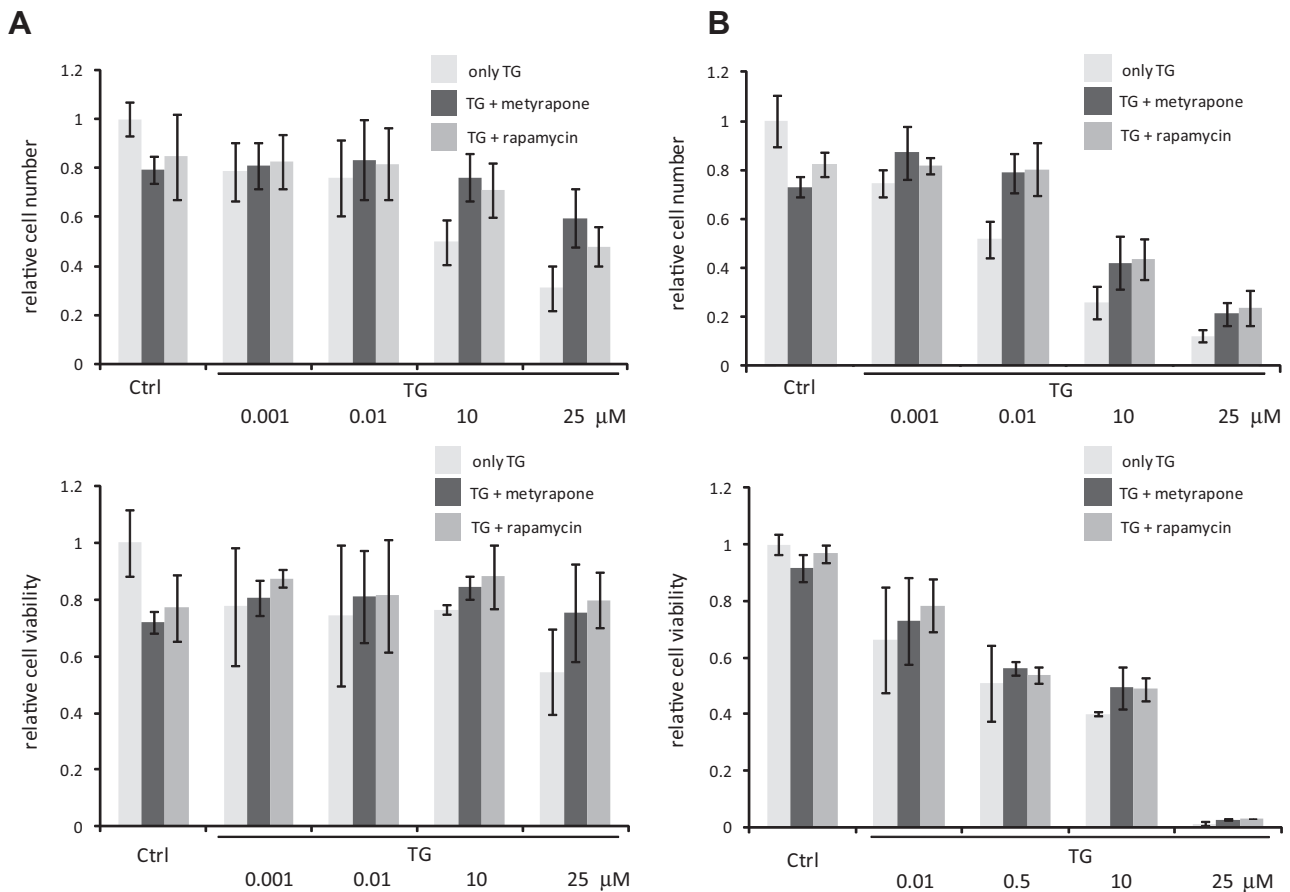


Fig. 2. mTOR inhibition increases cell viability in ER stress. Cell viability was assessed by both measuring the percentage of cells permeable to trypan blue (upper panel) and using CellTiter-Blue assay (lower panel). (A) HepG2 cells were treated with increasing concentration of TG for 2 h without/with metyrapone (100 μ M)/rapamycin (100 nM) for 2 h. (B) HepG2 cells were treated with increasing concentration of TG for 24 h without/with metyrapone (100 μ M)/rapamycin (100 nM) for 2 h.

(Figs. S1B–D). Altogether, the data supposed that autophagy induced at low concentration of TG or TM does not kill the cells, however a high level of ER stress results in apoptosis. An earlier study showed that TM- or TG-induced ER stress can activate both autophagy and apoptosis in a sequential manner and autophagy has a cytoprotective role [29]. This suggested that treating cells with higher concentration of TG for a shorter interval might only activate autophagy leading to increase in cell viability. Therefore, we performed the experiment for a shorter time interval of 2 h. The viability of cells treated for shorter time with high stress levels (TG = 10 μ M) did not decrease drastically (Fig. 2A). This observation coincides with an increase in autophagy activity as measured by the formation of LC3II and decrease in p62 levels (Fig. S2). A treatment of cells for shorter time with lower stress level (TG = 0.01 μ M) by itself was also sufficient to activate autophagy (Fig. S2). Thus, at higher stress level cells initiate autophagy to survive, however under longer duration of stress autophagy becomes inactive and the cells commit to apoptotic cell death suggesting mutual exclusion between the surviving and self-killing mechanisms. These data are consistent with our recent qualitative model proposed for crosstalk between autophagy and apoptosis. We demonstrated that the induction of apoptosis depends on the stress level crossing a specific activation threshold [45]. The signal-response curve of apoptosis inducer with respect to stress level is shown in Fig. 1C. Here, the stress level ('signal') has to reach a critical value to turn on apoptosis ('response'). If the treatment is shorter or the concentration of ER stressor is lower, cells induce autophagy to "self-eat" their damaged components instead of promoting apoptosis. Apoptosis is switched on only when cells are

treated with higher concentration of ER stressor for longer duration as observed by a drastic drop in viability, PARP cleavage induction and decrease of procaspase-3 level in our experiments. It can be seen from the signal-response curve that the stress threshold for the irreversible activation of apoptosis inducer arises due to the bistable characteristic of the autophagy – apoptosis regulatory network (Fig. 1C).

3.2. Metyrapone – similarly to rapamycin – improves cell viability under ER stress

Rapamycin induces autophagy by mimicking nutrient depletion via down-regulating the mTOR pathway [49]. Recent experimental data suggested that mTOR inhibition promotes cell survival under ER stress induced by TG or TM [37,38]. Consistently, we observed in HepG2 cells that rapamycin treatment alone induced the transformation of LC3I to LC3II indicating an increase in autophagosome formation, and a moderate decrease in cell viability was also present (Fig. 1B). Further, rapamycin treatment rescued the cells from cell death provoked by higher concentrations of TG. This was observed for both long and short treatment of cells with TG beside rapamycin (Fig. 2). Collectively, these results suggest that the activation of autophagy under rapamycin treatment might contribute towards decrease in cell death under ER stress. To further test this possibility, we used another ER stressor, metyrapone that is capable of activating autophagy via downregulation of mTOR pathway [22,25]. Fig. 1B shows that a 2-h long treatment of cells with metyrapone was sufficient to induce the transformation of LC3I to LC3II and cell viability decreased slightly. A decreased level of

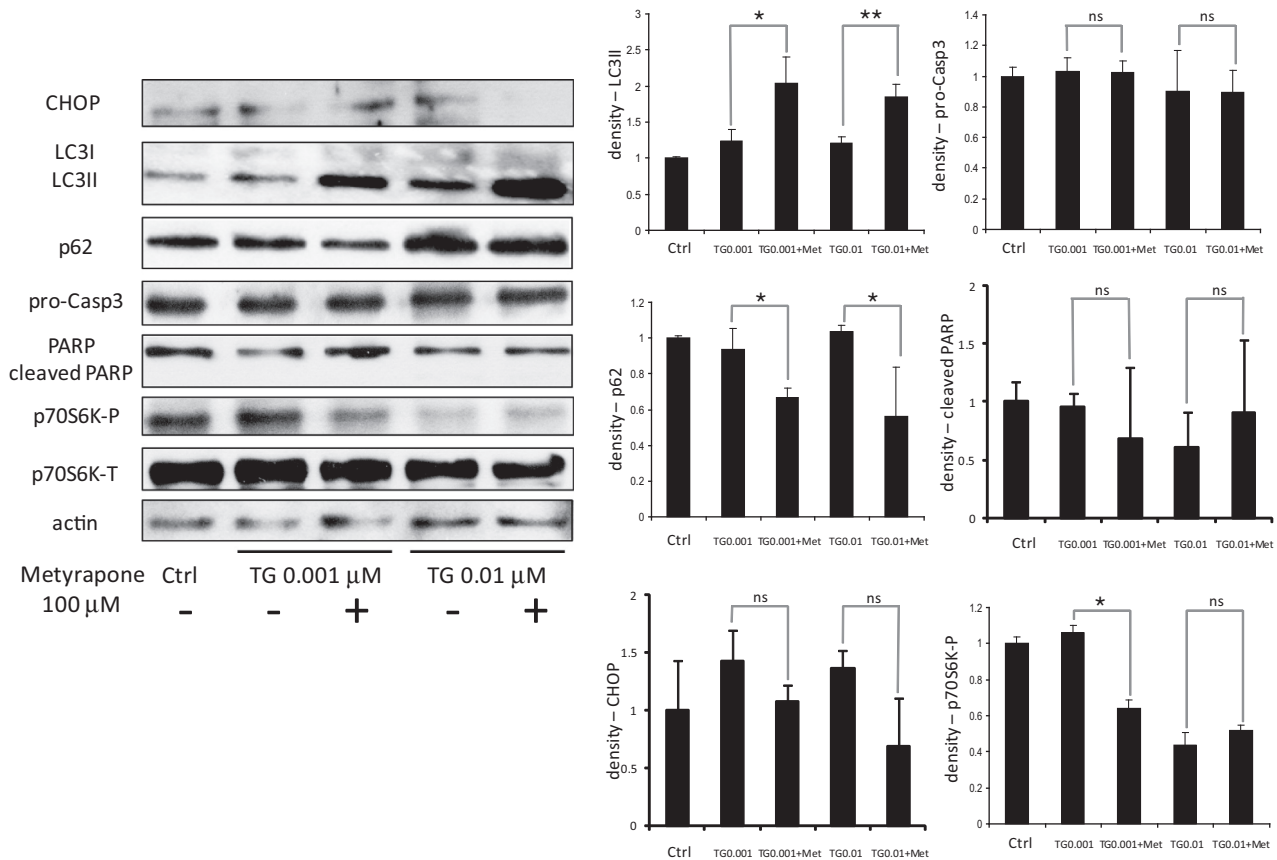


Fig. 3. Combined treatment with metyrapone and TG induces early autophagy. HepG2 cells were treated with increasing concentration of TG for 24 h without/with metyrapone for 2 h (100 μ M). Expression of GADD153/CHOP, LC3, p62, procaspase-3, PARP, p70S6K-P, p70S6K-T and actin was probed by immunoblotting. Densitometry data represent the intensity of LC3II, p62, GADD153/CHOP, procaspase-3, cleaved PARP and p70S6K-P bands in arbitrary units normalized for actin (asterisks indicate statistically significant difference: * $p < 0.05$; ** $p < 0.01$).

p62 indicated effective autophagosome formation while caspase-3 activation and PARP cleavage were not observed. Thus, metyrapone has similar effect to rapamycin in promoting autophagy (Fig. 1B). This similarity was also confirmed by using various autophagy inhibitors, such as 3-methyladenine (3-MA) and bafilomycin A (Fig. S3).

To investigate whether metyrapone is also capable of restoring cell viability under ER stress, we performed the experiments involving combined treatment of cells with both metyrapone and TG. Cells were treated with different concentration of TG together with metyrapone for 2 h and cell viability was quantified in two ways (see Section 2). A combined treatment of cells with TG and metyrapone for 2 h suppressed the occurrence of cell death at higher concentrations (10–25 μ M) of TG (Fig. 2A). We also studied the effect of metyrapone by treating the cells with TG for 24 h, while metyrapone was added only for 2 h. The short treatment of metyrapone was sufficient to increase the cell viability at high TG concentrations even after 24 h (Fig. 2B). Similar results were observed at simultaneous treatment of TM and metyrapone both in HepG2 and HEK cells (Fig. S4).

We characterized the positive effect of metyrapone on ER stressed cells by following the markers of both autophagy and apoptosis by immunoblotting under combined treatment (Figs. 3 and 4 and Fig. S2). Metyrapone promoted a significant transformation of LC3I to LC3II under ER stress caused by 0.001–10 μ M TG. Furthermore, p62 level did not increase so drastically even at a higher concentration of TG (10 μ M). Since apoptosis activation can be observed at 10 μ M TG treatment, the experiment was also carried out at a slightly lower TG concentration to catch the switch on of apoptosis (see 5 μ M TG on Fig. 4). Meanwhile the mTOR

marker p70S6 kinase got dephosphorylated. These data support an effective autophagosome formation and high autophagic activity at combined treatment. We also observed that procaspase-3 level remained higher and cleaved PARP level was lower with metyrapone treatment suggesting an inhibition of apoptosis at high ER stress (Fig. 4). Since both rapamycin and metyrapone induced autophagy and rescued cells from cell death under ER stress, it raises the question whether these drugs bring about their cytoprotective effect *via* the same pathway. We tested this by treating the ER stressed cell with both rapamycin and metyrapone. Our results suggest that these drugs – although they have different targets – might act through the same pathway of autophagy activation to suppress the effect of ER stress (Fig. S5). To confirm further that metyrapone-induced viability occurred via intensive autophagy at combined treatment the experiment was repeated by using 3-MA or bafilomycin A. It is well-known that 3-MA enhances cytotoxic effect of TG, but both inhibitors were able to block the positive effect of metyrapone (Fig. S6).

Our results indicate that autophagy and apoptosis can exhibit the characteristic of mutual antagonism, which could influence the behavior of cells under combined treatment. Therefore, an increase in autophagy by mTOR inhibition can delay cell death via feedback loops.

3.3. A minimal model of autophagy-apoptosis crosstalk under ER stress

We have previously shown by a mathematical model that the cellular decision of either to survive or die under stress is influenced by the dynamics of regulatory motif involving a mutual antagonism between autophagy and apoptosis inducers and a

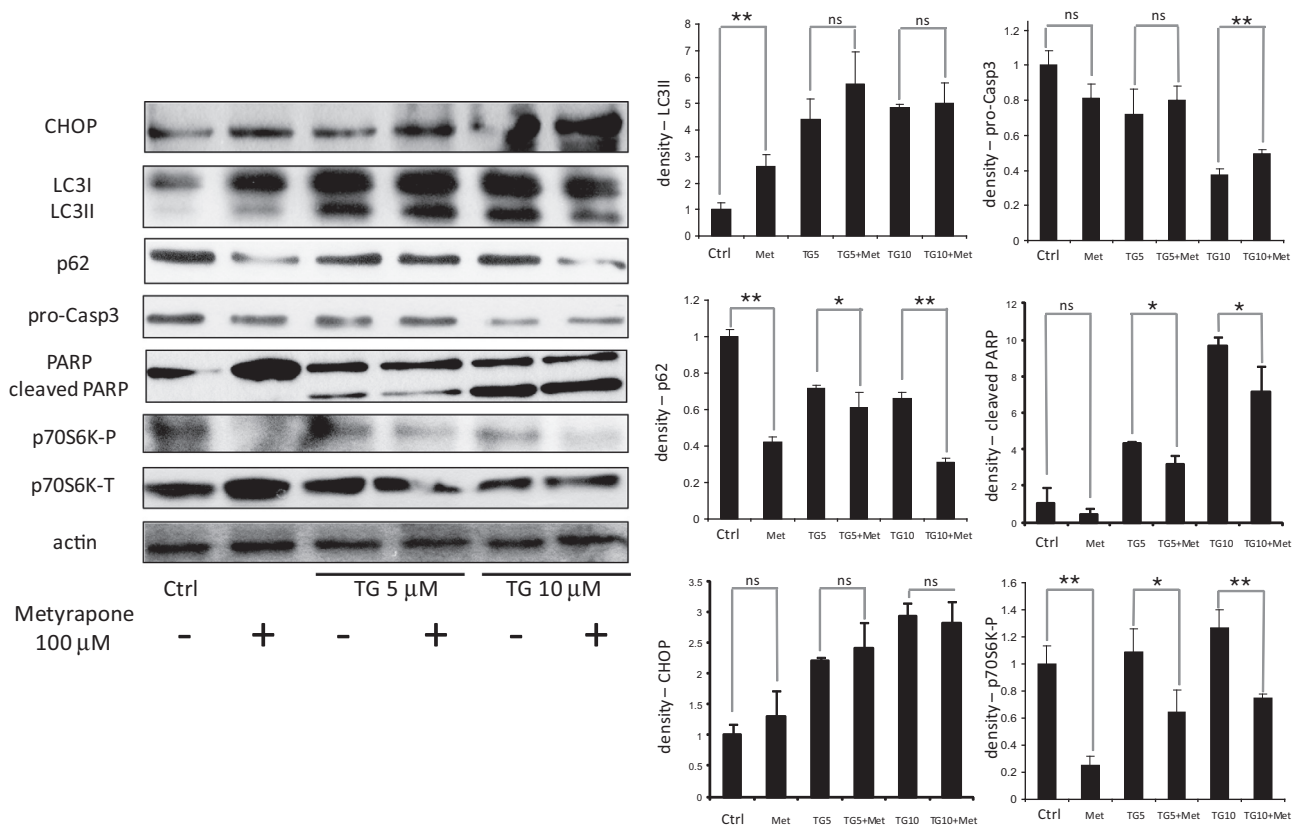


Fig. 4. Metyrapone treatment delays apoptotic cell death caused by TG. HepG2 cells were treated with increasing concentration of TG for 24 h without/with metyrapone for 2 h (100 μM). Expression of GADD153/CHOP, LC3, p62, procaspase-3, PARP, p70S6K-P, p70S6K-T and actin was probed by immunoblotting. Densitometry data represent the intensity of LC3II, p62, GADD153/CHOP, procaspase-3, cleaved PARP and p70S6K-P bands in arbitrary units normalized for actin (asterisks indicate statistically significant difference: * $p < 0.05$; ** $p < 0.01$).

crosstalk element Bcl2 [45]. Although such a regulation is already reported under nutrient starvation, we claimed that this motif might be relevant even under ER stress based on the experimental results obtained by us and relevant data published by other groups. To demonstrate this point further, we interpret our earlier model from the context of ER stress and include the interconnections of mTOR with autophagy and apoptosis inducers. This minimal model was independent of identity of molecular players and only includes the system level feedback loops relevant to autophagy – apoptosis crosstalk.

In our previous model, the crosstalk element Bcl2 inhibits both autophagy and apoptosis inducers and various cellular stressors activate both autophagy and apoptosis by inhibiting the crosstalk element [45]. It is well known that ER stress activates a crosstalk element involving three branches of unfolded protein response (UPR) to activate both autophagy and apoptosis. For simplicity, we assumed that an ER stress sensor (ERS) representing the three branches of UPR directly activates both autophagy and apoptosis inducers. ERS activity is very low under physiological conditions and increases rapidly under ER stress. Since autophagosome formation was disrupted in parallel to apoptosis induction under ER stress (Fig. 1), we considered that apoptosis inducer inhibits autophagy inducer. Such an interaction can be envisaged to happen through caspases-dependent cleavage of autophagic proteins as reported under nutrient starvation. Similarly, autophagic inducers could prevent apoptosis activation by degradation of proteins including apoptosis inducers. This could inactivate apoptosis directly or indirectly by relieving ER stress. Such interactions generated a double negative feedback loop between autophagy and apoptosis inducers. Beside this feedback loop, incorporating mTOR regulation of

autophagy and apoptosis under ER stress introduced more feedback loops into the regulatory system. Activation of mTOR is known to promote apoptosis (under ER stress) and inactivate autophagy [34,35]. On the other hand, autophagy is also shown to have a negative influence on mTOR activation [50,51]. These generated a double negative feedback loop between mTOR and autophagy inducers. The minimal model included four components namely ER stress sensor (ERS), mTOR, autophagy and apoptosis inducers (Fig. 5A).

To examine other qualitative behavior of the system, we translated the regulatory structure into a set of nonlinear differential equation and performed a phase plane analysis (see Section 2 and Suppl. data). A two dimensional phase plane of autophagy and apoptosis inducers was created by assuming that other two regulators (ERS and mTOR) were at steady state. The curves on the phase plane are called the balance curves (also called nullclines) of autophagy and apoptosis inducers, which represent how steady state of one inducer varies as function of other inducer. Intersections of the two balance curves show the steady state of system, which could be stable or unstable. The phase plane of autophagy (green) and apoptosis (red) inducers under physiological, low and high stress conditions are shown in Fig. 5. Under physiological condition, both balance curves intersected at low value of autophagy while apoptosis inducer was completely suppressed (Fig. 5B). At low stress level, the balance curves intersected at three points representing two stable steady states separated by an unstable steady state (Fig. 5C). A stable state with higher autophagy inducer activity but lower apoptotic inducer activity corresponds to autophagy. On the other hand, a stable state with higher apoptosis inducer activity but lower autophagy inducer activity corresponds to apoptosis.

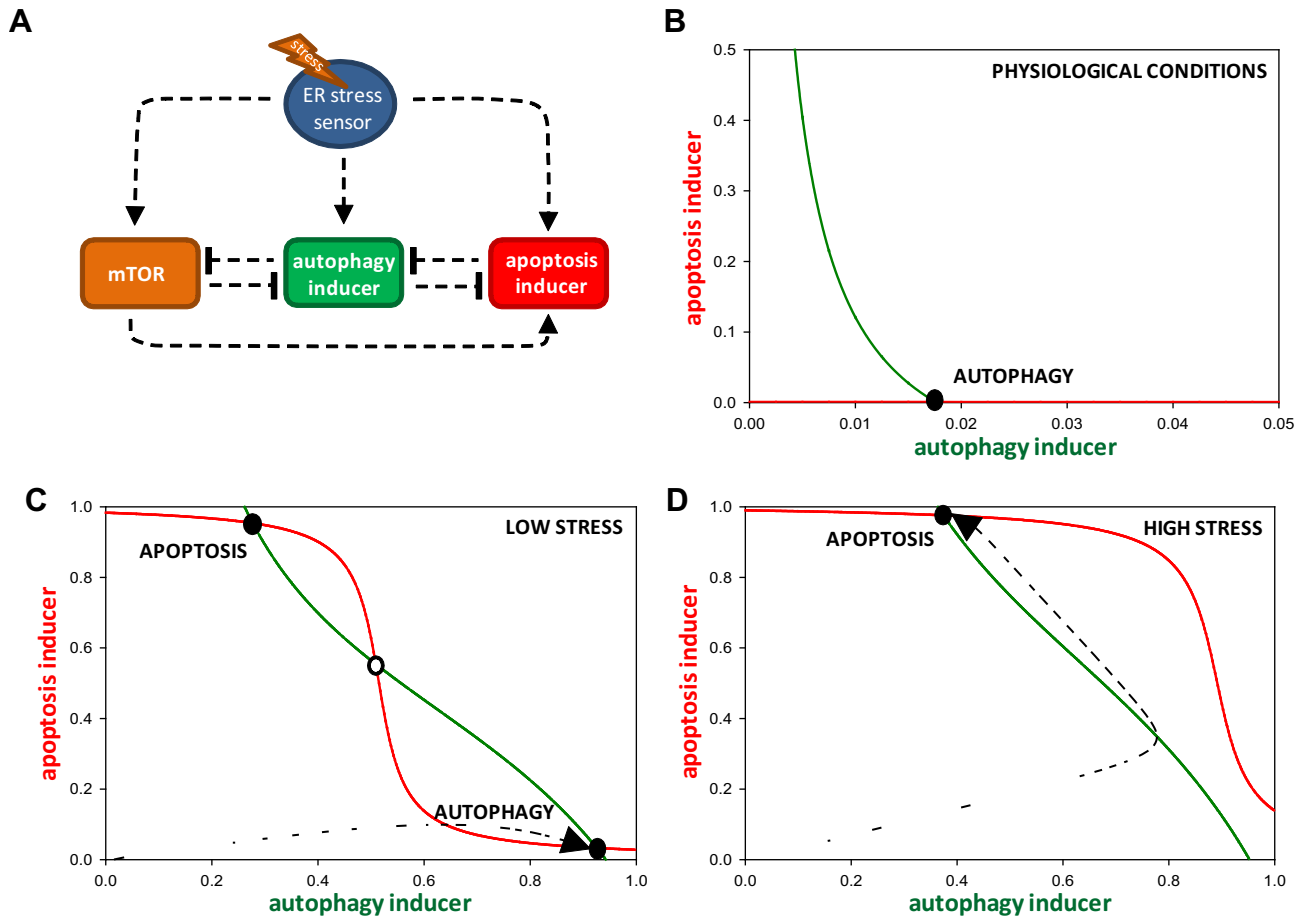


Fig. 5. The minimal model of cell fate decision during ER stress. (A) Wiring diagram of the regulatory network. The autophagy inducer, the apoptosis inducer, mTOR and the crosstalk element are denoted by isolated boxes. Dashed lines show how the molecules can influence each other. Blocked end lines denote inhibition. (B), (C) and (D) Phase plane portrait of crosstalk between autophagy and apoptosis. Red nullcline represents apoptosis inducer, while green nullcline denotes autophagy inducer. Intersections of nullclines represent the stable (filled circle) and unstable (open circle) steady states. The phase planes are shown for (B) physiological conditions (stress = 0), (C) low level of ER stress (stress = 25) and (D) high level of stress (stress = 100). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

At low (tolerable) level of stress, the system occupied the autophagic state (Figs. 1 and 5C). With increase in the stress level the balance curve of apoptosis (red) shifted to the right resulting in the loss of intersection of the curves at autophagic state (Figs. 1C and 5D). Therefore, the regulatory system fired to the sole remaining stable state, which corresponds to apoptosis.

3.4. The effect of autophagy on apoptosis under combined treatment of cells

In this section, we raise the question how the simultaneous treatment of rapamycin/metyrapone and ER stressor can affect the balance curves of the system. Our experimental data showed the following effects of rapamycin/metyrapone on ER stressed cells:

1. Autophagy got activated at lower concentration of TG in the presence of rapamycin/metyrapone compared to TG treatment alone suggesting that the activation threshold for autophagy shifted to lower stress level in the presence of combined treatment (Figs. 3 and 4).
2. Rapamycin/metyrapone increased cell viability by maintaining autophagic state at severe ER stress suggesting that the activation threshold of apoptosis inducer moved to higher stress values (Figs. 2–4).

Since both rapamycin and metyrapone act *via* mTOR inhibition, we studied the qualitative behavior of the system under combined treatment by decreasing the total level of mTOR to close to zero to mimic the effect of rapamycin/metyrapone (Fig. 6A). A combined treatment shifted the balance curve of autophagy inducer to right while the balance curve of apoptosis inducer shifted to left in comparison to the treatment of cells with only high TG concentration (Fig. 6B). The net effect of combined treatment was that the system was able to preserve its autophagic state even at higher concentration of TG. However, at much higher concentration of TG, the balance curve of apoptosis shifted towards right even in the presence of rapamycin/metyrapone suggesting that the system lost its surviving state at severely stressed conditions.

We also computed the effect of combined treatment on the signal response curve of autophagy and apoptosis inducers (Fig. 6C and D). The autophagy inducer followed a sigmoid activation characteristic with respect to low levels of stress, while apoptosis inducer underwent a switch-like induction at a higher threshold of ER stress. Apoptosis activation also led to the inhibition of autophagy. The combined treatment of cells resulted in the activation of autophagy inducer to reach its maximum value at much lower level of stress, while its inactivation threshold moved to a higher threshold suggesting that one of the main role of mTOR is to suppress autophagy dependent survival during intolerable ER stress (Fig. 6C and D). Thus, it could be seen that under combined treat-

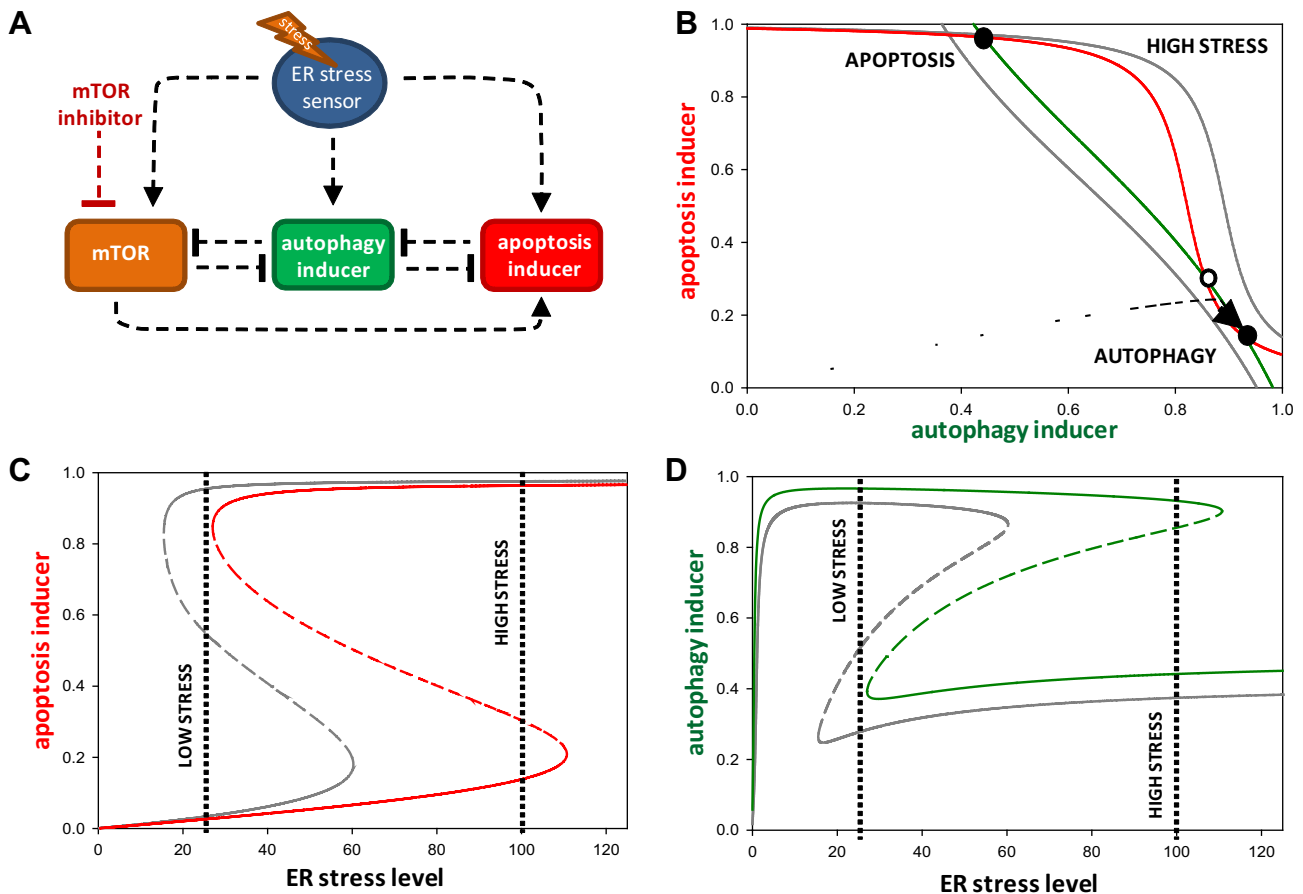


Fig. 6. The effect of combined treatment on cell fate decision. (A) Wiring diagram of the regulatory network when mTOR inhibitor is used. Autophagy inducer, apoptosis inducer, mTOR and crosstalk elements are denoted by isolated boxes. Dashed lines show how the molecules can influence each other. Blocked end lines denote inhibition. (B) Phase plane portrait of crosstalk between autophagy and apoptosis. Red nullcline represents apoptosis inducer, while green nullcline denotes autophagy inducer. Intersections of nullclines represent the stable (filled circle) and unstable (open circle) steady states. The phase planes are shown for (gray lines) high stress (stress = 25) and for (solid lines) high stress + mTOR inhibitor (stress = 100, mTORT = 0.05). (C) and (D) Signal-response curve of crosstalk between autophagy and apoptosis. The signal-response curve of (C) autophagy inducer and (D) apoptosis inducer are shown with respect to the increasing stress level. The signal-response curves are plotted with (colored lines) and without (gray lines) mTOR inhibitor. Solid lines denote stable states, while dashed line denotes the unstable state. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ment the activation and inactivation thresholds of autophagy inducers decreased and increased, respectively, to extend the stress window of autophagy-dependent survival. Increase in autophagy under combined treatment was manifested through increase in magnitude and duration of autophagy inducer to suppress apoptosis (Fig. S7). Our model analysis suggested that both “arms” of mTOR (inhibiting autophagy and activating apoptosis inducers) were lost under the combined treatment to block the activation of apoptosis. We observed comprising either of them is insufficient to block apoptosis at higher stress levels (Fig. S8).

4. Discussion

A cellular decision to survive or die under stress conditions is influenced by the crosstalk between signaling pathways that regulate these processes. Cells employ pleiotropic pathways or proteins to make mutually exclusive decisions. These aspects raise a question: how cells succeed to switch on or off robustly specific stress response using an integrated network. In this study, we addressed this question using experiments and mathematical modeling in the context when cells are subjected to ER stress.

An increase in misfolded proteins due to change in ER folding capacity is shown to be monitored by three branches (IRE1, PERK and ATF6) of unfolded protein response (UPR) [14]. These branches not only help towards maintaining homeostasis but also can

trigger apoptosis in the case of irresolvable stress. One of the mechanisms activated by UPR is autophagy, a catabolic process that helps towards cell survival under different stress conditions. Interestingly, both autophagy and apoptosis share mutual relationship as well as are under the positive control of UPR. Further, UPR activates mammalian TOR (mTOR) pathway, which in turn promotes apoptosis, while inhibits autophagy [35]. These paradoxical interactions make it difficult to understand regulatory principles of stress responses intuitively.

Our experiments showed that cells depending on the magnitude and duration of ER stress (by perturbing ER homeostasis using TG or metyrapone) temporally co-ordinate autophagy and apoptosis in a sequential and mutually exclusive manner (Fig. 1). We observed that autophagy is activated even at high stress conditions and contributes towards cell survival for few hours, after which only cell death mechanism prevails (Fig. 4 and Fig. S2). This is evident from an early event: increase in the conversion of LC3I to LC3II (an indicator of autophagy activation) and late events: induction of PARP cleavage and decrease of procaspase-3 level (indicators of apoptosis activation) and increase in p62 levels (an indicator of autophagy inhibition) (Figs. 3 and 4 and Fig. S2). Accordingly, cell viability is high at 2 h while low at 24 h (Fig. 2). On the other hand, at lower stress level, the activation of autophagy is observed, concomitantly with rise in cell viability. These results suggest that autophagy might have cytoprotective role dur-

ing ER stress and can delay or prevent UPR dependent apoptosis activation to relieve the stress. Such a role for autophagy in delaying cell death has been reported under nutrient starvation stress with both autophagy and apoptosis sharing antagonistic relationship.

Nutrient starvation condition is known to inhibit mTOR and increase autophagy activity [33]. We confirmed that under ER stress mTOR has dual roles of activating apoptosis and inhibiting autophagy. Thus, inhibiting mTOR by rapamycin or metyrapone in ER stress-induced cells leads to increase in cell viability (Fig. 2). We observed that this increase is accompanied by significant increase in autophagy due to mTOR pathway inhibition even at low level of ER stressor (Figs. 3 and 4). Previous studies claimed that inhibition of autophagy under ER stress accelerates cell death and loss of function mutation of ATG1 suppresses the positive effect of rapamycin on cell survival [29,38]. Based on these observations, we conclude that UPR activates autophagy as a control strategy to delay apoptosis since it also serve as an activator of apoptosis.

In our previous study, we showed by a mathematical model how stress modulates the activation of autophagy and apoptosis using common regulatory player Bcl2 and crosstalk between these cellular processes [45]. Although the regulatory design remains the same under different stress conditions, cell type- and stress-specific regulatory players additionally influence the dynamics of cell survival-cell death decision. One such example is mTOR, which is shown to promote cell death under ER stress. This is in contrast to its roles in cell growth and suppression of stress responses such as autophagy. We have analyzed with help of mathematical model the influence of mTOR on the dynamics of autophagy and apoptosis activation under ER stress. Both phase plane and bifurcation analysis of the model showed that the stress threshold for apoptosis activation is sensitive to mTOR activity and can increase with decrease in mTOR activity (Figs. 5 and 6). Thus, inhibition of mTOR activity with rapamycin or metyrapone increased the threshold for apoptosis activation to higher stress levels thereby suppressing cell death under ER stress (Fig. 6 and Fig. S7). In the minimal model, mTOR can influence the threshold in two ways: by controlling the apoptosis inducer directly and by inhibiting autophagy, which has negative effect on apoptosis inducer. We observed that perturbing either of them fails to suppress cell death under high ER stress suggesting that mTOR might contribute to cell death through different routes (Figs. S8 and S9). Another way that mTOR can promote apoptosis activation is by increasing the burden on ER through protein synthesis. As a consequence, both mTOR and ER stress sensor, UPR can form a dangerous positive feedback loop to amplify the stress level beyond the threshold required to induce apoptosis. Thus, altering the stress threshold (by enhancing autophagy) or preventing an increase in stress level (by protein synthesis inhibition) is sufficient to rescue cells from death under high ER stress.

It can be noted that the model also incorporates the mutual antagonism between mTOR and autophagy controlled by UPR. mTOR is active under stress free situation to promote growth and can inhibit autophagy. This creates a situation how autophagy is turned on under ER stress. The model explains this situation through rapid activation of autophagy by UPR in comparison to mTOR. As a result, autophagy overcomes its inhibition to suppress both mTOR and apoptosis at low stress level. On the other hand, at high stress level, the extent of UPR activation increases which lead to an increase in the rate of activation of apoptosis and mTOR to overcome autophagy. Thus, mTOR inactivation is only transient under high stress level. Therefore, mTOR inhibition by rapamycin or metyrapone results in sustained activation of autophagy that can prevent apoptosis activation. The model predicts that mTOR activity temporally varies as a function of ER stress levels to control cell fate decisions.

Our experimental and mathematical data highlight the contribution of UPR, mTOR and autophagy towards establishing the stress threshold for apoptosis activation. Therefore, the dynamics of apoptotic pathway is not only influenced by the feedback loops within this pathway but also by the crosstalk with UPR, mTOR and autophagy. Our study provides a framework to build a comprehensive model of crosstalk between pathways in the control of cell survival-cell death decision.

ER stress dependent cell death is an important factor in the pathomechanism of various human pathologies (e.g. neurodegenerative diseases, obesity-related diseases, proteinopathies). Stimulation of autophagy via mTOR inhibition either by lifestyle changes (nutrient starvation) or by pharmacological agents (calorie restriction mimetics, mTOR inhibitors) can be a tool to combat these diseases. Further development of our mathematical model formulated on an experimental ground can have a biomedical value in the identification of targets and in the evaluation of the efficacy of agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fob.2014.07.006>.

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