Surviving apoptosis: life-death signaling in single cells

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Abstract

Tissue development and homeostasis are regulated by opposing pro-survival and pro-death signals. An interesting feature of the Tumor Necrosis Factor (TNF) family of ligands is that they simultaneously activate opposing signals within a single cell via the same ligand-receptor complex. The magnitude of pro-death events such as caspase activation and pro-survival events such as NF-κB activation vary not only from one cell type to the next but also among individual cells of the same type due to intrinsic and extrinsic noise. The molecules involved in these pro-survival/pro-death pathways, and the different phenotypes that result from their activities, have been recently reviewed. Here we focus on the impact of cell-to-cell variability in the strength of these opposing signals on shaping cell fate decisions.

Keywords

Life-Death Decisions

During embryogenesis, development, and tissue turnover, some cells die by apoptosis while other cells avoid death and assume various cellular fates. What makes some cells die and others survive is not completely understood. In some cases, only specific cells receive the death signal, while in other cases, the signal is interpreted differently due to cell- or context-specific cues. Such cell-to-cell variability, which has various origins, has recently been shown to play an important role in cell fate decisions [1–3].

Similarly, stress-response signaling often has a dual role, activating survival pathways to buffer and repair damage, and death pathways to kill cells when the damage is beyond repair. Examples include pathways regulating heat shock proteins, p53, autophagy, and inflammation, and here, too, individual cells often respond with variable outputs [4–6]. Thus, protective stress pathways and death signaling are tightly linked, and many cellular proteins have evolved to exert both functions, often in parallel [7,8].

Proteins that regulate cell death are also essential for normal cellular processes, including metabolism, proliferation, and differentiation [9,10]. In some cases, these proteins "deviate" from their physiological function only when external cues point to cell death. In other cases, proteins exhibit both functions simultaneously (for example through interactions within different protein complexes), or play a survival role only when death is inhibited. Some of these proteins (e.g. caspases) belong to multiple signaling pathways (Box 1), while others, such as the TNF family of death ligands, activate parallel but opposing pathways through recruitment of different sets of signaling molecules. Thus, many proteins are essential for both life and death of cells, and the particular outcome may depend on cell type, exposure to external stimuli, or other context-dependent choices.

Box 1

Non-death roles for death proteins

Many proteins exhibit both pro- and anti-apoptotic activities. For example, cytochrome c is important for both mitochondrial homeostasis and execution of cell death [125]. The first caspase to be discovered, Interleukin-1-beta-Converting-Enzyme (ICE/Caspase-1), is responsible for cytokine processing and represents a subgroup of inflammatory caspases with functions in immune signaling [126]. TNF was discovered for its role in tumor necrosis, but also acts as an inflammatory cytokine [127]. Subsequently, non-apoptotic roles have been uncovered for most proteins associated with apoptosis.

Both initiator and effector caspases exhibit non-apoptotic functions [9]. Caspase-8 promotes cell migration [128–130], T cell proliferation [131], wound healing [132], stem cell reprogramming [133] and macrophage differentiation [134]. Caspase-3 plays a role in shaping cell morphology [135] and in differentiation of red blood cells, lens epithelial cells, and skeletal muscle cells, processes that involve degradation of intracellular organelles or substrates ("incomplete apoptosis") [136–140]. Caspases are required for spermatid differentiation, oogenesis, and wing development in *Drosophila* [141–143], and also play a role in neuronal sculpting, synaptic plasticity, and neural development [117,144–146].

Bcl-2 family members also have functions unrelated to apoptosis, such as regulation of mitochondrial homeostasis and glucose metabolism [147,148]. Mcl-1, an anti-apoptotic Bcl-2 family member, was first discovered as a differentiation marker for myeloid cells, and is required for embryonic development and immune system function [149]. In addition, Bcl-2 members allow rapid switching between states that favor life versus death. For example, alternatively-spliced isoforms and cleavage products of these proteins can promote either survival or death, and the fast degradation rate of Mcl-1 in particular allows cells to rapidly undergo cell death under conditions of stress [108].

Death-Inducing Signaling Complex (DISC) proteins such as c-FLIP, FADD and RIP also promote death or survival, and combinatorial regulation of these proteins may determine cell fate [150,151]. c-FLIP can be pro- or anti-apoptotic, depending on levels and the particular isoforms expressed [27,152,153], and cleaved FLIP (p43) regulates activation of survival pathways via NF-κB [154]. FLIP and FADD are both required for embryonic development and T cell proliferation [155], and FADD plays a role in cell cycle progression, differentiation, and innate immunity [151]. Moreover, FADD, Caspase-8, and

FLIP appear to promote cell survival during development through inhibition of necroptosis, inducing apoptosis only in response to certain stimuli [35]. RIP1 can activate survival, apoptosis, or necroptosis, depending on its post-translational modifications [150]. Finally, kinases associated with the DISC can have pro- or anti-apoptotic activity: p38, JNK, PKC and ERK either promote or inhibit apoptosis induced by death ligands, depending on context [59].

In this review, we examine how a "death" signal can lead to a non-death output, with a particular focus on the TNF family of death ligands. We also describe some non-apoptotic functions of "death" proteins and discuss potential advantages of this convergence. Finally, we review how the interplay between death and survival signaling has been studied at the level of single cells, how variability in these signals contributes to variability in cell fate, and the implications of these studies for understanding the roles of life-death signaling in development and disease.

Integrating Life-Death Signals

Cell-to-cell variability has been shown to play an important role in cell fate decisions [11]. This variability can result from differences in cellular state (genetic, epigenetic, phenotypic, or due to stochastic fluctuations) as well as from cell cycle differences or effects of the cellular microenvironment [1–3]. Cues external to the cell, such as death or survival stimuli, can be viewed as variable inputs acting on already variable cellular states. Together, these different sources of variability lead to downstream heterogeneity in phenotype.

The following simplified scenarios illustrate several ways in which competing pro-death/pro-survival signals can lead to variable cell fates. On the one hand, the relative strength of distinct and opposing stimuli may tip the balance in favor of survival or death, as in the case of a growth factor protecting cells from a death-inducing agent (Figure 1A). On the other hand, a single stimulus may induce both death and survival signals within a single cell; the internal state of the cell then determines which pathway is dominant at a given time (Figure 1B). For example, a block in apoptosis may unmask pro-survival signals triggered by a death ligand, or vice-versa; this may be true at the cell population level, or may vary among individual cells (Figure 1Ci). Alternatively, pro-death and pro-survival signaling may actively compete to determine whether a cell lives or dies, leading to cell-to-cell variability in response (Figure 1Cii). Finally, in cells exposed to a death-inducing agent, counter-balancing adaptive pathways may become activated to varying degrees in individual cells in a population, protecting against a future death stimulus (Figure 1D). Thus, a cell's choice between life and death can be a function of both external context (e.g. signals from other ligands or cells) and its own internal state.

Death Ligands and Death Receptors

Evasion of apoptosis is a hallmark of cancer cells and contributes to both cancer progression and resistance to chemotherapeutic drugs. Traditional chemotherapy targets the "intrinsic" pathway of cell death, activating apoptosis from within cells through induction of DNA damage or other cellular stresses. In contrast, the "extrinsic" apoptosis pathway is mediated

by death ligands that bind to death receptors expressed at the surface of target cells [12]. Apoptosis triggered by death ligands is thought to represent a form of innate immunity.

The three most-studied members of the death ligand family are TNF, Fas-Ligand (FasL), and TRAIL (TNF-Related Apoptosis-Inducing Ligand), in part because of their potential as cancer therapeutics. However, death ligands also activate non-apoptotic pathways, including inflammation and metastasis [13]. The extent of activation of each of these pathways depends in part on the ligand: TNF is a strong inducer of inflammation, whereas FasL primarily induces apoptosis but exhibits hepatotoxicity, precluding its use as a cancer drug [12]. The promise of TRAIL and agonist TRAIL receptor antibodies as cancer therapeutics reflects their specificity in targeting cancer cells for death. Nevertheless, many cancers exhibit resistance to these agents, and TRAIL can also activate "non-death" pathways under certain conditions [14,15]. Thus, even the most potent apoptosis-inducing death ligand may successfully kill some cancer cell types but lead to unwanted effects in others.

Cell Death by Death Ligands

Death ligands bind to their cognate receptors and induce cell death via recruitment of a Death-Inducing Signaling Complex (DISC) and consequent activation of a caspase cascade that leads to permeabilization of mitochondria, further activation of caspases, and degradation of the proteome and genome (Box 2).

Box 2

Cell death by death ligands

In the extrinsic apoptosis pathway, cell death is initiated by binding of ligand to receptors (DR4/5 for TRAIL and FasR for FasL) and subsequent recruitment of proteins to intracellular receptor tails. Ligand-receptor engagement leads to receptor aggregation, followed by a conformational change in receptor tails that promotes recruitment of the adaptor protein FADD and initiator caspases, and formation of the DISC [156,157]. At the DISC, initiator caspases-8 and -10 (denoted as C8 in the figure) become activated via dimerization and autocatalytic cleavage, a process that can be either blocked or promoted by the presence of FLIP [26,158,159]. In some cell types (Type I cells), initiator caspases directly cleave and activate effector caspases-3/7 (denoted as C3), leading to cell death. In contrast, Type II cells require mitochondrial outer membrane permeabilization (MOMP) for activation of effector caspases and cell death [160]. In these cells, effector caspases are held in check by inhibitor of apoptosis protein XIAP [161,162], and this inhibition is relieved in a pathway involving MOMP [163,164]. Initiator caspases cleave the pro-apoptotic BH3-only protein Bid into truncated Bid (tBid), which translocates to the mitochondria and activates pro-apoptotic Bcl-2 family members Bax and Bak. When the amount of activated Bax/Bak (denoted as Bax*) exceeds a threshold such that it overcomes the inhibitory effect of anti-apoptotic Bcl-2 family proteins, Bax/Bak forms pores in the mitochondrial membrane, leading to MOMP and release of Smac and cytochrome c into the cytosol [165]. Cytochrome c promotes assembly of the caspase-9 (C9)-containing apoptosome, while Smac displaces XIAP from its inhibitory effect on caspase-3, leading to rapid cleavage of caspase-3 substrates and cell death [166] (see figure

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I, part A, shown here as a simplified representation of TRAIL-induced apoptosis). Each of these parts of the pathway (receptors, DISC, mitochondria, and IAP/C3) exhibits multiple levels of regulation that affect the strength of apoptotic signaling (see figure I, part B, and main text).
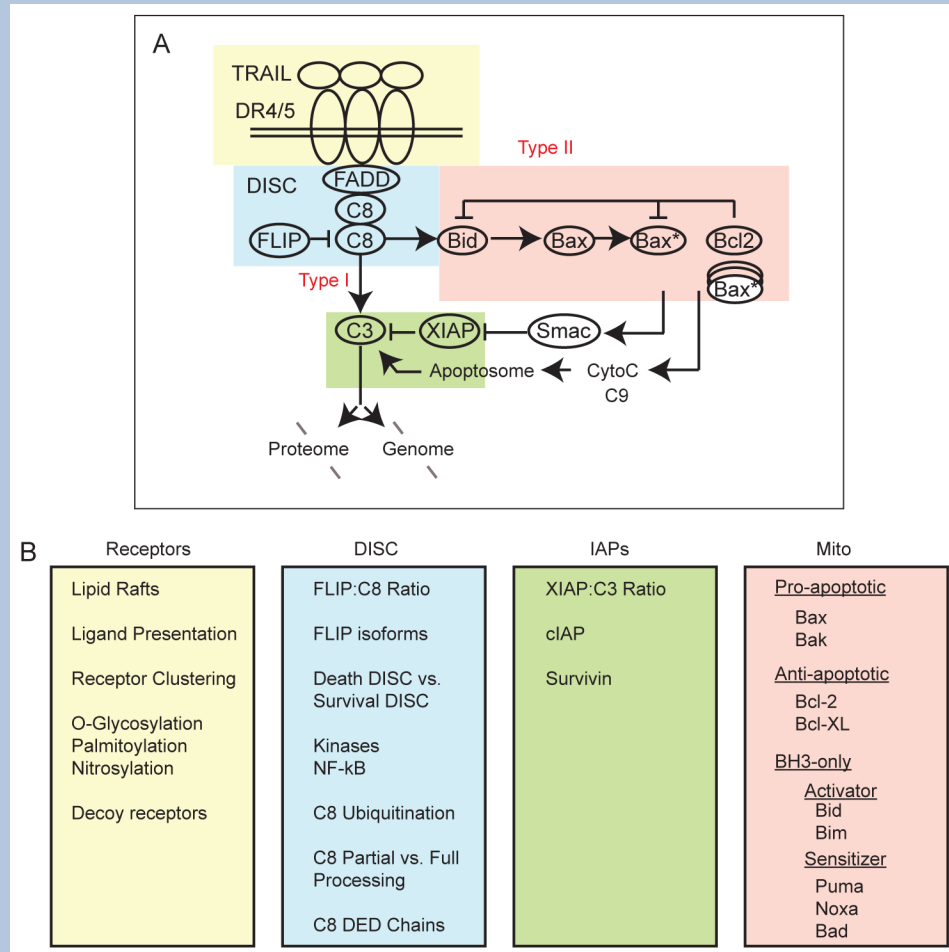

Figure I.
Cell death by death ligands.

Receptor-mediated apoptosis can be regulated and fine-tuned at many levels. Interactions among receptors (including anti-apoptotic decoy receptors for TRAIL), receptor post-translational modifications and localization to different membrane compartments determine the strength of pro-apoptotic signaling, for example by promoting or preventing the formation of higher-order receptor aggregates [16–22]. The DISC is a complex structure containing multiple components that together determine whether apoptosis becomes activated in response to different stimuli in different cell types [23–25]. c-FLIP, a DISC protein that has homology to caspase-8 but lacks proteolytic activity, is an anti-apoptotic regulator of initiator caspases, although it can exert pro-apoptotic activity in some contexts [26,27]. The Bcl-2 family, which comprises more than 20 members, also undergoes complex

regulation [28,29]. Finally, extrinsic cell death is regulated by inhibitor of apoptosis proteins (IAPs), in particular XIAP. When XIAP levels are sufficiently high, cell death may be blocked; conversely, a low XIAP:caspase-3 ratio promotes cell death, diverting cells from Type II, mitochondrial outer membrane permeabilization (MOMP)-dependent death, to a Type I pathway in which caspase-3 is directly activated by caspase-8, leading to cell death even in the absence of MOMP (see Box 2) [30–32]. It is likely that the relative levels of multiple proteins in the apoptosis pathway (and the interactions among them), rather than variation in a single protein, determines whether apoptosis occurs in response to death ligand. Thus, cells treated with death ligand may die or survive depending on their location in a multidimensional phase space of protein expression levels [31,33,34].

Alternative Pathways Induced by Death Ligands

The extrinsic cell death pathway has been described as a "node of coordination in immune signaling networks" because of its multifaceted role in the immune system [15]. Thus, it is not surprising that death receptors can induce both apoptosis and cell survival, processes that are equally important in the regulation of inflammation and immunity (Figure 2A). TNF is a strong inducer of the pro-inflammatory transcription factor NF-κB and leads to upregulation of both inflammatory and pro-survival genes that inhibit caspase activation; TNF is thus generally pro-apoptotic only under conditions in which the NF-κB pathway is blocked [35] (Figure 2B). In contrast, TRAIL and FasL are strongly pro-apoptotic, and are thought to activate NF-κB relatively weakly, such that alternative signaling emerges mainly under conditions in which apoptosis is attenuated [23,36–39] (Figure 2C). Moreover, NF-κB may mediate inflammatory signaling in response to TRAIL and FasL more strongly than survival signaling per se [40–42].

In spite of being strongly pro-apoptotic, TRAIL and FasL have been shown to exhibit non-apoptotic activities in many settings [13,43]. FasL promotes branching of cultured neurons, as well as liver regeneration following hepatic injury [44,45]. TRAIL is involved in non-apoptotic forms of cellular differentiation, with reported roles in hematopoiesis and intestinal and skeletal muscle differentiation [46–48]. TRAIL promotes survival, migration, and proliferation of human vascular endothelial cells and smooth muscle cells via a pathway involving ERK and AKT, suggesting roles in vascular physiology and promotion of angiogenesis [49–51]. Both FasL and TRAIL also exhibit tumorigenic activities in cancers that have acquired resistance to apoptosis. FasL induces migration and invasion of glioblastoma and other apoptosis-resistant tumor cells, through activation of PI3K, ERK, NF-κB, and caspases, and also leads to secretion of inflammatory cytokines [39,52]. Tumor growth in a mouse xenograft model has been shown to depend on constitutive FasL secretion by tumor cells, in a pathway involving JNK [53]. TRAIL induces cell proliferation, migration, invasion, and secretion of pro-inflammatory cytokines in apoptosis-resistant cell lines, and promotes metastasis in a mouse xenograft model of pancreatic adenocarcinoma [42,54–58]. Thus, both TRAIL and FasL appear to play physiological roles other than activation of apoptosis, and these pathways may be coopted by cancer cells that have become TRAIL-resistant [59].

What determines whether a particular cell type or tumor undergoes apoptosis or activation of non-apoptotic pathways in response to treatment with a death ligand? The relative apoptotic

sensitivity of a particular cell type following treatment is tuned at many levels (see Box 2). In addition, many of these same components determine the extent to which alternative/pro-survival pathways become activated. For example, stimulation with TNF, FasL, or TRAIL initiates recruitment of different protein complexes to receptor tails, leading to different cell fates [20,36]. These fates include not only apoptosis and survival/inflammation phenotypes, but also alternative forms of cell death such as necroptosis [60,61]. TNF-receptor binding initially promotes recruitment of a "survival/inflammation" complex (TNF complex I) that contains the proteins TRADD and RIP1 and leads to activation of IKK, NF-κB, and transcription of pro-survival and pro-inflammatory genes; this is followed by recruitment of pro-apoptotic proteins FADD and caspase-8 in a secondary complex (TNF complex II) that is inhibited by anti-apoptotic NF-κB targets, thereby promoting cell survival. When NF-κB is inhibited, however, activity is switched toward complex II signaling and apoptosis; the apoptosis and survival pathways activated by TNF thus negatively regulate each other [23,62]. When apoptosis is also blocked, an alternative complex (IIb) leads to cell death by necroptosis, a process that is dependent on RIP1 and RIP3 [60].

In contrast, Fas agonists and TRAIL primarily lead to formation of pro-apoptotic caspase-8-containing DISCs, but subtle changes in DISC structure and composition can shift cells to a survival pathway that also involves activation of inflammatory signals, or to necroptosis [61,63]. Which proteins are recruited to the DISC appears to depend on ligand presentation and strength as well as on the extent of receptor clustering and internalization, receptor post-translational modifications, and the availability of intracellular DISC proteins [16–20,64,65]. For example, receptor sub-membrane localization and subsequent recruitment of distinct DISC complexes can determine TRAIL-mediated death vs. survival, with cell death favored when receptors localize to lipid rafts, and c-FLIP- and RIP1-dependent survival signaling when receptors are sequestered in non-raft fractions [66]. Low FasL doses or receptor expression levels are sufficient to promote FasL-mediated survival but not cell death, presumably through a sub-threshold-dependent recruitment of pro-survival DISC components [67,68]. Ligand presentation is also important since *in vivo*, membrane-bound FasL induces apoptosis whereas soluble FasL leads to NF-κB signaling and inflammation [69]. In some cell types, E-cadherin-mediated cytoskeletal coupling is necessary for optimal TRAIL receptor clustering and pro-apoptotic DISC formation, and cells that have lost this coupling are resistant to cell death [21]. Notably, expression levels of TRAIL receptors are generally not as good at predicting apoptotic sensitivity as is their ability to form high molecular weight complexes upon stimulation [19].

Within the DISC, multiple factors can affect the activation of caspase-8 and whether cells live or die. For example, small changes in the levels of different c-FLIP isoforms can shift cells toward apoptosis or survival [24,70]; a variety of kinases (e.g. p38, JNK) that either promote or inhibit apoptosis may also be recruited and activated [52,71,72]. In the case of TRAIL, increased levels of anti-apoptotic DISC components may shift cells toward activation of a secondary pro-inflammatory DISC complex, promoting activation of NF-κB, p38, and JNK [37]. Ubiquitination of caspase-8 is required both for its activation and for subsequent dampening of its activity through degradation [73,74], and the extent of death effector domain (DED)-mediated caspase-8 chain formation at the DISC can influence the

degree of apoptosis induction and potentially affect the activation of alternative pathways [75,76]. Finally, it has been proposed that caspase-8 dimerization at the DISC, in the absence of proteolytic cleavage, can lead to survival signaling by processing a limited repertoire of substrates in the absence of cell death; intra- vs. inter-dimeric caspase-8 cleavage may also regulate this process [77,78]. Thus, the decision between life and death is mediated by fine-tuning the extent of both cell-death pathway activation and survival pathway induction. Both the type of ligand-receptor interaction and the balance of pro- and anti-apoptotic proteins that come together as a result can determine cell fate by favoring one pathway over another (Figure 2D).

Cell Fate Decisions in Individual Cells

The fine-tuning of apoptotic and non-apoptotic pathway activation in response to treatment with death ligands impacts not only the overall behavior of cell populations, but also cell fate decisions of individual cells within a population. For example, in response to treatment with TRAIL or other death-inducing agents, many cell types exhibit a fractional response in which only a subset of cells dies even at saturating ligand doses [40,79]. Such cell-to-cell variability has been observed both in tumors *in vivo* and in cultured cells and is a contributor to the challenges of using these agents as anti-cancer drugs [20,80,81]. The origins of this variability have been extensively reviewed and contribute not only to fractional killing, but also to variable activation of non-apoptotic pathways leading to alternative cell fate outcomes (Box 3).

Box 3

Cell-to-cell variability and cell fate

Populations of cells are inherently heterogeneous, and cell fate differences among cells are observed in diverse cellular processes including cell proliferation, differentiation, and cell death [167–169]. Sources of variation can be genetic or non-genetic; non-genetic cell-to-cell variability includes both epigenetic differences and stochastic fluctuations in the levels or activities of factors within cells [1,2,170–172]. Stochastic fluctuations result from either extrinsic noise, loosely defined as fluctuations in gene expression external to a particular pathway, or intrinsic noise, defined as fluctuations in levels of proteins in the pathway itself [173]. Stochastic differences among cells typically have a "remixing time," defined as the time it takes for a cell to switch from a given state back to the population average, which usually occurs on the order of hours or days [107]. This is in contrast to epigenetic variation, with typically longer switching times between states (on the order of weeks or months), although these switches can also be caused by stochastic factors [81,174]. In between these two types of variation are transiently induced or adaptive responses affecting changes in gene and protein expression or protein phosphorylation; these responses typically last longer than stochastic fluctuations but are shorter in duration than most epigenetic cell states (although this distinction is often not clearly defined, and both transiently-induced and epigenetic differences may be described as examples of phenotypic variation).

Each of these sources of variability also leads to temporal variation in the activity or localization of signaling proteins that ultimately affects cell fate decisions. This has been demonstrated not only for p53 and caspase activation dynamics during cell death [30,175,176], but also for proteins involved in transcription and cell survival. NF-κB exhibits cell-to-cell variability through an inhibitor of kappa B protein (IκB)-mediated negative feedback loop that drives oscillations in NF-κB nuclear translocation [177]. The set of genes activated in an individual cell is determined by dose of the stimulus, fold-changes in the levels of nuclear NF-κB, frequency of pulsatile stimulations, and temporal patterns of the oscillations [178–181]. Variability in expression and in phosphorylation and localization dynamics of ERK, PI3K and AKT also affect cell fate, influencing cell cycle and differentiation decisions as well as tuning the response to inducers of cell growth and death [182–185]. Thus, a multitude of factors can affect, at any given time, a cell's decision to live or die in response to a death stimulus, and if it survives, whether it takes on a new phenotype.

In the case of TRAIL, transient variation in the levels of proteins or other factors within cells has been shown to determine both the timing of cell death as well as whether cells live or die [79,82,83]. This variation is non-genetic in origin, with pre-existing differences among cells (prior to treatment) determining, in large part, the timing of cell death as well as the fate of individual cells [79,84]. Sister cells that have recently divided are highly correlated in their death times, but de-correlate within hours after cell division [79]. Importantly, experiments involving the use of cycloheximide as well as modeling studies have demonstrated that such cell-to-cell variability and cell fate bifurcations can occur even in the absence of induced pro-survival signaling, resulting simply from differences in protein expression and degradation among cells, leading to differences in the strength of apoptotic signaling [30,85].

Other studies show, however, that cell-to-cell variability affects not only the extent of death pathway activation, but also the magnitude of survival pathway induction, and that both can contribute to fractional killing within a cell population. For example, cells that survive an initial TRAIL treatment exhibit a period of transient resistance one day later in which 90% of cells are completely resistant to a subsequent TRAIL treatment[40]. Cells enter this transiently resistant state even when cell death is inhibited during initial TRAIL exposure using caspase inhibitors, demonstrating that resistance is at least partially induced by TRAIL itself, rather than being a consequence of selection for a relatively resistant cell subpopulation. Resistance can be sustained by periodic TRAIL treatments, even in the absence of cell killing, but cells reset to a sensitive state following outgrowth in the absence of TRAIL [40]. Such induced survival signaling may also play a role in responses to FasL, which has been shown to variably activate life-death pathways depending on the relative expression of different DISC proteins within individual cells [24,70]. In these studies and others [86,87], death and survival signals (e.g. caspases and NF-κB/kinases) are activated simultaneously in individual cells treated with Fas agonists or TRAIL, and presumably the relative strength of pro- and anti-apoptotic factors determines whether a cell lives or dies.

Several other studies have also suggested that the balance between survival and death pathway signaling following treatment of cells with death-inducing agents may contribute to

differences in cell fate among cells in a population. For example, one group measured the dynamic regulation of multiple fluorescently-labeled endogenous proteins in cancer cells treated with a topoisomerase inhibitor and showed that individual cells responded heterogeneously, leading to different life-death outcomes [88]. In these cells, upregulation of an RNA helicase correlated with cell survival. Another group showed that PI3K/mTOR inhibition in matrix-attached mammary epithelial cells led to death of inner matrix-deprived cells but to activation of an adaptive survival program in outer cells [89]. In yet another study, compensatory survival signaling protected a subset of an oncogene-addicted cell population from drug-induced death [90]. Thus, cell fate in response to death-inducing agents likely depends, at least in some cases, on variable activation of both death and survival pathways. Moreover, variably-induced survival or adaptive pathways may re-enforce cell fate decisions in cells that initially survive a treatment due to prior variation in factors affecting sensitivity to death (Figure 3A–C).

A combination of pre-existing cell-to-cell variability (leading to differences in apoptotic sensitivity) and induced survival or adaptive pathway signaling in cells that survive is also likely to play a role in reversible (dynamic) drug resistance of cancer cell subpopulations. For example, cells have been shown to switch between drug-sensitive and drug-resistant states, or to develop resistance following a drug treatment and then become sensitive again when given a "drug holiday" [91–95]. Dynamic TRAIL-resistance has similarly been observed in several studies, and has been collectively attributed to changes at the level of receptors, DISC, or Bcl-2 family proteins [40,56,96–98]. In some of these cases, cells in a population may pre-exist in a dynamic equilibrium of sensitive and resistant states and switch back and forth over time, whereas in other cases, reversible resistance in a fraction of cells results from a drug-induced adaptive state [90–95,99–101]. Notably, this distinction is not always clear unless specific experiments are designed to probe the origins and durability of resistance. In the absence of live-cell reporters, it is technically challenging to observe both the initial state of a cell and its ultimate fate, and to determine whether markers that characterize survivors pre-date drug treatment or arise in response to it [88,102].

When changes in cell state are induced by a drug treatment, they may not only affect survival but also lead to development of new phenotypes. For example, cells surviving fractional killing by TRAIL take on an inflammatory phenotype in which cells migrate and differentiate as though responding to an injury [40]. Such plasticity in cell subpopulations can be rationalized with reference to a protein expression "landscape," in which individual cells lie in different parts of the landscape and therefore exhibit differential responses to stimuli. Phenotype switching of an individual cell would thus depend on its "initial conditions" (position in the landscape) and how susceptible it is to a stimulus-induced adaptive response. Memory of a previous stimulus could determine the response of cells to a subsequent stimulus by shifting a cell to a different area of protein expression space [103]. Once pushed into a pro-survival region of the landscape (e.g. after surviving an initial TRAIL treatment), continued stimulation might keep cells in this region via activation of positive feedback loops or other regulatory networks, thereby preventing cells from relaxing back to the apoptosis-sensitive position they occupied prior to stimulation (Figure 3D). It will be interesting to ascertain the identities of these feedback loops using live-cell markers, ideally

tagged at the endogenous locus, or by comparing time courses of transcript or protein profiles in populations of cells following treatment [104–107].

Advantages of Simultaneous Induction of Opposing Life-Death Signals

It has been suggested that in order for cell death to have evolved, proteins necessary for initiating death must originally have had non-apoptotic roles, making cells dependent on them for survival; hence, the close relationship between death and survival mechanisms. Alternatively, death and survival pathways may be coupled such that a chronic pro-death signal is inhibited until a survival signal is turned off, as occurs during the rapid degradation of anti-apoptotic Bcl-2 family proteins in stressed or infected cells [108,109]. Indeed, evidence for both of these evolutionary strategies appears to exist [7,110].

The activation of opposing pathways by a single stimulus may be advantageous on several levels. First, induction of inflammation by death ligands may act as a "second line of defense" to eliminate damaged or infected cells (by recruiting engulfing phagocytes) when the primary outcome (death) has failed to transpire [35]. Second, cells faced with a sub-lethal stress or injury may induce protective mechanisms against a future exposure to that stress, a concept known as preconditioning or disease tolerance [111,112]. At the level of individual cells, a memory of the first stress (activated only in cells that respond with induction of a stress response rather than death) leads to preferential protection of some cells over others when a future stressor arises. Moreover, cells that survive an injury may activate not only signals that protect themselves against death by a future insult but also inflammation and differentiation programs that promote repair of damage, clearance of apoptotic cells, and healing of wounds caused by death of neighboring cells [37,113–115]. Thus, depending on need, a secondary signal may function either to heal damage caused by cell death or to carry cell death to completion by an alternative mechanism.

In the case of tissue sculpting, it may be highly efficient for a single stimulus to kill some cells and induce differentiation in others. This may occur, for example, during lumen formation, mammary gland involution, neuronal differentiation, and immune responses [8,9,116,117]. TNF family members are involved in many of these processes, and non-apoptotic physiological responses to death ligands may have been co-opted by cancer cells to protect themselves against death by these agents [35,59]. Thus, alternative fates of cancer cells following TRAIL or FasL treatment may be a carry-over from higher order differentiation systems *in vivo*, as well as a form of "bet-hedging" by cells to survive in a dynamic environment. Such "bet-hedging" through non-genetic heterogeneity has been demonstrated in bacteria[118] and has also been proposed to occur in cancer cells evading drug treatment [94,119]. It seems likely that cancer cells exposed to death ligands may use a combination of bet-hedging and environmental sensing as a strategy to evade the immune system, by generating a fraction of cells that not only resist death, but also co-opt death signals for promotion of their own survival (via activation of death ligand-induced survival pathways and protection against future death stimuli). At any given time, a subset of cells may be predisposed to survive treatment due to natural fluctuation in protein levels; these cells then adapt (following death ligand exposure) and induce transient phenotypic changes associated with survival, inflammation, and tumorigenesis. The presence of such cells

results in fractional killing by death ligands and therapeutic drugs and promotes residual disease.

Concluding remarks

Many proteins and pathways in cells have evolved to exhibit both death and non-death functions in different contexts. Non-death roles include signaling in inflammation, differentiation, proliferation, metabolism, survival, or any pathway integral to the life of a cell. These same proteins also regulate cell death, in the context of maintaining balance in cell populations.

Cell death-inducing agents have shown promise as cancer therapeutics because of their ability to eliminate damaged, "primed", or rapidly proliferating cancer cells. However, these agents also paradoxically activate a variety of non-death signals that are likely to reduce the effectiveness of treatment. Alternative signals can become activated in apoptosis-resistant cell populations, or in subpopulations of cells exhibiting cell-to-cell variability. Cells that survive treatment may undergo an adaptive program that ensures their survival when faced with subsequent treatments, leading to a "memory" of their resistant state. This memory can occur on various timescales, depending on the type of adaptation.

The idea that a cell "remembers" previous exposure to a treatment implies that with long-term exposure, cell states can be sustained for extended periods. The question then arises as to when "acquired resistance" is actually a manifestation of transiently induced resistance that is reversible upon drug removal, versus when it results from a stable genetic mutation or from a more slowly reversible epigenetic adaptation. This and other interesting questions will need to be addressed in future studies, to better understand the contribution of transiently-induced factors to the development of drug-resistant states. Predicting the emergence of these states through measurements of heterogeneity within cell populations should help improve strategies for identifying biomarkers of drug resistance, as well as suggest how drug holidays and drug combinations can reduce resistance-promoting mechanisms [94,100,120,121]. In addition, it will be important to understand how cell-to-cell variability impacts treatment schedules that aim to avoid the emergence of undesirable adaptive states [40,93,122].

Life-death decisions are influenced by multiple factors converging on a single cell, where each factor can itself support various outcomes. Understanding signaling systems as interacting components can thus be utilized to exploit therapeutic selectivity to the system rather than to single components. For example, targeting a protein complex or pathway rather than an individual protein may give better results if that protein takes on different functions under different conditions [123,124]. A deeper understanding of these protein interactions and pathways will aid us in designing better therapeutics that take into account this complex regulation.

Acknowledgments

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Highlights

- TNF family death ligands activate both death and non-death signaling pathways.

- Cells surviving death stimuli may enter adaptive, death-resistant states.

- Cell-to-cell variability in life-death signaling impacts cell fate decisions.

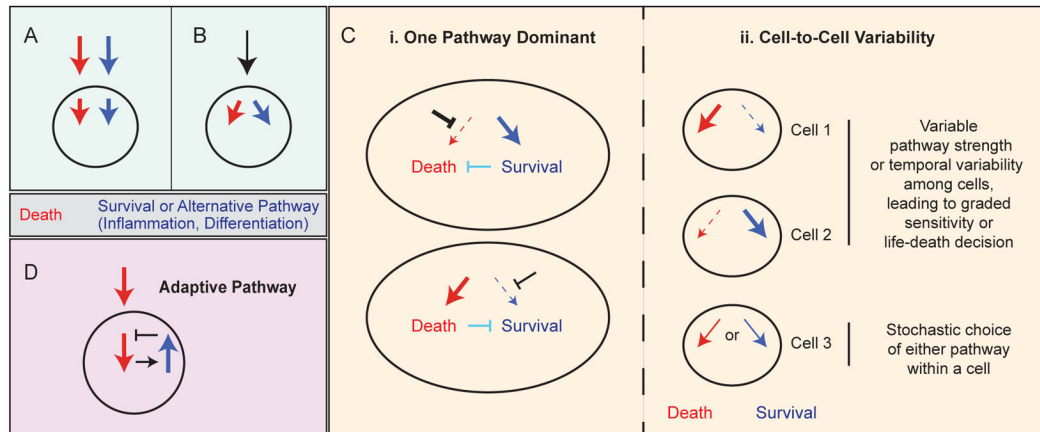- The evolutionary advantages of variability in life-death signaling are discussed.

Figure 1. Scenarios illustrating how parallel or competing death and survival signals may influence cell fate decisions in individual cells

A, Separate death and survival signals (e.g. an inhibitor drug and growth factor ligand) activate parallel pathways within a cell. B, A single stimulus, such as a death ligand, activates both pro-death and pro-survival pathways. C, In the case of a single stimulus activating two pathways, one pathway might be dominant within a cell population, for example if the other pathway is not functioning (as in very high expression of an anti-apoptotic protein; bold black inhibition arrow), or if the first pathway negatively regulates the second pathway (aqua inhibition arrows)—leading to activation of only one of the pathways within the cell population. In this case, the strength of a single pathway would presumably vary among individual cells, leading to variability in cell fate (i). Alternatively, both pathways may be activated within cells, and cell-to-cell variability affects the relative strength or duration of each pathway, further leading to differences in cell fate (ii). In cells 1 and 2, variable pathway strength, or temporal variability among cells, leads to graded sensitivity within the cell population and variable life-death fate outcomes. In cell 3, one pathway or another is activated stochastically within a cell, also leading to variable life-death fate outcomes among cells. D, A drug treatment that can induce cell death leads to activation of a compensatory adaptive pathway that mediates resistance to the death signal in cells that escape death initially. The difference between B–C and D is that in B–C the death signal directly activates an alternative pathway alongside the death pathway (as in the case of death ligands), whereas in D, the adaptive pathway is indirectly activated in response to the death signal via a separate pathway. It should be noted that these two outcomes are not necessarily mutually exclusive.
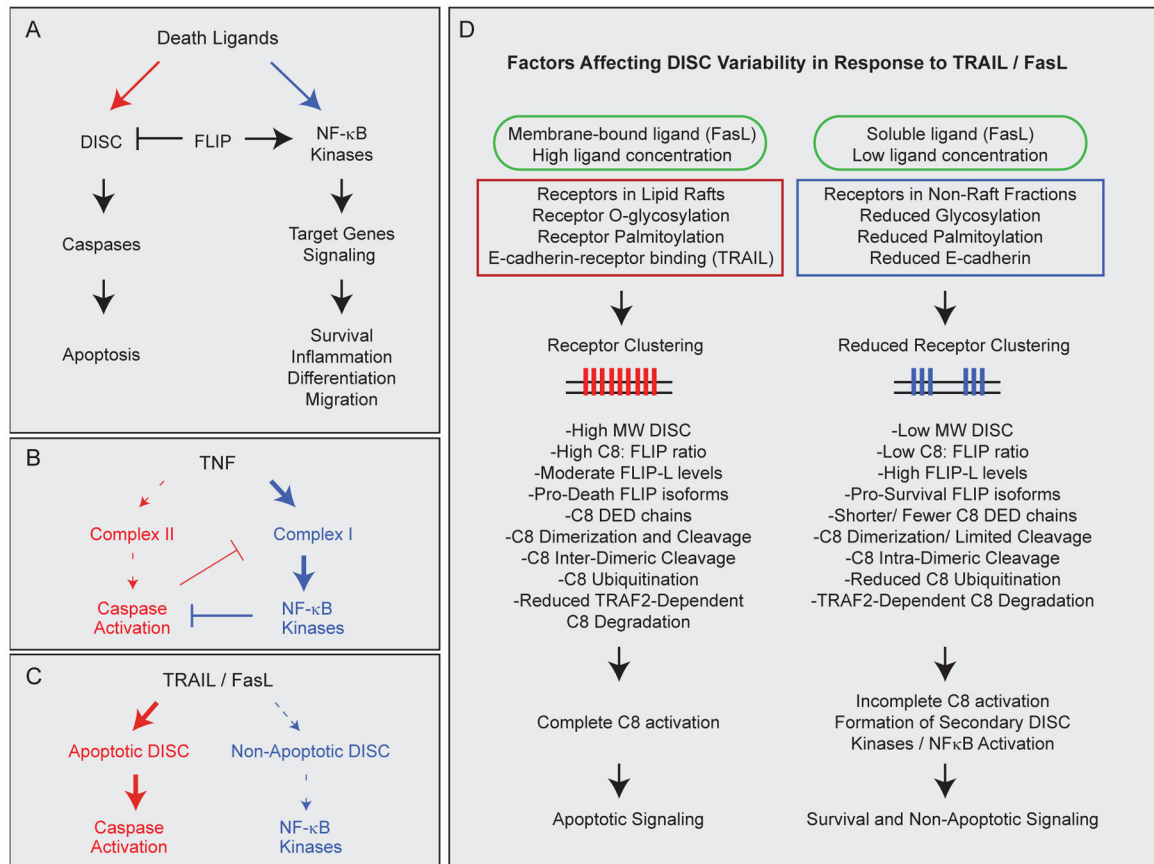
Figure 2. Activation of opposing pathways by death ligands

A, Death ligands activate opposing signals leading to apoptosis via a caspase pathway, or to alternative pathways via kinases and NF-κB. Alternative pathways include both survival and phenotypes such as inflammation, cell migration, and differentiation, and are mediated at least in part via transcription of target genes. B–C, Different ligands lead to sequential recruitment of distinct signaling complexes to receptor tails, with the primary complex promoting cell survival/inflammation in the case of TNF but apoptosis in the case of TRAIL/FasL. B, TNF leads to the formation of a primary signaling complex (complex I; bold blue arrow) that activates the NF-κB pathway, and induces cell death via the delayed formation of a secondary complex (complex II; dashed red arrow) that becomes active only when NF-κB signaling is inhibited. Apoptosis, in turn, negatively regulates the NF-κB pathway through cleavage of complex I proteins such as RIP1. When apoptosis is also inhibited, necroptosis may ensue through formation of an alternative secondary complex (IIb; not shown). C, TRAIL and Fas agonists primarily activate apoptotic DISCs (bold red arrow), but also activate NF-κB and kinase signaling through formation of secondary or alternative DISC structures (dashed blue arrow). In some cases NF-κB signaling in response to TRAIL/FasL is weak relative to apoptotic signaling, or becomes dominant only in cases when apoptotic signaling is dampened or blocked. Necroptosis may ensue when both apoptosis and survival signaling are inhibited in certain settings (not shown). D, Summary of factors affecting apoptotic vs. non-apoptotic DISC formation and signaling in response to TRAIL and FasL. Receptor clustering, which is controlled by ligand concentration,

membrane vs. soluble ligand presentation, receptor localization in lipid rafts, and receptor post-translational modifications, reaches a threshold of activation that promotes pro-apoptotic DISC assembly, caspase-8 (C8) activation, and cell death (left). In contrast, sub-threshold receptor clustering leads to reduced formation of pro-apoptotic DISC structures and/or formation of anti-apoptotic DISCs (right). Stimulation strength and extent of receptor clustering can influence subsequent DISC structure and components, which are also regulated by the availability of different DISC factors. Some or all of these factors may impact signaling choices in different contexts and may vary with regards to TRAIL vs. FasL signaling. In some cases, cells may fail to die due to insufficient apoptotic signaling even in the absence of additional survival pathway activation.
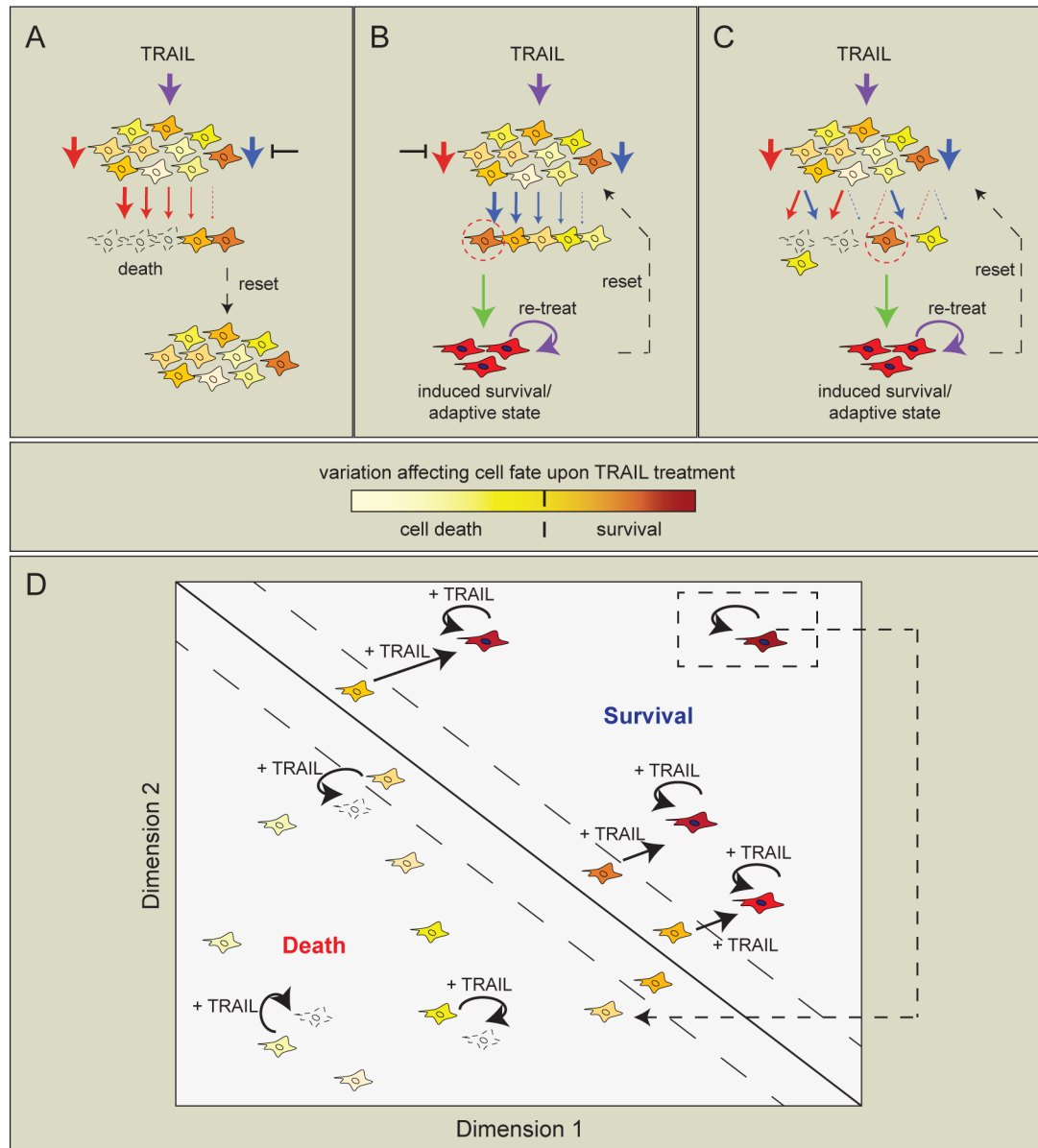
Figure 3. Pre-existing variability in cell state and subsequent variability in induced pathways mediate cell fate decisions in cells treated with death ligands

In this schematic, yellow-orange shadings depict non-genetic heterogeneity in protein levels or other factors in a naïve cell population. A, Following treatment, individual cells activate apoptosis signaling (red arrows) to varying degrees (depending on their initial cell state), in the absence of induced survival/alternative signaling (blue arrow, not activated in this cell population). The sensitive fraction of cells dies by apoptosis (unfilled cells) and the less sensitive fraction survives (dark yellow-orange cells). When the treatment is removed, protein levels re-distribute within several days, such that the new cell population is equivalent to the starting control population (dashed black arrow; reset). B, When apoptosis is blocked, survival/alternative signals (blue arrows) may become variably activated within the cell population, either maintaining cell survival, or leading to transient induction (green

arrow) of alternative cell phenotypes or adaptive states (red cells with filled nuclei, indicating activation of a transcriptional state). Re-treating the cell population with death ligand may sustain these induced states (curved purple arrow), whereas removing treatment allows the induced signals to decay as cells divide, returning cells to their pre-treatment population distribution (dashed black arrow). C, Treatment with a death ligand activates both death and survival/alternative pathways to varying degrees in individual cells (red and blue arrows). If the signals are of equal strength, other factors within cells may influence cell fate (left, unfilled vs. yellow cell); if the signals are of unequal strength, the stronger signal may prevail to determine life or death of the cell. Similar to the scenario described in B, cells that survive may take on alternative phenotypes or adaptive states that are transiently resistant to death by a future stimulus (green arrow and red cells with filled nuclei), either through further activation of the initial survival signal or through compensatory survival signaling from another pathway. Again, the survival/adaptive state can either be sustained by continued treatment during the resistance stage or lost when treatment is removed, and this may occur across different timescales (taken with permission and adapted from Flusberg et al., MBOC, 2013). The schematic illustrates cell responses to treatment with TRAIL but is also relevant to signaling by other death ligands as well as more generally to any death-inducing agent that activates parallel or compensatory survival signals. D, Individual cells inhabit different areas in a multidimensional phase space, here shown for illustrative purposes as a projection in two dimensions. A cell's location in this space is determined by multiple factors, including transcriptional states and protein expression levels or activity. The phase space can be divided into regions (separated by solid diagonal line) in which cells treated with TRAIL (filled arrows) either undergo apoptosis or survive. Some cells lying near the division line may rely on chance to determine whether their fate is death or survival (denoted by cells bounded by dashed diagonal lines). However, once "pushed" into the survival region following TRAIL treatment, surviving cells respond to repeated TRAIL treatments with further upregulation of survival pathways and remain in this region until TRAIL is removed and survival signals have decayed. The decay period (dashed arrow) may take hours, days, or weeks-months, depending on the type of survival signal initiated (e.g. kinase cascade: hours, transcriptional program: days, epigenetic change: weeks-months; see also Box 3). Thus, cell fate results from both random variation in initial cell state and subsequent response to a stimulus.