

A cellular stress-directed bistable switch controls the crosstalk between autophagy and apoptosis†

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Decision-making between life and death is one of the most important tasks of cells to maintain their genetic integrity. While the surviving mechanism is driven by Beclin1-dependent autophagy, the suicide processes are controlled by caspases-mediated apoptosis. Interestingly, both these processes share regulators such as Bcl2 and influence each other through feedback loops. The physiological relevance of the crosstalk between autophagy and apoptosis is still unclear. To gain system level insights, we have developed a mathematical model of the autophagy–apoptosis crosstalk. Our analysis reveals that a combination of Bcl2-dependent regulation and feedback loops between Beclin1 and caspases robustly enforces a sequential activation of cellular responses depending upon the intensity and duration of stress levels. The amplifying loops for caspases activation involving Beclin1-dependent inhibition of caspases and cleavage of Beclin1 by caspases (Beclin1 + caspases + Beclin1; caspases → cleaved Beclin1 → caspases) not only make the system bistable but also help to switch off autophagy at high stress levels. The presence of an additional positive feedback loop between Bcl2 and caspases helps to maintain the caspases activation by making the switch irreversible. Our results provide a framework for further experiments and modelling.

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Introduction

Maintaining homeostasis is crucial for the survival of multicellular organisms. It depends on the ability of cells that build up the organism to sense and respond to the intracellular and extracellular signals. Cells are able to keep a balance between synthesis, degradation and recycling of different cellular components at a physiological level.¹ Autophagy plays an essential role in promoting the cellular-survival mechanism under tolerable stress conditions (such as transient nutrient starvation and growth factor withdrawal) which forces the return to the homeostatic state. However, severe or continuous stress conditions stimulate the well-known apoptotic cell death pathways leading to self-elimination.²

The Greek word “autophagy” means “self-eating” referring to the ability of cells to “digest” their own parts of the cytoplasm and intracellular organelles. Cellular components get sequestered into a vesicle, called autophagosome, whose contents are

then delivered to and degraded by lysosomes.^{3,4} Cells have residual autophagic activity even under physiological conditions; however the process gets more efficient during increasing stress levels.⁵ Excessive level of autophagy is also known to cause cell death in apoptotic-deficient cells.^{3,4} Due to its crucial role in controlling cellular homeostasis the turning on or off of autophagy is tightly regulated. The crucial regulator of autophagy is Beclin1, the mammalian homolog of yeast Atg6.^{6,7} Beclin1 forms a multiprotein complex with other molecules (such as UVRAG, AMBRA-1, Atg14L, Bif-1, Rubicon, SLAM, IP3R, PINK and survivin) to activate the class III phosphatidylinositol-3-kinase.⁸ Beclin1 depleted cells cannot induce the autophagosome formation.⁴

The suicidal cascade typically involves the activation of a family of cysteine proteases called caspases.^{2,9} First the initiator caspases (2; 8; 9; 10) get activated which in turn cleave the effector caspases (3; 6; 7) into their active forms. Effector caspases are synthesized in inactive pro-caspase forms, and these forms have to be cleaved at a particular residue to become active. The cleavage of target proteins of active effector caspases during apoptosis leads to the typical biochemical and morphological changes, such as chromatin condensation, DNA fragmentation, cell shrinkage and membrane blebbing.^{2,9,10} Depending on the origin of stress stimuli, caspases activation can be mediated by intrinsic or extrinsic pathways.¹¹

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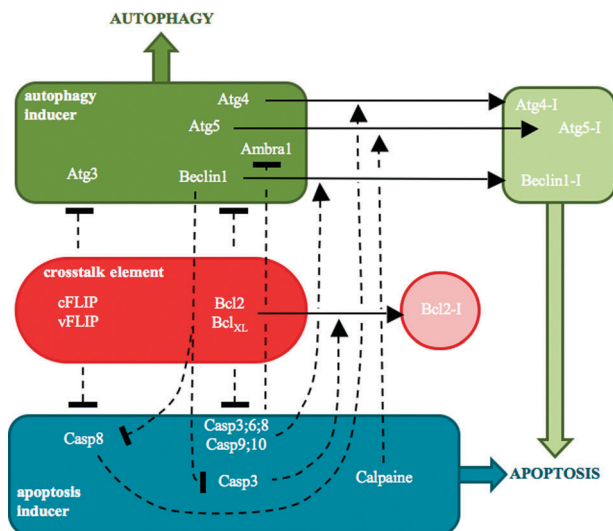


Fig. 1 The regulatory components and their inter-connections in the stress-induced autophagy–apoptosis crosstalk. The autophagy inducers, the apoptosis inducers and the crosstalk elements are grouped together in isolated green, blue and red boxes, respectively. The active forms of the molecules are grouped in dark-coloured boxes, while light-coloured boxes denote the inactive forms. Solid arrows represent biochemical reactions, dashed line shows how the molecules can influence each other. Blocked end lines denote inhibition.

Cells respond to the stress stimuli that originated within the cell such as DNA damage through an intrinsic pathway by promoting the release of factors such as cytochrome *c*, SMAC from the mitochondria to cytosol through membrane permeabilization. On the other hand, the extracellular ligand binding to death receptors initiates the extrinsic pathway, which also crosstalk with the intrinsic pathway.^{12,13}

Interestingly, different studies have revealed the existence of a crosstalk between autophagy and apoptosis regulatory mechanisms at the molecular level.^{14–17} Fig. 1 shows the interactions among the key molecular components which are classified based on their function into different groups such as apoptosis inducer, autophagy inducer and crosstalk element. It is well known that an anti-apoptotic factor Bcl2 (B-cell lymphoma-2) keeps the caspases in their inactive forms under physiological conditions.¹⁰ However, recent studies provide the evidence that Bcl2 (as well as its homologue Bcl_{XL}) also inhibits autophagy by binding to Beclin1 and forming an inactive complex.^{18,19} The Beclin1-bounded form of Bcl2 is still capable of inhibiting the caspases activation.²⁰ Another negative regulation includes cFLIP and vFLIP mediated inhibition of both extrinsic pathway of apoptosis (through Caspase-8) and autophagy (through protein conjugated enzyme, Atg3).^{17,21} A crucial question arises: how the two qualitatively different stress responses can be regulated by the same inhibitor molecules? Although different experiments have shown a strict order of activation where the survival mechanism is followed by the suicide loop,^{22–28} it is still unclear how this is achieved robustly.

Another crosstalk is shown to exist through caspases-dependent cleavage of autophagy related proteins, which terminates autophagic response.¹⁷ In addition to that the cleaved proteins of

autophagy are shown to promote apoptosis.¹⁷ For instance, it is shown that some of the caspases inhibit autophagy by cleaving Beclin1 following interleukin-3 (IL-3) depletion.^{15,29} The elimination of IL-3 resulted in autophagy measured by increasing the LC3II level in the cell, however after 8 hours the activation of caspases was observed.^{16,29} Meanwhile the level of the autophagy indicator also diminished completely due to the Caspase-3 and -7-dependent Beclin1 cleavage.²⁹ Beclin1 is also cleaved during nutrient starvation, oxidative stress, treatment with staurosporine, TRAIL-induced apoptosis and chemotherapy.^{15,30–32} Further, it is shown using a non-cleavable Beclin1 that full length protein does not, but the cleaved C-fragment of Beclin1 enhances the apoptotic cell death.³² A similar regulation involving caspases- or calpaine-dependent cleavage of other autophagy inducer proteins such as Atg4 and Atg5 has been reported.^{33,34} All these evidences support the notion that the caspases can amplify their own activity by their effect on the autophagy-related proteins. Caspase-3 is also known to promote its own activity by cleaving proteins in the intrinsic/extrinsic pathway such as Bcl2, inhibitors of apoptosis, Bid and other caspases.^{17,35} Studies have shown that overexpression of autophagy related proteins inhibits apoptosis and while their knockdown sensitizes the cells to apoptosis in response to death stimuli.^{4,5,25} Autophagic degradation of a subunit of active Caspase-8 has also been reported.³⁶ These studies suggest that autophagy regulators can antagonize apoptosis by promoting autophagy or by directly inhibiting it.

In the current work, we study the characteristic features of the autophagy–apoptosis network that is sufficient to enforce and maintain the crucial cell-fate decisions. A mathematical model of a minimal network including a system level crosstalk is developed. Our analysis using the recent experimental findings demonstrates how Bcl2 works together with other molecular players in the network depending on stress levels (a) to ensure that only one cellular response gets activated, (b) to control transition from one cellular response to another one and (c) to make irreversible cellular decision. We show that multiple feedback loops within the integrated network are required to achieve all the desired characteristics.

Model description

Although different molecular components and their inter-connections in autophagy and apoptosis pathways have been identified, there are variations in regulation depending upon the cellular settings and nature of stress stimuli. Such differences make them to work in any of these scenarios: as a partner, antagonist and enabler.¹⁶ Our analysis mainly focuses on the results when both autophagy and apoptosis are involved in mutual inhibition. This leads to the situation that either one of them is activated or sequential activation of cellular responses depending upon the stress levels or duration. Starvation, endoplasmic reticulum stress, growth factor depletion and DNA damage are all known to give rise to such cellular responses (Tables S1 and S2, ESI†). However, only in the case of nutrient depletion it is shown that the mechanism requires the

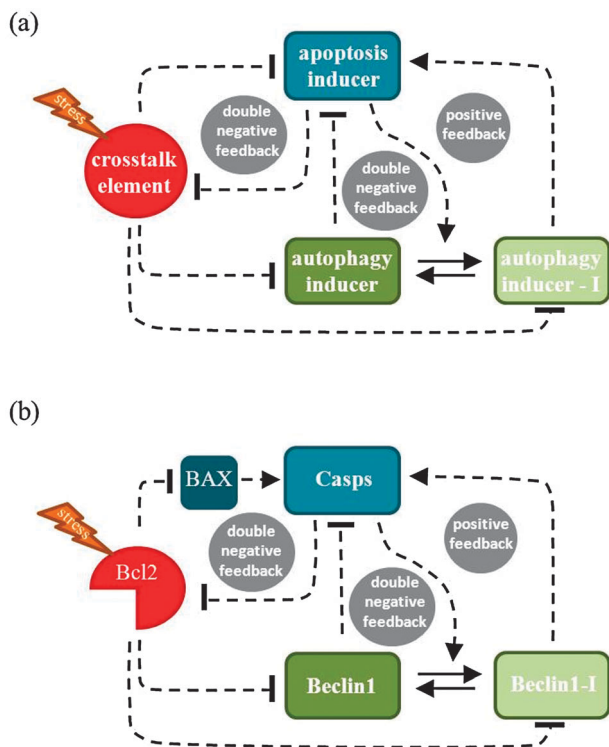


Fig. 2 The minimal network of stress-induced autophagy–apoptosis crosstalk. (a) The key network motifs controlling the autophagy–apoptosis crosstalk. (b) The wiring diagram of the Bcl2–Beclin1–Casps model. The autophagy inducer, the apoptosis inducer and the crosstalk element are denoted by isolated green, blue and red boxes, respectively. The active forms of the molecules are grouped in dark-coloured boxes, while the apoptosis inducer (Casps)-cleaved form of the autophagy inducer (Beclin1) is depicted by a light green box. Solid arrows represent biochemical reactions, dashed line shows how the molecules can influence each other. Blocked end lines denote inhibition.

release of Beclin1 from the inhibition of Bcl2. It is still unclear whether other stress signals operate through Bcl2 or other relevant targets.

We assemble based on Fig. 1 a minimal network that is independent of identity of molecular players and it only includes the system level feedback loops relevant to the crosstalk between autophagy and apoptosis (Fig. 2a). It can be seen that inducers of both processes are inhibited by crosstalk element and stress stimuli control them by down-regulating the crosstalk element. Autophagy and apoptosis inducers mutually inhibit each other forming a double negative feedback loop. Apoptosis inducers can promote their own activation by cleaving autophagy-related proteins which not only eliminates the negative effect of autophagy on apoptosis but also generates their cleaved products that promote apoptosis through a positive feedback loop. On the other hand autophagy can keep itself active by inhibiting apoptosis through autophagic degradation of apoptosis inducers. Further, apoptosis inducers can also promote their own activation by inhibiting the crosstalk element thereby forming another double negative feedback loop. Our analysis focuses on the system of crosstalk between autophagy and apoptosis that is characterized in response to starvation. This network includes the inter-connections between Bcl2,

the autophagy inducer Beclin1 and the apoptosis inducer caspases (called Casps in the model) (Fig. 2b). Each of these components fall under the different categories described above. The crosstalk element Bcl2 is high under physiological conditions to arrest the activation of both “self-eating” and “self-killing” pathways. However, in the presence of stress Bcl2 levels drop or they become inactive and the cell can respond to the unfavourable conditions by autophagy and/or apoptosis induction.^{19,37} The control of Bcl2 involves regulations at the level of synthesis, degradation and activity.³⁷ Bcl2 undergoes post-translational modifications (such as JNK-dependent phosphorylation³⁸) which aid in the dissociation of the Beclin1–Bcl2 complex. In our model, Bcl2 regulation is simplified by considering a constant rate of its synthesis and degradation with stress decreasing the stability of the protein (see the model in ESI†).

Both Beclin1 and Casps have active and inactive forms in the model. Casps activation depends on the pro-apoptotic proteins BAX/BAK¹⁰ (called BAX in the model). Bcl2 inhibits both Beclin1 and BAX by forming inhibitory complexes with them (Fig. S1, ESI†). The Bcl2–Beclin1 complex also inhibits BAX. We consider that the release of BAX activates Casps directly instead of its effect on mitochondria (through cytochrome *c* release¹⁰). While Beclin1 can be directly inactivated in two different ways (through inhibitory complex formation with Bcl2 and Casps-dependent cleavage), the inactivation of Casps is solely by Beclin1 in our model. Besides free BAX, the cleaved form of Beclin1 also promotes Casps activation (Fig. S1, ESI†).

The stress is used as an input (bifurcation parameter) to the model which facilitates the dissociation of both Beclin1–Bcl2 and BAX–Bcl2 complexes. In the context of this analysis we categorized stress based on its ability to activate only autophagy or autophagy followed by apoptosis (Table S1, ESI†). For instance, a low concentration of rapamycin or a transient nutrient starvation leads to only autophagy. Therefore, we classify the low concentration or transient induction of stress into a category of “low stress” condition. On the other hand, the sustained induction of stress (such as 16 h long starvation) leads to autophagy followed by apoptosis and we classify it as “high stress” condition.

Results

Autophagy–apoptosis transition mediated by a bistable switch

At first the contribution of Bcl2 regulation and feedback loops in the network towards achieving a strict order of activation of two qualitatively different stress response mechanisms were analyzed. Both stoichiometric binding and positive feedback regulation present in the network are common motifs that can provide an expeditious change in the activity of the regulated molecule in response to a stimulus.³⁹ To study the effect of regulatory motifs on ordering of the events we have computed the overall steady state response of the system which is given as a signal response curve. The input signal is the stress level rising from zero to a high value, while the steady state level of both Beclin1 and Casps is traced as the output of the control network.

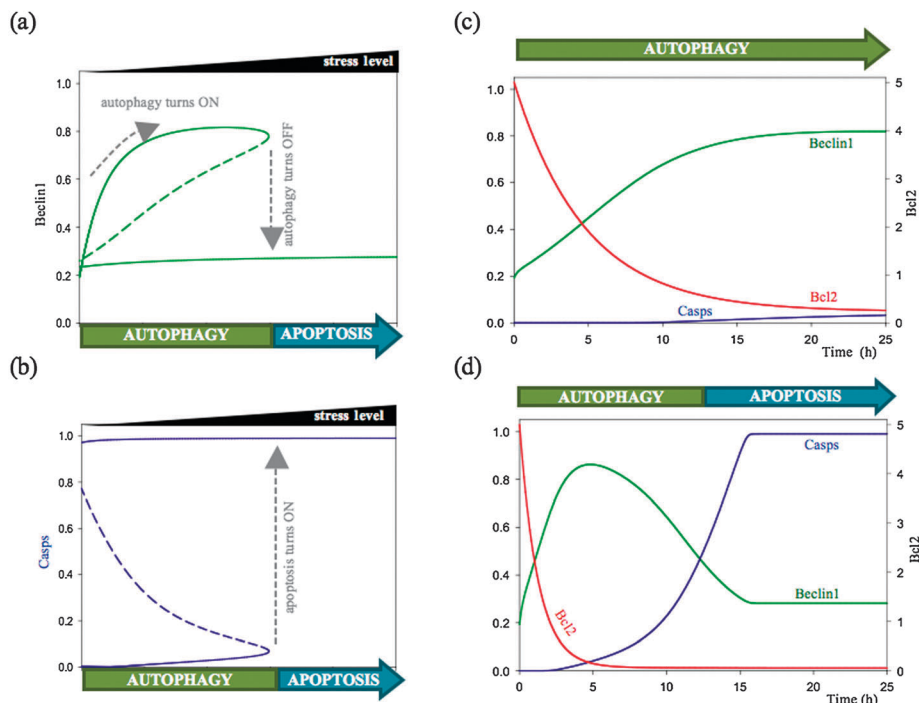


Fig. 3 The characteristic properties of the Bcl2–Beclin1–Casps regulatory network. The signal–response curve of (a) Beclin1 and (b) Casps are shown with respect to the increasing stress level. Solid lines denote stable states, while dashed line denotes the unstable state. The dynamic simulation of the model with (c) continuous low stress conditions (stress = 0.5) and (d) continuous high stress conditions (stress = 2). The dynamics of Bcl2, active Beclin1 (free form) and Casps are shown.

Fig. 3a and b shows that both Beclin1 and Casps activities exhibit discontinuous bistable behaviour with respect to stress levels. At lower stress levels, Beclin1 activity follows a hyperbolic response curve (Fig. S2a, ESI[†]) but as the stress level increases the curve turns back to become a Z-shaped curve (Fig. 3a). The initial activation of Beclin1 is the consequence of its stress-directed stoichiometric binding to Bcl2 to form an inactive Beclin1–Bcl2 complex. Although inhibitory stoichiometric complex formation is favourable under physiological conditions, the reversible binding always generates some Beclin1 activity which accounts for the residual autophagic activity observed under physiological conditions.⁵ On the other hand, the response curve of Casps is S-shaped with respect to stress levels (Fig. 3b). Both the S- and Z-shaped region of the response curves indicate that for each stress value the systems have two possible stable steady states (solid line) separated by an unstable state (dashed line). The one stable state represents the active state of autophagy: Beclin1 is active, Casps are inactive while the other stable state represents the active state of apoptosis: Beclin1 is inactive, Casps are active. The unstable state with intermediate levels of active Beclin1 and Casps cannot be reached physically by the control system, thereby resulting in a discontinuous switch between the two stable states. It can be observed that the Casps activity occupies a lower stable steady state until the stress level reaches the limit point (Fig. 3b, ESI[†]) of stable and unstable steady states to activate Casps. At this critical threshold, the activation of Casps switches to the higher stable steady state, which is accompanied by the inactivation of Beclin1 (Fig. S2b, ESI[†]).

This indicates that both autophagy and apoptosis cannot coexist together during different stress levels.

Furthermore, a portion of the S-shaped response curve of Casps is pushed onto the negative regime of the diagram therefore the inactivation threshold of Casps is a negative number (Fig. 3b, ESI[†]). This indicates that once the Casps are turned on by the stress signal their activity does not decrease even if the stress signal is turned off. As the stress level decreases the activity of Casps moves to the left of the bifurcation diagram along the upper stable state and remains in an active state for the stress value equal to zero (Fig. 3b, ESI[†]). This might be a desirable property for such a system because once cells have committed to undergo apoptosis; there should not be a possibility to go back to the surviving state. Therefore the control system has to render the switch of Casps activation (and Beclin1 inactivation) to be an irreversible one-way switch. A similar characteristic can be observed for the Beclin1 response curve with a portion of it on the negative regime of diagram (Fig. 3a, Fig. S2a, ESI[†]). Besides irreversible activation of Casps this network guarantees the irreversible inactivation of Beclin1 under continuous high stress conditions.

Further the dynamics of such a control network was also studied by following the time evolution of the activity or level of three main components (Bcl2, active Beclin1 and Casps) in the presence of a continuous low or high stress level. Fig. 3c shows the effect of a sustained low stress level on the system, which activates Beclin1 gradually by promoting the degradation of Bcl2 and dissociation of the Beclin1–Bcl2 complex. However Casps remain inactive because the suicide feedback loop fails to turn on thereby protecting cells from death. If the stress

connected events can be eliminated the system returns to its homeostatic state (data not shown). However, a constant high level of stress has dramatic effects on the system (Fig. 3d). Beclin1 gets activated rapidly first, but under these circumstances the Bcl2 level diminishes completely. This facilitates the activation of Casps by turning on the positive feedback loop. Although Beclin1 induces autophagy and inhibits apoptosis, Casps can still be turned on. The increasing Casps activity rapidly down-regulates Beclin1 by transforming it into an inactive form. This form of Beclin1 fails to induce autophagy, but it promotes apoptosis by Casps activation. It can be noted that there is a short window of autophagic activity at high stress levels. The regulatory system has a chance to rescue itself by healing damages, but continuous intolerable stress results in apoptosis. Such a situation is possible because of the time delay in the appearance of Casps.

The positive feedback regulation is shown to be crucial to establish an irreversible cell-fate decision between two qualitatively different states in other systems.^{40,41} In the autophagy–apoptosis crosstalk network considered here there are multiple feedback loops which can provide this system level property. This includes both double negative feedback loops (Beclin1 \uparrow Casps \uparrow Beclin1 and Casps \uparrow Bcl2 \uparrow Casps), and a positive feedback loop (Casps \rightarrow cleaved Beclin1 \rightarrow Casps) (Fig. 2b).

It is known that in the apoptosis regulatory network itself there are different positive feedback loops which can potentially yield a bistable response.^{42,43} However, there is no clear evidence for the contribution of an individual feedback loop towards bistable response. On the other hand, the experimental

results have indicated that apoptotic inducers have to accumulate to reach a threshold for promoting the switch-like activation of the “self-killing pathway”.^{42–47} While the contribution of different positive feedback loops towards bistable response remains to be elucidated, we demonstrate that such a property can also potentially arise from the crosstalk with autophagy regulation. Another importance of such a crosstalk is the efficient prevention of autophagy, when apoptosis gets activated depending on the stress levels. This is through a feedback loop that has a positive effect on apoptosis while a negative effect on autophagy both of them mediated through cleavage of Beclin1 by Casps.

Feedback mechanism at the crosstalk between autophagy and apoptosis

In order to study the effect of feedback loops on the steady state and dynamics of the autophagy–apoptosis crosstalk network, the interaction of feedback loops was systematically perturbed. The full length Beclin1 is inhibited by Casps-dependent cleavage and the nascent C-fragment of Beclin1 obtained after cleavage lacks autophagic activity.²⁹ Interestingly, translocation of Beclin1-C-fragments into mitochondria induces apoptosis by enhancing the release of mitochondrial pro-death factors.²⁹ Also, non-caspase-cleavable Beclin1 restored autophagy in BAX-overexpressed cells.³² Therefore we studied the effect of modulating the Casps mediated cleavage of Beclin1, which in principle should perturb two feedback loops (Beclin1 \uparrow Casps \uparrow Beclin1 and Casps \rightarrow cleaved Beclin1 \rightarrow Casps) on Casps activity (Fig. 2b).

Fig. 4a shows the response curve for the situations involving non-cleavable Beclin1 ($k_{ibc}' = 0$). It can be seen that with

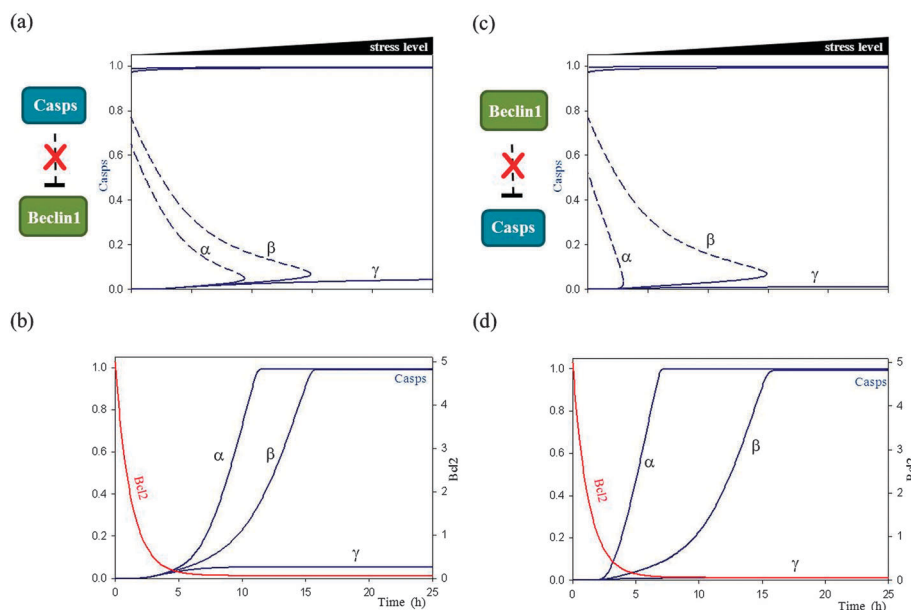


Fig. 4 The effect of feedback loops involving Beclin1 and Casps on the network properties. (a) Signal response curve of Casps and (b) dynamics of the network are shown with different rates of inhibition of Beclin1 by Casps-dependent cleavage. Symbols γ : $k_{ibc}' = 0$, β : $k_{ibc}' = 5$ and α : $k_{ibc}' = 10$ represent the situations without inhibition, control and higher rate of inhibition of Beclin1 by Casps, respectively. (c) Signal response curve of Casps and (d) dynamics of the network are shown with different rates of Casps inhibition by Beclin1. Symbols α : $k_{icp}' = 0$, β : $k_{icp}' = 0.35$ and γ : $k_{icp}' = 0.7$ represent the situations without inhibition, control and higher rate of inhibition of Casps by Beclin1, respectively. The solid lines in the signal response curve denote stable states, while dashed line depicts the unstable state. The temporal dynamics is simulated under continuous high stress conditions (stress = 2). The dynamics of Bcl2 and Casps are shown.

non-cleavable Beclin1 the activation of Casps (Fig. 4a, symbol γ) is affected while the activation of Beclin1 follows a hyperbolic response curve with respect to stress levels and remains uninhibited (see Fig. S3a, ESI_{\uparrow} , symbol γ). On the other hand on increasing the Casps-dependent Beclin1 cleavage, the critical threshold for the activation of Casps decreases to lower stress levels (Fig. 4a, symbol α) in comparison to the control situation (Fig. 4a, symbol β). Accordingly Beclin1 gets inhibited at lower stress levels (Fig. S3a, ESI_{\uparrow} , symbol α). This analysis reveals that the bistable characteristic and the corresponding threshold for Casps activation are influenced by Beclin1–Casps interaction. We have also computed the dynamics under these situations for continuous high stress conditions. Under a non-cleavable situation, Beclin1 activity increases and remains high (Fig. S3b, ESI_{\uparrow} , symbol γ), while Casps activity is low (Fig. 4b, symbol γ). Increasing Casps-dependent Beclin1 cleavage leads to premature activation of Casps by decreasing the time delay in its activation (Fig. 4b, symbol α). This restricts the duration of autophagic activity in comparison to the control situation (Fig. S3b, ESI_{\uparrow} , symbols α and β).

Since non-cleavable Beclin1 eliminates both feedback loops acting on Casps, we analyzed the situation when only one feedback loop is compromised. This can be done by removing Beclin1-dependent inhibition of Casps ($k_{icp}' = 0$) which retains the positive feedback regulation of Casps mediated by cleavage of Beclin1 (Fig. 2b). In the absence of Beclin1-dependent inhibition of Casps, the response curve of Casps is still an irreversible bistable switch but the activation threshold of Casps shifts to a lower threshold (Fig. 4c, symbol α) in comparison to the control situation (Fig. 4c, symbol β). On the other hand increasing the strength of this interaction completely blocks the activation of Casps by counteracting the positive feedback loop (Fig. 4c, symbol γ), while Beclin1 activity remains high (Fig. S3c, ESI_{\uparrow} , symbol γ). This indicates that the strength of the positive feedback loop determines the threshold to overcome the negative effect of autophagy. Therefore in the situation with no positive feedback loop such as non-cleavable Beclin1, we observed Casps inactivation due to the negative effect of Beclin1 on Casps (Fig. 4a, symbol γ). If the Beclin1-dependent inhibition of Casps is weaker then a switch-like activation of Casps can be observed (data not shown). This suggests that the phenotype of non-cleavable Beclin1 can vary depending on the magnitude of Beclin1-dependent inhibition of Casps.

Fig. 4d shows the dynamic simulation in the absence (symbol α) or hyper-activation (symbol γ) of Beclin1-dependent inhibition of Casps, which in principle resembles the situations with hyper-activation (Fig. 4b, symbol α) or absence of (Fig. 4b, symbol γ) Beclin1 cleavage, respectively.

The analysis was also performed by retaining the double negative feedback loop (Beclin1 \uparrow Casps \uparrow Beclin1) involved in Casps activation and compromising the positive feedback loop that is mediated through the Casps-cleaved form of Beclin1 ($k_{acp}' = 0$). Experimentally this mutant phenotype can be mimicked by blocking the cleaved Beclin1-dependent apoptotic events (e.g. by blocking the re-localization of Beclin1-C-fragments to the mitochondria).

In this mutant, the response curve of Casps shows irreversible bistable behaviour with an activation threshold at higher stress levels in comparison to the control phenotype (data not shown).

Furthermore, we show that the bistable characteristic of the system is not specific for the chosen parameter set but can also be observed over the broad range of parameter values (see Fig. S4, ESI_{\uparrow}). In the model, the threshold for the activation of Casps can be associated with the presence of positive feedback loops and zero order ultrasensitivity. However, we note that the latter condition is not a strict requirement for observing the bistable response but can contribute towards extending the region of bistability to higher stress levels. As the effect of zero order sensitivity is reduced by increasing the Michaelis constant (J_{cp}) value of Casps activation and inactivation, the system is still bistable but for smaller stress values.

Feedback regulation of the apoptosis pathway

Kirsch *et al.* (1999) have shown that Caspase-3 has a negative effect on Bcl2 thereby promoting apoptotic activation.³⁵ Caspase-3-dependent cleavage of Bcl2 during apoptosis inhibits the pro-apoptotic effect of Bcl2.³⁵ This creates a double negative feedback loop in the system, where Bcl2 inhibits Casps and Casps in turn inhibit Bcl2. We perturbed the Casps-dependent inhibition of Bcl2 (k_{db2}'') to study the contribution of the double negative feedback loop on the overall response curve of Casps (Fig. 5). We show that in the absence of Casps-dependent Bcl2 inhibition, the irreversible bistable response curve of Casps activation becomes reversible (Fig. 5a, symbol γ and Fig. S4, ESI_{\uparrow}). Therefore, once Casps become active, they can be turned off if the stress signal is eliminated. This analysis suggests that although the interactions between Beclin1 and Casps are sufficient to make the system bistable, they are insufficient to enforce the one-way directionality of the system. In the model, the reversible characteristic of the Beclin1–Casps bistable switch is the result of Bcl2 re-activation with the elimination of stress. Bcl2 re-activation down-regulates both Beclin1 and Casps activity, which is prevented in the control situation (Fig. 5a, symbol β). However the activation threshold for Casps (and the inactivation threshold for Beclin1) has roughly the same value in each case (Fig. 5a, Fig. S4, ESI_{\uparrow}). This suggests that the mutual inhibition of Casps and Bcl2 does not change the S-shape of the Casps response curve with respect to stress levels, but it pushes the inactivation threshold of Casps to the left on the diagram. Therefore increasing the strength of the feedback loop pushes a portion of the S-shaped curve further into the negative regime (Fig. 5a, symbol α). The dynamic simulation shows that Casps become active after 10 hours of continuous treatment with a high stress level in both control and in the absence of Casps-dependent inhibition of Bcl2 (Fig. 5b). However, when the stress-dependent term is zeroed out at 20 h, the irreversible switch maintains Casps activity (symbol β), whereas its activity decreases in the absence of Casps-dependent inhibition of Bcl2 (symbol γ).

It is important to note that the irreversibility in the control system prevents the cell to return to its homeostatic state only

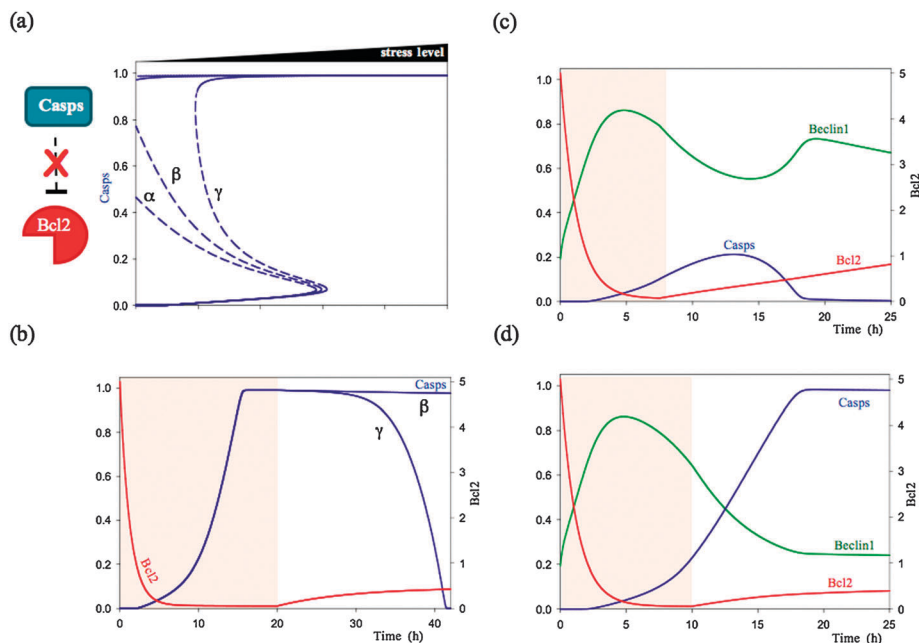


Fig. 5 Irreversible one-way switch of apoptosis induction. (a) The response curve of Casps and (b) dynamics of the network are shown with different rates of Casps-dependent inhibition of Bcl2. The temporal dynamics is simulated for 20 hours long treatment with high stress (stress = 2) depicted by a brown background. Symbols γ : $k_{db2''} = 0$, β : $k_{db2''} = 0.1$ and α : $k_{db2''} = 0.2$ represent the situations without inhibition, control and higher rate of inhibition of Bcl2 by Casps, respectively. The solid lines in the response curve denote stable states, while dashed line depicts the unstable state. The dynamics of Bcl2 and Casps are shown. The temporal dynamics under transient high stress conditions (stress = 2) is shown for (c) 7.5 hours long treatment and (d) 10 hours long treatment. The dynamics of Bcl2, active Beclin1 (free form) and Casps are shown with a duration of stress depicted by a brown background.

under continuous high stress conditions. The system can revert back to its original state if the high stress conditions get diminished before reaching the activation threshold of Casps. It can be observed that if the intolerable stress is eliminated at 7.5 hours in the numerical simulation, Beclin1 remains active (Fig. 5c). Although Bcl2 disappeared due to a high stress level and started to turn on Casps, stress was eliminated before Casps could get activated by helping themselves through Beclin1-Casps feedback loops. Under these circumstances, the re-accumulation of Bcl2 is relatively slow and therefore Beclin1 activity is high. If the duration of intolerable stress is extended then this pushes the system to the Casps activation state and the system cannot revert back to its original state (Fig. 5d). These results suggest that there is a time window after which intolerable stress turns on the irreversible switch.

The negative role of Bcl2 in the activation of both autophagy and apoptosis pathways

We also analyzed the effect of regulating the autophagy–apoptosis crosstalk in the absence of feedback loop regulation. This situation can be mimicked by blocking the Casps-dependent inhibition of both Beclin1 and Bcl2. In this case, the network is left with Bcl2 inhibition of both Beclin1 and BAX and Beclin1 inhibition of Casps. This forms an incoherent feed-forward loop in the network, where Casps get inhibited through both Bcl2-BAX and Beclin1 (Fig. 2b). Therefore the response curve of Casps with respect to stress levels depends on the extent of inhibition of Casps by both Bcl2 and Beclin1 (Fig. 6a and c).

At lower stress levels Beclin1 gets activated, since Bcl2 inhibition of Beclin1 is weaker compared to Casps (Fig. S6a, ESI[†]). As the stress level increases, Casps inhibition by Bcl2 is relieved and it becomes activated only if Beclin1 inhibition of Casps is removed or weak. In the absence of inhibition of Casps by Beclin1, Casps get activated at a lower stress threshold in a sigmoidal manner (Fig. 6a, symbol α) compared to the control situation (Fig. 6a, symbol γ). However, with an increase in the inhibition of Casps, the activation threshold moves to higher stress levels (Fig. 6a, symbol β) and beyond a critical value it can inhibit the activation of Casps. The dynamic simulation shows that Casps activation is delayed with respect to Beclin1 activation (Fig. 6b, Fig. S6b, ESI[†]). The time delay arises from the Bcl2 regulation of Casps through BAX even in the absence of Beclin1 inhibition of Casps and feedback loops in the network. This could vary depending upon how fast Bcl2 gets inactivated by stress (Fig. 6d, symbol α) or how fast its effect reaches the effector caspases through a pathway involving several components (Fig. 6d, symbol γ). The threshold for the activation of Casps is also influenced by the magnitude of the Bcl2 inactivation rate (Fig. 6c, symbol α) and the Casps activation rate by BAX (Fig. 6c, symbol γ). These results suggest that the sequestration of activators by inhibitors such as Bcl2 can contribute towards controlling the order of events.

Discussion and conclusions

Recent studies have shown that besides apoptosis autophagy is crucial in normal human physiological processes, and defects

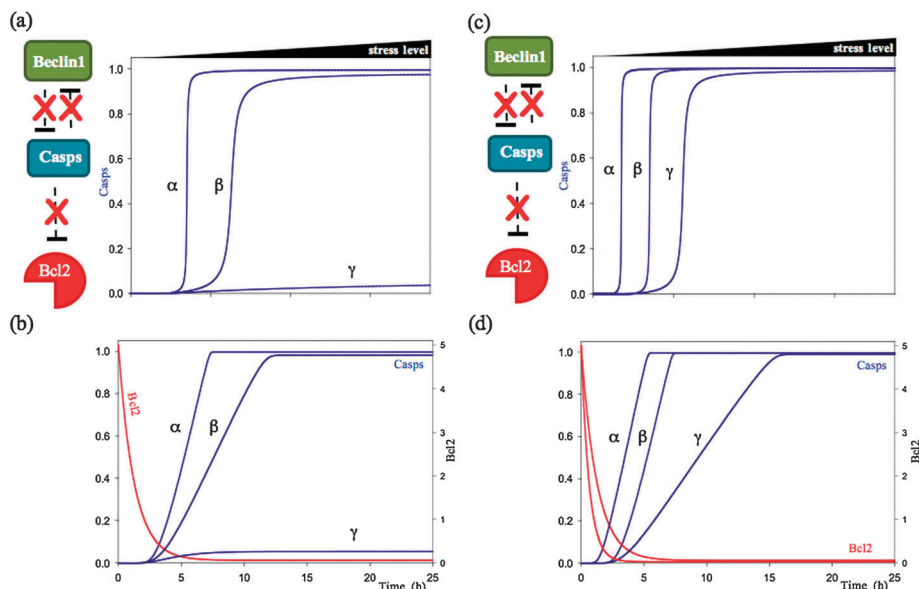


Fig. 6 The key roles of Beclin1 and Bcl2 in Casps activation, when all the feedback loops are compromised. The effect of feedback loops is removed by setting the parameter values of $kdb2'$ and $kicb'$ to zero. (a) The response curve of Casps and (b) dynamics of network are shown with different rates of inhibition of Casps by Beclin1. Symbols α : $kicp' = 0$; β : $kicp' = 0.15$ and γ : $kicp' = 0.35$ represent the situations without inhibition, low and high rate (control) of inhibition of Casps by Beclin1, respectively. (c) The response curve of Casps and (d) dynamics of the network are shown in the absence of inhibition of Casps (symbol β : $kicp' = 0$) by Beclin1 with either increased rate of Bcl2 degradation (symbol α : $kdb2' = 0.8$) or decreased rate of Casps activation by BAX (symbol γ : $kacp'' = 0.2$). The solid lines in the signal response curve denote stable states, while dashed line depicts the unstable state. The temporal dynamics is simulated for continuous high stress conditions (stress = 2). The dynamics of Bcl2 and Casps are shown.

in autophagy can result in different types of diseases (e.g. cancer, neurodegeneration).^{3,4,14} Therefore, it is important to elucidate the role of regulatory components involved in both autophagy and apoptosis. A better understanding of the crosstalk between autophagy and apoptosis may help us to stop or promote fatal cell decisions, with the long-term aim of therapeutic intervention. In this work we have explored the system level properties of a network comprising of crosstalk between autophagy and apoptosis using a mathematical model. Since both autophagy and apoptosis are regulated by an integrated network, it is difficult to explore their effects using a reductionist approach. However, a computational model of the network serves as a useful tool to predict the system response under different situations, which in turn can be tested using experiments. Our first step was to build a minimal model using the crucial components of the network to determine how the qualitative behaviour of the system changes with respect to stress levels.

The model of crosstalk between autophagy and apoptosis involves Bcl2-dependent inhibition of Beclin1 and BAX working together with feedback loops involving Beclin1 and Casps. Our analysis shows that such a minimal network is sufficient to turn on robustly appropriate cell-fate decision depending on the stress levels. We demonstrate that the stress threshold required for Beclin1 activation (*i.e.* Bcl2 inactivation) should be lower compared to Casps activation; therefore autophagy gets activated at lower stress levels whereas Casps activation is restricted to only higher levels of stress. It can also be seen that Casps activation is delayed in comparison to Beclin1 activation. Although a simple network involving only Bcl2-dependent

regulation is sufficient to establish a threshold and bring about sequential activation of stress responses, our analysis suggests that these properties can be robustly achieved in the presence of feedback regulations between stress responses. The amplifying loops involved in Casps activation (Beclin1 \uparrow Casps \uparrow Beclin1 and Casps \rightarrow cleaved Beclin1 \rightarrow Casps) not only make the system bistable but also help to switch off the autophagy at high stress levels (Fig. 3). Such a crosstalk ensures that both autophagy and apoptosis cannot coexist together at the same time resulting in two discrete events. Further, a discontinuous bistable response prevents 'chattering' between autophagy and apoptosis when the stress level is fluctuating around the Casps activation threshold. This is likely to happen with a system only having Bcl2-dependent regulation. However, a combination of Bcl2-dependent regulation and feedback regulation of both Beclin1 (negative) and Casps (positive) provides a flexibility to adapt depending on the intensity and duration of stress levels. For example, at lower stress levels, autophagy is turned on whereas apoptosis is suppressed by the remaining Bcl2 activity (Fig. 3c). At intermediate stress levels, autophagy is turned on as an initial response, which provides a time window for survival, however apoptosis gets turned on with firing of the positive feedback loops and inactivates autophagy (Fig. 3d). On the other hand, at a very high stress level apoptosis turns on instantaneously and inactivates autophagy. The time interval between autophagy and apoptosis activation decreases with an increase in the stress level, which controls the decay of Bcl2 activity and turning on of the feedback loops between Beclin1 and Casps.

We have also shown that presence of feedback loops between Beclin1 and Casps is insufficient to stop the cells to go back from apoptosis to survival mode with the elimination of stress. The presence of an additional positive feedback loop such as Bcl2 + Casps + Bcl2 loop³⁵ is required to ensure irreversible cellular decision (Fig. 5). In principle it can be noted that Casps activation and inactivation are controlled by feedback loops. Its activation is controlled by feedback loops Casps → cleaved Beclin1 → Casps and Bcl2 + Casps + Bcl2, while inactivation is controlled by Beclin1 + Casps + Beclin1. Such a feedback regulation of opposing reactions is shown to produce a robust all or none bistable responses.⁴⁸ The two parameter bifurcation diagrams demonstrated that only the threshold for the activation of Casps increases or decreases with parametric variations (Fig. S4, ESI†). This does not alter the qualitative behaviour of the system but shifted the response curve to lower or higher stress levels. However, we also observed in few cases the existence of other situations such as inhibition of apoptosis by autophagy at high stress levels (either increasing k_{icp}' or decreasing k_{ibc}' as shown in Fig. S4, ESI†) or apoptosis is a sole response (increasing k_{acp}).

Our study also shows that the key requirements for the observed qualitative features rely on the strength of Bcl2-dependent inhibition of Beclin1 and Casps and on the strength of their mutual inhibition. Bcl2 indirectly controls the activation of Casps by strongly inhibiting its activator BAX in comparison to direct inhibition of Beclin1 ($k_{dsbc}/k_{asbc} \gg k_{dsbx}/k_{asbx}$). Further, the inactivation rate of Beclin1 by Casps is higher in comparison to the inactivation rate of Casps by Beclin1 ($k_{ibc}' \gg k_{icp}'$). The mutual inhibition of Beclin1 and Casps is illustrated on the phase plane computed with the model (Fig. 7). Beclin1 nullcline shows that its steady state concentration decreases with an increase in Casps concentration due to the Casps-dependent cleavage of Beclin1. On the other hand, Casps nullcline is Z-shaped with respect to Beclin1 due to the positive feedback loop acting on Casps and a high concentration of Beclin1 inhibits Casps. The intersection of two nullclines results in two stable (autophagy and apoptosis)

and one unstable steady state. At a low level of stress the system occupies the stable state of autophagy. However with an increase in the stress value Casps nullcline shifts to a higher concentration of Beclin1 and only one state is present corresponding to apoptosis. Therefore the system fires to that sole remaining stable state.

Interestingly, the apoptosis network also consists of other explicit and implicit positive feedback loops, which might work redundantly to enforce an irreversible bistable response.^{43,49,50} Similarly, the positive feedback loop through Casps mediated cleavage of the autophagic protein not only includes Beclin1 but also other proteins such as Atg5 and Atg4.^{33,34} In addition to multiple feedback loops, inhibitors are also known to influence both autophagy and apoptosis responses. Bcl2 and Bcl_{XL} form an inhibitory complex with Beclin1 and BAX to keep a check on both stress responses and its inhibition is relieved in multiple ways depending upon the cellular setting and stress.^{10,19,51} Besides JNK mediated regulation of the complex, phosphorylation of Beclin1 by DAPK and binding of pro-apoptotic proteins such as Bad is also shown to promote the dissociation of the Beclin1–Bcl2–Bcl_{XL} complex.^{52,53} Further, it is shown that Naf1 binding to Bcl2 stabilizes the Bcl2–Beclin1 interaction and inhibits autophagy.⁵⁴ However, Bcl2 is not the only inhibitor to control autophagy and apoptosis. cFLIP/vFLIP is also known to inhibit both stress responses.²¹ Recently another pro-apoptotic protein Bim1 has also been shown to inhibit Beclin1 by sequestering it to microtubules. The dissociation of the Beclin1–Bim1 complex leads to autophagy activation but also suggests that released Bim1 can translocate to mitochondria to drive apoptosis.⁵⁵ Similarly, Casps activation is regulated by inhibitors of apoptosis such as X-linked IAP (XIAP).⁵⁶ On the other hand, several Beclin1 binding proteins have been identified and their interactions positively influence autophagosome formation.⁵ These additional layers of regulation might eventually contribute towards ordering of cellular responses depending upon stress intensity and duration.

Our analysis using the minimal model predicts the dynamics of autophagy and apoptosis inducers when the crosstalk interactions

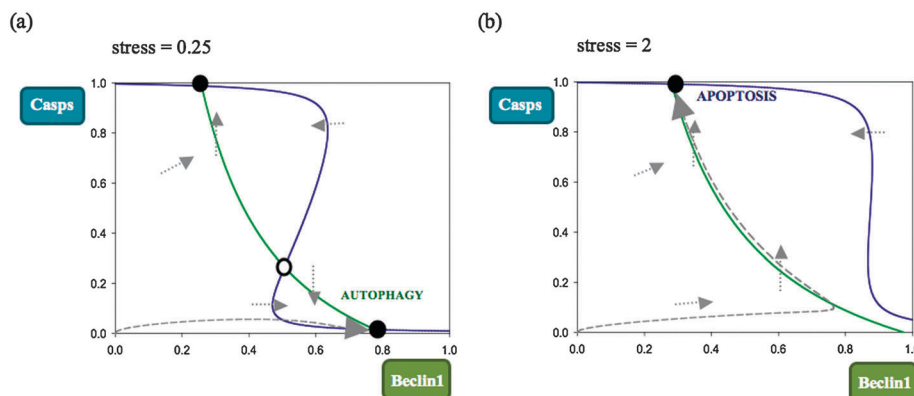


Fig. 7 Phase plane portrait of crosstalk between autophagy and apoptosis. Casps and Beclin1 nullclines were computed as one parameter bifurcation diagrams and combined to create a phase plane portrait. The phase planes is shown for (a) stress = 0.25 and (b) stress = 2. Intersections of nullclines represent the stable (filled circle) and unstable (open circle) steady states.

are perturbed. In certain situations we show that autophagy can overcome apoptosis or apoptosis can terminate autophagy. Such information can be used to test (and to identify) experimentally the situations that alter the balance between autophagy and apoptosis. Our analysis suggests that cells might depend on the antagonistic relationship between autophagy and apoptosis to make timely decisions with the inactivation of an inhibitor (crosstalk element in Fig. 1) of both processes by different stress stimuli (Table S2, ESI†). The decision involving transition from autophagy to apoptosis can be addressed by the experiments of inhibiting autophagy (e.g. by 3-methyladenine) or using non-cleavable Beclin1 in the presence of stress stimuli, which are predicted to bring about opposite effects on the dynamics of apoptosis activation (Fig. 4). Similarly, the non-cleavable Beclin1 is predicted to bring about a pronounced effect on apoptosis activation than blocking the cleaved Beclin1 (Beclin1-C fragment) translocation to mitochondria.

The minimal model proposed in this work serves as a starting point to build a comprehensive integrative model with the availability of quantitative data. In the literature, different mathematical models^{49,50,57} have been proposed for apoptosis but these models do not examine the influence of other signalling cascades such as autophagy under stress. Most of the anti-cancer drugs are known to induce autophagy in cancer cells while autophagy inhibitors sensitize cells to chemotherapy.³⁰ Therefore, a system level understanding requires inclusion of a crosstalk between pathways together with feedback regulation within individual pathways.

Methods

An ordinary differential equation (ODE) based model is used to describe the Bcl2–Beclin1–Caspases minimal network. The model is used to explore how the qualitative behaviour of the dynamical system changes with respect to stress levels and the role of key motifs in the network towards enforcing these changes. The temporal profiles and signal response curves were computed numerically using XPP-AUT. This program is freely available from G. Bard Ermentrout, Department of Mathematics, University of Pittsburgh (<http://www.math.pitt.edu/~bard/xpp/xpp.html>). The list of ODEs, parameter values and a detailed method for computation are provided as part of ESI.† We also provide the XPP code that can be used to compute the figures presented in this work.

Abbreviations

Bcl2 B-cell lymphoma-2
Beclin1 Bcl2-interacting protein-1
Caspases caspases

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