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Caspase-3 and RasGAP: a stress-sensing survival/demise switch

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Abstract

The final decision on cell fate, survival versus cell death, relies on complex and tightly regulated checkpoint mechanisms. The caspase-3 protease is a predominant player in the induction of apoptosis. However, this protease is more than a blind killer. It can gauge the degree of cellular stress and damage by differential processing of p120 RasGAP to generate the appropriate cellular response (survival or cell demise). Partial cleavage of RasGAP by caspase-3 initiates the anti-apoptotic Akt pathway. In contrast, full cleavage of RasGAP abrogates this survival response, hence favoring cell death. Therefore, rather than relying on separate sensors, cells can modulate a given set of proteins to generate, depending on the intensity of the input signals, opposite outcomes (survival vs. death).

Introduction

Proper functioning of cells requires that they maintain their biochemical parameters within rather narrow limits. This process, called homeostasis, is vital for cells and they spend a considerable energy to maintain it. For example, the appropriate gradient of sodium and potassium ion concentrations between the inside and the outside of brain cells via the action of the sodium/potassium ATPase pump consumes close to half of the ATP used at the basal state [1]. If the extracellular conditions are changing too much or if the cellular biochemistry is exceedingly altered, such as following viral infection [2,3] or oncogene expression [4], than homeostasis maintenance is compromised. This perturbation is sensed by cells as cellular stress. Situations and conditions that can generate cellular stress are extremely diverse. They include environmental pollutants, metabolic stress induced by toxic lipids in obese individuals, UV-mediated stress generated by prolonged exposure of the epidermis to sun exposure, endoplasmic reticulum stress caused by sustained chronic increase in insulin production by pancreatic beta cells in type 2 diabetes patients, chemical or ionizing stress that cancer patients experience when treated with chemo- or radiotherapy, or physicochemical stress provoked by heat or cold exposure.

When encountering a stress condition, cells have two options, either to cope with it, which requires an adaptive response, or to give up and die, which involves the initiation of an active and programmed cell death process. Cells are in fact well equipped to sense the extent of stress and this allows them to decide whether they should try to restore homeostasis or whether they should commit suicide because stress is evaluated as too extensive and potentially deleterious. Various switch mechanisms can be used by cells to evaluate stress and insults to either

promote cell survival or apoptosis. We will focus here on a newly described physiologic stress-sensing cellular system based on the differential cleavage of a specific caspase-3 substrate.

Caspase-3, from reaper to friendly protease

At the end of the nineties, a consensus emerged that apoptosis (Box1) was carried on by the executioner members of the caspase family (Box1) (caspase-3, -6, and -7) [5-8]. Caspase-3 has been considered as the central executioner protease due to its high catalytic turnover and greediness to cleave substrates [9]. Gene targeting in mice has revealed a certain level of redundancy in the function of executioner caspases in apoptosis induction [10], and more importantly that these executioner caspases fulfill other non-apoptotic functions as well [11].

Historically however, non-apoptotic functions of pro-apoptotic caspases were first discovered for caspase-8, an initiator caspase. It was shown in 1998 that mice lacking caspase-8 were embryonic lethal [12]. This lethality was later shown to result from degeneration of the vasculature of the yolk sac [13]. This suggested that caspase-8 is required for the proper development and/or the maintenance of vascular structures in the early embryo, which is most certainly distinct from its ability to mediate apoptosis via the extrinsic cell death pathway. Also in 1998, it was shown that cell expressing CrmA, a caspase-8 inhibitor, were paradoxically sensitized to necrotic cell death [14], suggesting that caspase-8 could also promote cell survival [15]. Non-apoptotic functions were then progressively reported for other caspases and a series of reviews have been describing these in the past decade [11,16-21]. Caspase-3 in particular appears

to regulate the differentiation of skeletal muscle cells, osteoblasts, lens epithelial cells, neural stem cells, and several hematopoietic lineages (T and B lymphocytes, erythrocytes, macrophages, platelets). Caspase-3 has also been reported to participate in stem cell maintenance, inhibition of B-cell proliferation, dendritic cell maturation, and proliferation of brain cells and keratinocytes [22-25].

The levels of activated caspase-3 that stimulate the non-apoptotic biological responses should obviously be lower than during apoptosis otherwise this cell death process would not be avoided. Numerous substrates of caspase-3 have been determined [9,26-28]. However their role in apoptosis has only been explored in a few cases. Additionally, the impact of caspase-mediated cleavage for the vast majority of substrates has not been explored by studying the phenotype of cells or mice in which the wild-type substrate is replaced with a non-cleavable knock-in form.

Different executioner caspases can target the same substrates while a given caspase can cleave some substrates better than others [9,29]. In particular, caspase-3 appears to cleave most substrates more efficiently than other caspases [29]. It is conceivable that the high K_m value of caspase-3 for some of its substrates is related to its non-apoptotic functions while apoptosis may imply the less effective substrates. In other words, low activation levels of caspase-3, leading to restricted cleavage of high affinity substrates, would allow survival while higher levels of caspase-3 activation would result in the cleavage of a much wider spectrum of substrates, including low affinity ones, ultimately leading to the induction of apoptosis.

In addition to the possible differential affinity of caspase-3 toward the anti- and pro apoptotic substrates, there are other mechanisms that can keep caspase-3 activation levels in check during stressful situations. One possibility is that cells have a built-in system that prevents amplification of caspase-3 activation. The best-known family of proteins that can negatively control executioner caspases is the inhibitor of apoptosis (IAPs) protein family. IAPs are characterized by one to three baculovirus IAP repeat (BIR) zinc-binding domain, which, at least for some IAP family members, is required for their ability to bind caspases [30]. However, contrary to initially speculated, X-linked IAP (XIAP) is the only IAP that can function as a direct inhibitor of caspase-3, -7 and -9 [31,32]. Cellular IAP1 and 2 (cIAP1/2) instead promote cell survival by functioning as E3 ubiquitin ligases that promote the degradation of caspase-3 and -7 [33] and positively regulate activation of the canonical NF-kB pathway [34-36]. In addition, cIAP1/2 function as regulators of survival signaling in cancer cells by preventing RIPK1 from becoming a pro-death stress sensing molecule [37]. Hence, IAP family members allow cells to cope with stress to a certain limit if they are expressed at sufficiently high levels either by activating NF-kB, preventing RIPK1 from becoming a pro-death molecule or by keeping caspase-3 activity in check.

Another possibility that can explain why activation of caspase-3 does not lead to apoptosis in all cases is that caspase-3 itself, when activated at low levels, turns on an anti-apoptotic response that acts as a negative feedback loop preventing further caspase-3 activation (i.e. an amplification of caspase-3 activity). A recent study [38] provides genetic evidence for this second possibility. In the C57BL/6 background, mice lacking caspase-3 are viable and fertile [39]. The vast majority of development programs occur normally in these mice. They do however

display abnormal inner ear development [40]. It is not known whether this defect results from a defect in apoptosis or from alterations of other functions controlled by caspase-3. Apoptosis in these mice can proceed normally or be inhibited, depending on which tissues and organs are investigated. For example, female germ cell apoptosis is not affected by the lack of caspase-3 while granulosa cell apoptosis is not taking place during follicular atresia when this caspase is missing [41]. Caspase-3 knock-out mice are unable or strongly impaired in activating the anti-apoptotic Akt kinase (box 2) in response to a variety of stresses such as UV-B exposure of the skin, doxorubicin-mediated cardiac damage, and experimentally-induced colitis, an inflammation of the large intestine. Akt activation is also compromised in wild-type mice injected with the Q-VD-OPh caspase inhibitor suggesting that the activity of caspase-3, and not a potential adaptor function of the protein, is required for its capacity to activate Akt. As detailed below, the defect in Akt activation in mice lacking caspase-3 is accompanied by increased cell death responses. Caspase-3 therefore appears to stimulate survival responses in some situations.

As caspase-3 is a main executioner caspase, its absence generally compromises or abrogates apoptosis induction by various stress inducing conditions. For example, pancreatic beta cells lacking caspase-3 are fully resistant against streptozotocin-induced death [42]. Even though absence of caspase-3 generally reduces the apoptotic response, this does not mean necessarily that the death response is lowered. For example, UV-B-induced apoptosis is decreased in the epidermis of caspase-3 KO mice but the overall death response is not, as in the absence of apoptosis induction cell death proceeds in caspase-independent cell death with necrotic morphology [38].

153 In some cases, caspase-7 can compensate, at least partially by enhanced
154 expression levels in C57Bl6 strains for the lack of caspase-3 and allow apoptosis
155 to proceed [10]. Doxorubicin is an efficient anti-cancer drug that is currently
156 used against various tumors. However, this drug also induces serious cardiac
157 side effects. Cardiomyocytes are indeed rather sensitive to doxorubicin and
158 readily undergo apoptosis when exposed to the drug. Unexpectedly, caspase-3
159 knock-out mice experience even more cardiomyocyte apoptosis than wild-type
160 mice when treated with doxorubicin, leading to increased mortality [38]. This
161 suggests that the apoptotic role of caspase-3 is redundant while its prosurvival
162 role in stressed tissues and organs is not.
163
164 **Cellular sensor of caspase-3 activity**
165 There is only a handful proteins including p120 RasGAP[43], $p27^{kip1}$ [44], Lyn
166 [45], Synphilin-1 [46], nucleophosmin [47], Rb [48], and the Drosophila IMD
167 protein [49] that may activate survival pathways once cleaved by caspases. The
168 first of these, RasGAP, is of particular interest in the present context because the
169 N-terminal moiety generated by cleavage of RasGAP at position 455 by caspase-3
170 activates Akt and protects cells *in vitro* [50,51]. In cells, RasGAP does not seem to
171 be cleaved by caspases other than caspase-3 [51]. RasGAP bears another
172 caspase-3 cleavage site at position 157 that is made accessible only following the
173 first cleavage event or that is much less efficiently recognized by caspases [43].
174 Consequently, the second site is not used when caspase-3 is mildly activated [52]
175 (Figure 1, upper left panel). However, after an apoptotic insult, concentrations of
176 activated caspase-3 further increases, reaching a threshold allowing the N-
177 terminal fragment of RasGAP to be cleaved at position 157, abrogating Akt

activation. The second RasGAP cleavage thus favors death [52] (Figure 1, upper right panel). RasGAP is the only caspase-3 substrate that shows this differential cleavage-mediated control of two opposite outcomes (survival vs apoptosis). Interestingly, RasGAP has no obvious anti-apoptotic activities as a full-length protein [53]. It only acquires cell protective functions when cleaved at position 455. The physiological role of caspase-3-mediated cleavage of RasGAP has recently been assessed using a knock-in mouse strain homozygous for a mutation in RasGAP at position 455 that prevents its cleavage by caspase-3. The RasGAP knock-in mice phenocopied the caspase-3 knock-out stress-induced Akt activation defect in response to various patho-physiological stresses (Figure 1, lower panels). The knock-in mice also experienced increased tissue damage and organ dysfunction in response to these insults in comparison to wild-type mice [38]. These results provide genetic evidence for the importance of caspase-3-mediated p120 RasGAP cleavage as a defense mechanism to protect organisms against damage induced by diverse pathological conditions.

It can therefore be proposed that caspase-3 itself activates the anti-apoptotic Akt kinase following certain types of stresses (e.g. starvation, DNA damage)[51] and this seemingly operates via the efficient cleavage of only one of its substrates, the ubiquitous p120 RasGAP protein. The model drawn for these studies is that RasGAP bears two cleavage sites with differential sensitivities for caspase-3 proteolytic activity. RasGAP acts as a "sensor like" protein that reflects the levels of caspase-3 activation, which correlates with the extent of cellular stress. This differential cleavage aids determining the fate of stressed cells: activation of a protective Akt-dependent pathway following the first cleavage of RasGAP or

inactivation of this protective pathway following the second cleavage of RasGAP (Figure 1, upper panels).

Perspectives: one set of signaling proteins, different outcomes

There are other comparable decision-making systems assessing the magnitude of cellular stress, where within a given signaling pathway, differential regulatory events dictate distinct cell signaling events. These "sensor-like" responses include the unfolded protein response (UPR) in the endoplasmic reticulum and the activation of p53 transcription factor pathway by DNA damage. What determines the cellular outcome once a given switch is triggered relies on the duration and the extent of the stress that allows or not the accumulation of pro-apoptotic genes, either as a consequence of differential stability of the anti- and pro-apoptotic proteins or as a result of post-translational modifications of the switch components (Figure 2). For instance, the goal of the UPR initially is to try restoring the folding capacity of the ER while diminishing protein load in order to reestablish ER homeostasis [54-56]. Only a strong and sustained ER stress allows for the accumulation of pro-apoptotic labile proteins such as the transcription factor CHOP [56,57]. Similarly, distinct post-translational modifications (e.g. acetylation on lysine 150 or phosphorylation of serine 46) generated by various degrees of DNA damage, determines whether p53 preferentially stimulates a repair pathway versus a cell death response [58-60]. From these examples one could propose a general paradigm were cellular stress is sensed by molecules and pathways that stimulate either cell survival or apoptosis based on the extent of their stimulation (as in the case of the UPR) or as a result of stress-mediated differential post-translational modifications (e.g.

cleavage of RasGAP, acetylation/phosphorylation of p53). Hence, cells may use the same set of sensor proteins to fine-tune an appropriate cellular response either resulting in cell survival or cellular demise, rather than relying on separate sensors for either response.

Figure legends

Figure 1. RasGAP cleavage, a sensor of caspase-3-activity controlling the survival and the death of cells.

RasGAP bears two cleavage sites with different sensitivities towards caspase-3 activity. Site 1 at position 455 is used at low levels of caspase-3 activity, while site 2, at position 157, is only recognized at high levels of caspase-3 activity. **Upper left panel**. In situations of mild stress, caspase-3 is activated to low levels and this leads to the partial cleavage of RasGAP into fragment C and fragment N. The latter activates an Akt-mediated anti-apoptotic response that inhibits further amplification of caspase-3 activity. **Upper right panel**. When cells are facing higher stress, caspase-3 is more strongly activated resulting in further cleavage of fragment N and abrogation of Akt activation. This favors cell death. **Lower panels**. When cleavage of RasGAP cannot occur as a result of a mutation at its first caspase-3 cleavage site, the prosurvival kinase Akt is not stimulated. In this condition, the feedback loop that prevents an initially mild caspase-3 activity is not activated and this results in an amplification of caspase-3 activity. Consequently, mild stresses generate elevated caspase-3 activation comparable to when cells experience stronger stresses. This eventually leads to cell death.

Figure 2. Examples of mechanisms allowing single stress sensors to activate survival or death responses

A given protein or set of proteins can determine the fate of a cell (survival vs death). This can result as a consequence of a differential cleavage of a protease substrate (e.g. cleavage of RasGAP by caspase-3) (**upper panel**), the

accumulation above a certain threshold of a labile transcription factor (e.g CHOP translation following ER stress) (**middle panel**), or differential post-translational modifications (e.g. as occurring on p53 in response to varying degrees of DNA damage) (**lower panel**).

Box 1. Apoptosis and caspases

Apoptosis (apo=for, ptosis=falling) is a type of cell death that was formally described by Kerr, Wyllie and Currie in the early 1970s [61]. Apoptosis participates in the control of tissue size and shape during development. It is involved in the elimination of cells that may represent a threat to the organism such as pre-malignant cells and activated immune T cells. Apoptosis is characterized by a series of organized and finely regulated biochemical and cellular modifications that process the dying cells for efficient elimination by phagocytosis by neighboring cells or macrophages. This insures that no cytoplasmic compounds leak from the cells that would otherwise generate an inflammatory response [62]. The hallmarks of apoptosis include cell shrinkage, membrane blebbing, nuclear and cytoplasmic condensation, DNA fragmentation, cytoskeleton proteolysis and breakup of organelles. Cellular components are sorted into a number of vesicles known as apoptotic bodies. Only a few of these events are diagnostic for apoptosis (DNA cleavage, formation of apoptotic bodies). Intact apoptotic cells display “eat-me” flags on their surface such as phosphatidylserine that tag them as targets for phagocytosis. Phosphatidylserine is usually confined to the inner side of the plasma membrane but becomes exposed on the surface of apoptotic cells [63,64].

The cellular and biochemical features of apoptosis are triggered by members of the caspase family of proteases. Caspases are a family of cysteine aspartic acid proteases present in healthy cells as inactive precursor enzymes (zymogens). All caspases have a similar domain structure (pro-polypeptide, large and small subunits) [65]. Domains within the pro-polypeptide, such as caspase recruitment domain (CARD) and death effector domain (DED), by homophylic interactions,

can recruit caspases to activation platforms. Not all caspases are implicated in apoptosis but those that are can be divided into initiator (caspase-2, -8, and -9) or executor caspases (caspase-3, -6, and -7). The former auto-activate when brought together by receptors belonging to the death receptor family (e.g. Fas). The latter are activated by the former by proteolysis and execute the proteolysis events that are seen during the demolition phase of apoptosis [62,66]. Executioner caspases cleave hundreds of substrates during the process of apoptosis but only for a minority of these is the physiological function of their cleavage understood. These include ICAD, the inhibitor of CAD, the DNAse that cleaves the DNA between nucleosomes. Caspase-mediated cleavage of ICAD leads to CAD activation, generating ~180 base pair multimeric DNA fragments, the diagnostic laddering in apoptotic cells.

Box 2. The generally pro-survival Akt pathway

Akt is a serine/threonine kinase involved in the regulation of cell survival, proliferation, and metabolism and is activated by phosphorylation. Three isoforms exist (1, 2, and 3) that all contain an N-terminal pleckstrin homology (PH) domain, a central kinase domain containing a phosphorylation site within the activation-loop (threonine 308 in human Akt1), and a conserved regulatory serine phosphorylation site in a hydrophobic motif near the C terminus (position 473 in human Akt1). The interaction of the Akt PH domain with 3'-phosphoinositides causes Akt translocation to the plasma membrane, inducing conformational changes that allow Akt to expose its phosphorylation sites [67].

Phosphoinositide-dependent kinase-1 (PDK-1) is also recruited to the plasma membrane after PIP3 generation. PDK-1 is the kinase that phosphorylates Akt on

311 threonine 308, which stabilizes the activation loop in an active conformation.
312 Serine 473 is then phosphorylated by the PDK2 kinase activity which is
313 predominantly carried out by mammalian target of rapamycin (mTOR) complex
314 2 (mTORC2) or DNA-PK. Phosphorylation of threonine 308 is a prerequisite for
315 kinase activation. Phosphorylation of serine 473 appears to further increase Akt
316 kinase activity. Recent evidence suggests that this phosphorylation event also
317 controls the target specificity of the kinase [67].
318 Among the Akt substrates that have been identified in mammalian cells, many
319 are regulators of apoptosis or cell growth [67]. The Akt substrates are
320 phosphorylated within the same basic motif RXRXXS/T. The anti-apoptotic
321 response induced by Akt involves NF-κB- or CREB-mediated up-regulation of
322 anti-apoptotic proteins such as c-IAP1/2, Mcl-1 and Bcl-2, or the direct
323 phosphorylation and inhibition of pro-apoptotic proteins such as Bad and
324 caspase-9. It is worth noting however that the Akt phosphorylation site in
325 caspase-9 is not conserved in mammals [68]. Akt also inhibits members of the
326 Forkhead family of transcription factors (FOXO transcription factors). This
327 prevents the expression of pro-apoptotic genes such as Fas ligand. Additionally,
328 murine double minute 2 (MDM2), an E3 ubiquitin ligase targeting p53 for
329 degradation, is stabilized by Akt-mediated phosphorylation. This will therefore
330 diminish p53-induced pro-apoptotic signaling in cells. Therefore, Akt promotes
331 survival in most cells either through direct phosphorylation of targets, or
332 through the induction of anti-apoptotic genes [67]. There are some cell types
333 however in which Akt activation can lead to death. In pancreatic beta cells for
334 example, Akt activation leads to NF-κB-dependent apoptosis [69].
335

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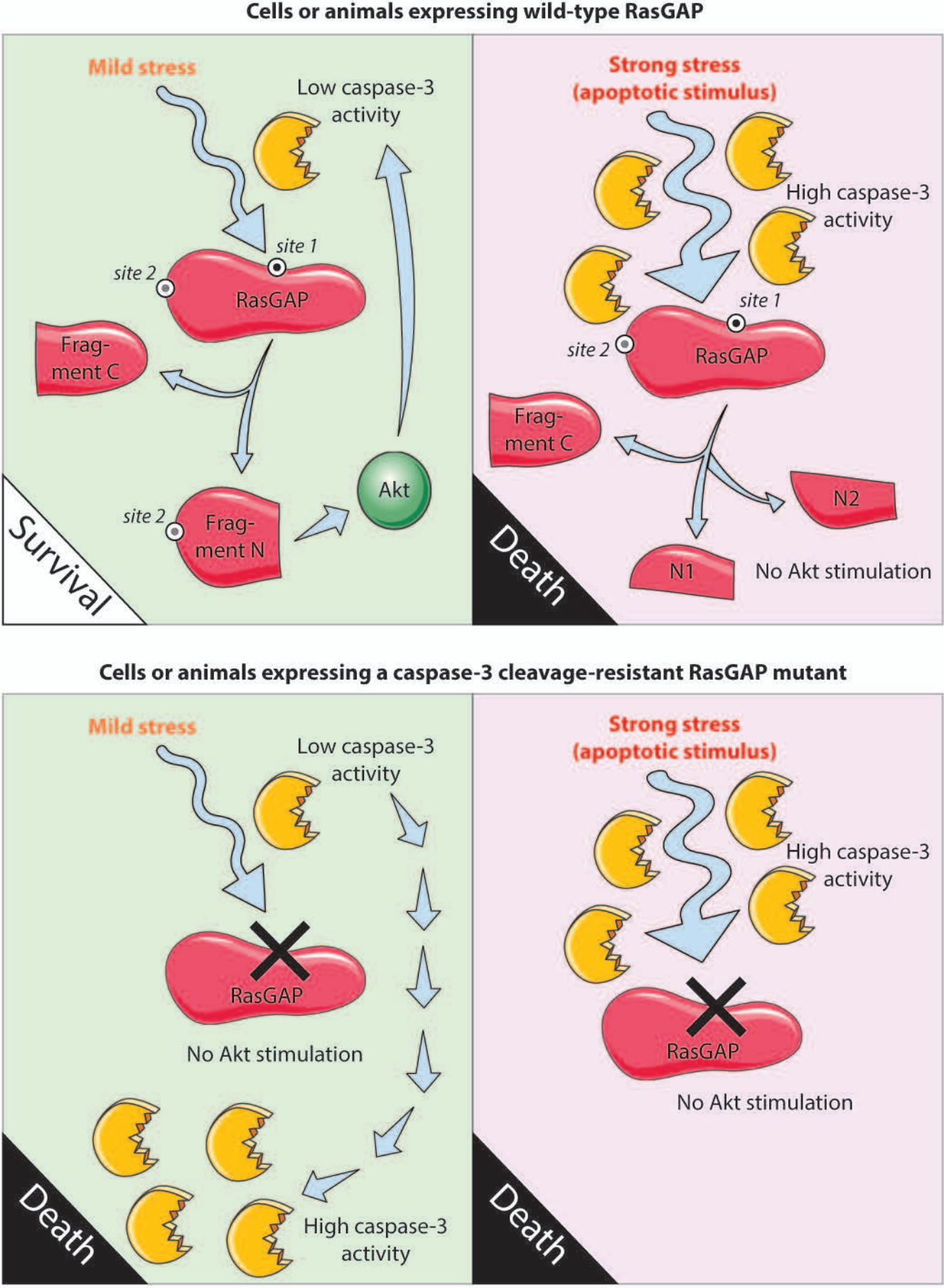
Cells or animals expressing wild-type RasGAP
Mild stress
Low caspase-3 activity
site 1
site 2
RasGAP
Frag-ment C
site 2
Frag-ment N
Akt
Survival
Strong stress (apoptotic stimulus)
High caspase-3 activity
site 1
site 2
RasGAP
Frag-ment C
N2
N1
No Akt stimulation
Death
Cells or animals expressing a caspase-3 cleavage-resistant RasGAP mutant
Mild stress
Low caspase-3 activity
RasGAP
No Akt stimulation
High caspase-3 activity
Death
Strong stress (apoptotic stimulus)
High caspase-3 activity
RasGAP
No Akt stimulation
Death

Figure 1

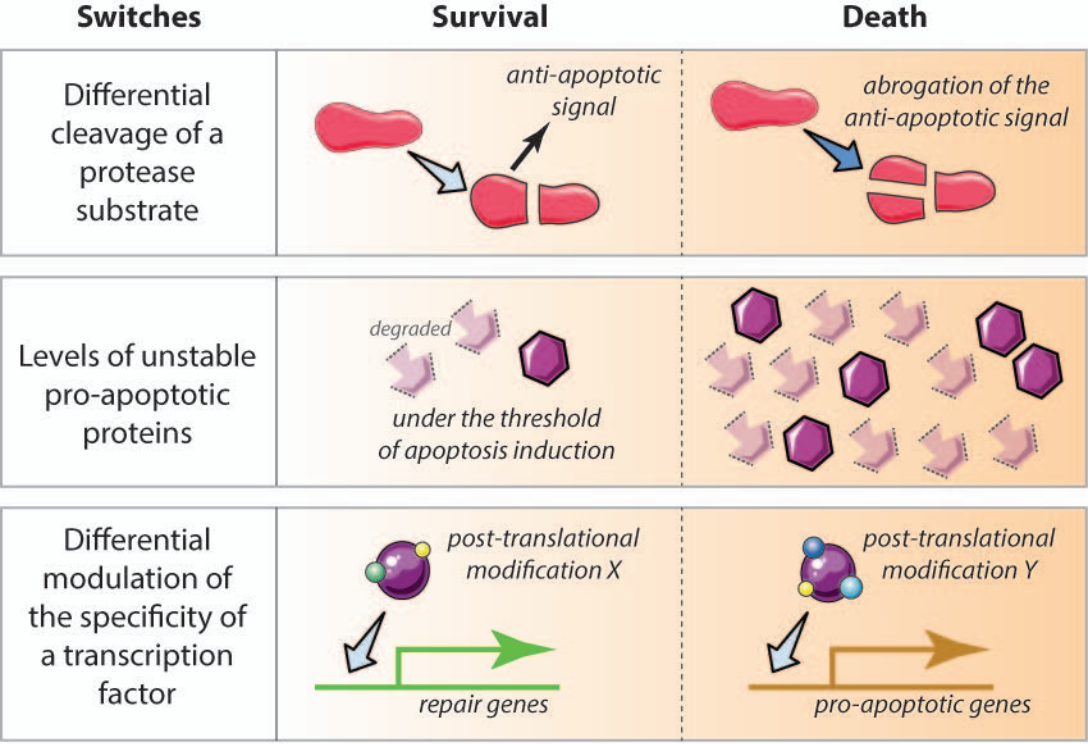
Switches
Survival
Death
Differential cleavage of a protease substrate
anti-apoptotic signal
abrogation of the anti-apoptotic signal
Levels of unstable pro-apoptotic proteins
degraded
under the threshold of apoptosis induction
Differential modulation of the specificity of a transcription factor
post-translational modification X
repair genes
post-translational modification Y
pro-apoptotic genes

Figure 2