Transcription-dependent and -independent control of neuronal survival by the PI3K–Akt signaling pathway

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The PI3K–Akt signaling pathway plays a critical role in mediating survival signals in a wide range of neuronal cell types. The recent identification of a number of substrates for the serine/threonine kinase Akt suggests that it blocks cell death by both impinging on the cytoplasmic cell death machinery and by regulating the expression of genes involved in cell death and survival. In addition, recent experiments suggest that Akt may also use metabolic pathways to regulate cell survival.

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Abbreviations

APAF1	apoptosis protease activation factor-1
BDNF	brain-derived neurotrophic factor
CREB	cAMP-responsive element binding protein
FasL	Fas ligand
FOXO	Forkhead box transcription factor, class O
GSK-3	glycogen synthase kinase-3
IAP	inhibitor of apoptosis
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor-κB
NGF	nerve growth factor
PI3K	phosphatidylinositol-3-OH kinase
PIP	phosphoinositide phosphate
PKA	protein kinase A
PKC	protein kinase C
RSK	ribosomal S6 kinase
SGK	serum glucocorticoid inducible kinase

Introduction

During the development of the mammalian nervous system, half of all generated neurons undergo a pre-determined program of cell death. As the nervous system develops, neurons that are wired together appropriately and are in contact with target-derived molecules — either neurotransmitters or peptide trophic factors — suppress this intrinsic apoptotic program and survive [1].

The physiological interplay between neuronal death and survival that occurs during the development of the nervous system has been recapitulated in several *in vitro* culture models of primary neurons, including sympathetic, hippocampal, cortical, cerebellar granule and motor neurons. All of these neuronal types can be successfully cultured in the presence of defined neurotrophic stimuli, and die through an apoptotic process after withdrawal of trophic support. For example, brain-derived neurotrophic factor (BDNF) is a potent *in vitro* survival factor for cerebellar granule neurons. Mice that are genetically deficient for the BDNF or BDNF receptor genes display an excess of apoptotic cells in the cerebellum [2,3], indicating that the *in vitro* primary culture system may accurately recapitulate cellular events that occur during normal nervous system development *in vivo* [4].

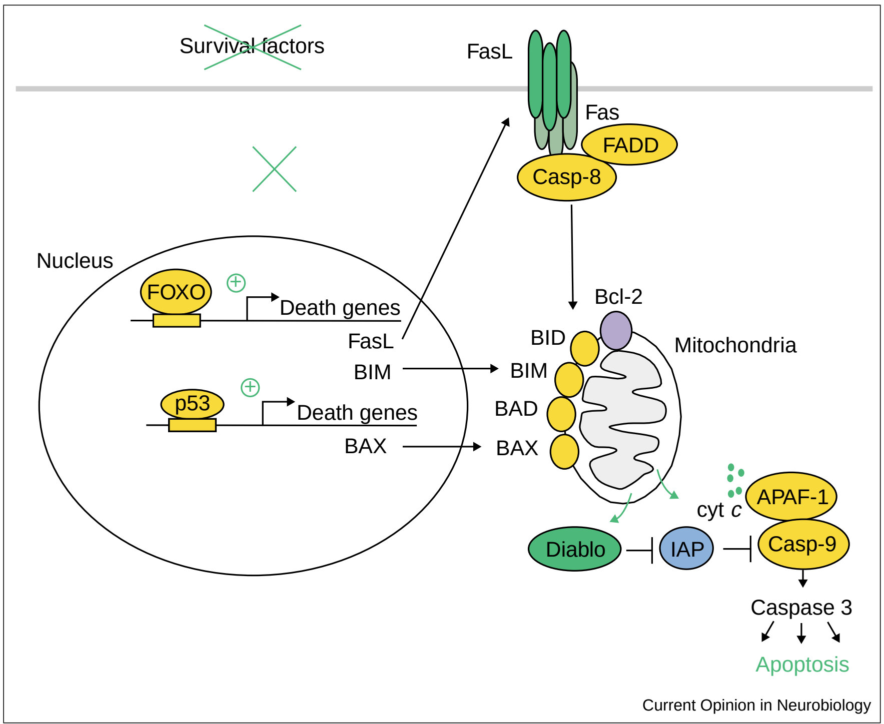
In recent years, these neuronal culture systems have been used to elucidate several molecular mechanisms by which survival factors prevent programmed cell death. In this review, we discuss recent discoveries of transcription-dependent and -independent mechanisms by which survival signaling pathways suppress neuronal apoptosis.

The process of cell death: possibility for regulation at many levels

Many of the components of the apoptotic machinery pre-exist in cells in a latent form. However, initiating apoptosis in neurons upon withdrawal of survival factors requires *de novo* gene expression, suggesting that some components or regulators of the apoptotic machinery need to be newly synthesized before apoptosis occurs [5]. Although the complete subset of genes that is induced by survival factor withdrawal is not yet known, several inducible genes have been identified that encode critical components of the apoptotic machinery. For instance the pro-apoptotic Bcl-2 family member BAX is upregulated during apoptosis of sympathetic neurons [6]. Other genes that are upregulated in response to apoptotic stimuli encode extracellular or transmembrane ligands, such as Fas ligand (FasL), which in turn regulate the cell death machinery in a paracrine or autocrine manner [7•,8•]. Alterations in gene expression are the first detectable changes in neurons deprived of survival factors, and thus the suppression of the transcription of specific death genes may be one mechanism by which survival factors block cell death.

The execution of apoptosis *per se* is controlled at least in part, by Bcl-2 family members that localize to the outer mitochondrial membrane and control mitochondrial permeability [9]. One prevailing model proposes that when the molecular ratio of pro-survival to pro-death Bcl-2 family members is biased towards pro-death Bcl-2 family members (either through changes in expression level, localization or activity), the outer mitochondrial membrane becomes permeable to proteins, including cytochrome *c*. Cytochrome *c*, when released from the mitochondria to the cytoplasm, participates in the formation of a complex, known as the 'apoptosome', that is composed of dATP, apoptosis protease activation factor-1 (APAF1), and the

Figure 1



In the absence of survival factors, transcription factors such as FOXO and p53 induce the expression of target death genes, including FasL or the pro-apoptotic Bcl-2 family members BIM and BAX. FasL, by binding to its cognate receptor Fas, triggers, through the adaptor molecule FADD (Fas-associated via death domain), the recruitment and activation of caspase 8, which in turn either directly activates caspase 3 or induces the translocation of the Bcl-2 family member BID to the mitochondria. When pro-apoptotic Bcl-2 family members such as BAX, BIM, BAD or BID are in excess over anti-apoptotic members, they promote the release of at least two proteins: cytochrome *c* and Diablo/Smac from the mitochondria. Cytochrome *c* binds to APAF1 which leads to the activation of caspase 9 and, subsequently, to the activation of caspase 3. Diablo binds to and inhibits the IAPs, thereby preventing them from inhibiting the caspases. Casp, caspase; cytc, cytochrome *c*.

cysteine protease caspase 9. Formation of the apoptosome results in the activation of caspase 9, which sets in motion the activation of a cascade of effector caspases, such as caspase 3, that kill the cells by irreversible proteolysis of critical cellular constituents [1,10] (Figure 1).

Recent experiments have demonstrated that apoptosis can still be inhibited even after cytochrome *c* has been released into the cytoplasm. Proteins of the inhibitor of apoptosis (IAP) family prevent apoptosis by specifically binding to and inhibiting the caspases [11]. Du *et al.* [12••] and Verhagen *et al.* [13••] have recently shown that the pro-survival function of the IAPs is inhibited by Diablo/Smac — a mitochondrial protein that is released into the cytoplasm under apoptotic conditions. The release of Diablo prevents IAPs from suppressing caspase activity, and thereby promotes cell death (Figure 1). In sympathetic neurons, the release of cytochrome *c* from the mitochondria is not sufficient to trigger apoptosis, and a second event is required for nerve growth factor (NGF) withdrawal to induce apoptosis, possibly the release of Diablo, or other closely related proteins, from the mitochondria [12••,14]. In addition to controlling Diablo release from the mitochondria, one can speculate that survival factors may also regulate the expression or activity of Diablo.

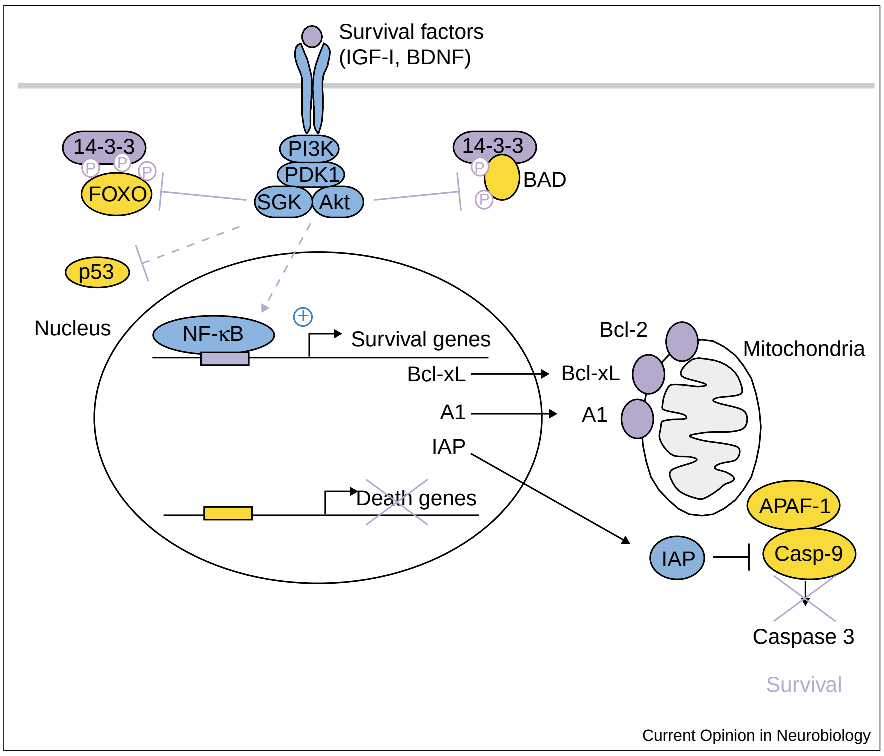
The PI3K–Akt pathway: a major pathway mediating neuronal survival

Trophic factors such as NGF, insulin-like growth factor I, or BDNF activate a variety of signaling cascades, including the phosphatidylinositol-3-OH kinase (PI3K)–Akt (Akt; protein kinase identified in the AKT virus [also known as protein kinase B]), the Ras–mitogen-activated protein kinase (MAPK), and the cAMP/protein kinase A (PKA) pathways [15]. Each of these pathways contributes to cell survival under certain conditions that depend on the neuronal cell type and the survival factor. We discuss the PI3K–Akt pathway here, as this pathway seems to be particularly important for mediating neuronal survival under a wide variety of circumstances.

Survival factors, by binding to their cognate tyrosine kinase receptors, elicit the recruitment of PI3K to the vicinity of the plasma membrane. The catalytic subunit of PI3K generates the phosphoinositide phosphates PIP_2 and PIP_3 at the inner surface of the plasma membrane. PIP_2 and PIP_3 in turn lead to the activation of several serine/threonine kinases, including Akt/protein kinase B, serum glucocorticoid inducible kinase (SGK), ribosomal S6 kinase (RSK), and atypical forms of protein kinase C (PKC) [16]. Akt is recruited to the inner surface of the plasma membrane through the interaction of its pleckstrin homology domain with the phospholipid products of PI3K. At the plasma membrane, the activation of Akt is dependent on phosphorylation, which is achieved at least in part by the protein kinase PDK1 (phosphoinositide-dependent protein kinase-1).

Over the past five years, the PI3K–Akt pathway has been found to be sufficient and, in some cases, necessary for the trophic-factor-induced cell survival of several neuronal cell types [17–24]. Ginty and co-workers [25••] have shown

Figure 2



In the presence of survival factors, the PI3K–Akt/SGK pathway is activated. Akt and SGK prevent the execution of apoptosis at several levels, in both transcription-dependent and independent manners. Akt and SGK phosphorylate and inhibit the transcription factor FOXO, and Akt indirectly inhibits p53, thereby preventing the expression of their target death genes. Akt also indirectly activates NF-κB, leading to the expression of survival genes, such as A1, Bcl-x_L and IAPs. In addition, Akt acts at a step before cytochrome *c* release, preventing the association of the pro-apoptotic family member BAD with Bcl-x_L, which allows Bcl-x_L to promote cell survival. Furthermore, Akt may act at a step subsequent to cytochrome *c* release, possibly by phosphorylating caspase 9, APAF1 or the IAPs. Casp, caspase.

recently that the ability of neurotrophins to promote neuronal survival requires a functional PI3K–Akt pathway both inside the cell body and in the distal axons that are in contact with the dendrites of target neurons.

An important point is that the experimental approach used in most studies to show that Akt is necessary for cell survival relies on the expression of dominant-interfering alleles of this protein kinase in neurons. This approach has substantial limitations because expressing dominant-negative alleles of Akt probably affects the activity of other closely related kinases, such as SGK, that are activated by the same upstream kinase, PDK1 [16]. Indeed, SGK, a protein kinase related to Akt and activated by PI3K, is also involved in mediating survival signals in cerebellar granule neurons [26•], as well as in other cell types [27]. Because the selective disruption of Akt or SGK remains a significant challenge owing to the presence of three distinct genes for each kinase, the identification of chemical inhibitors of this family of kinases will prove useful in future efforts to define the specific functions of Akt, SGK and other PDK1-regulated kinases, such as the RSKs and PKCs [14,28].

The identification of Akt substrates has been significantly aided by the characterization of a consensus peptide motif (RXRXXpS/T) that is preferred by Akt [29]. Database searches indicate that this motif is present in a large number of proteins; several of these proteins have been shown to be Akt substrates *in vitro* and *in vivo*, including transcription factors that may regulate the expression of components of the cell death machinery, Bcl-2 family members, a regulator of translation (4E BP-1) [30], endothelial nitric oxide synthase [31], the telomerase reverse transcriptase subunit [32], the tumor suppressor BRCA1 [33], and protein kinases such as Raf [34], IκB kinase [35,36] or glycogen synthase kinase-3 (GSK-3) [37]. As the PI3K–Akt pathway regulates cell proliferation and metabolism as well as cell survival, however, it will be important to distinguish which particular Akt targets mediate the neuronal survival effects of Akt.

Forkhead transcription factors of the FOXO family are directly controlled by phosphorylation by Akt

A series of recent studies indicate that Akt controls a major class of transcription factors — the Forkhead box transcription factor, class O (FOXO) subfamily of Forkhead transcriptional regulators (FKHR, FKHRL1 and AFX), which are homologous to Daf-16 of *Caenorhabditis elegans*. In the nematode, Daf-16 is negatively regulated by the PI3K–Akt pathway and controls metabolism and lifespan in this organism [38,39].

Several groups [40••–42••] have independently shown that Akt directly phosphorylates FOXOs and inhibits their ability to induce the expression of death genes. In the absence of survival factors, when Akt is inactive, FOXOs are localized in the nucleus and activate gene transcription (Figure 1). In the presence of survival factors, Akt becomes activated, phosphorylates FOXOs at several regulatory sites, and elicits the relocalization of FOXOs from the nucleus to the cytoplasm, away from their target genes (Figure 2) [40••–42••]. Recent evidence shows that SGK also phosphorylates the FOXO family member FKHRL1, but that Akt and SGK differ with respect to the efficacy with which these kinases phosphorylate the three regulatory sites of FKHRL1. As the phosphorylation of each regulatory site of FKHRL1 appears to be critical for the efficient exclusion of FKHRL1 from the nucleus, it is likely that SGK and Akt cooperate to promote cell survival by coordinately regulating FOXO transcription factors [26•].

When they are not phosphorylated, FOXOs can induce apoptosis of cerebellar granule neurons [40••], as well as other cell types [43,44], in a transcription-dependent manner, suggesting that FOXOs act by inducing death genes. A search of the promoter database using FOXO-binding sites reveals that the gene encoding FasL contains several FOXO-binding sites in its promoter, indicating that this gene may be regulated by FOXOs [40••]. Indeed, FasL mRNA is strongly induced upon removal of survival factors in cerebellar granule neurons [7•] — a condition that correlates with the presence of FOXOs in the nucleus. In addition, FOXO-induced cell death is diminished when FasL signaling is blocked in these neurons [40••]. Other potential genes that have FOXO-binding sites in their promoters are TRAIL (TNF-related apoptosis inducing ligand), tumor-necrosis factor-α and its receptor, and Fas, raising the possibility that FOXOs upregulate death cytokines as well as their cognate receptors. This in turn may trigger apoptosis, and possibly propagate the apoptotic signals to neighboring neurons.

In addition to suppressing the expression of death genes that might act in a paracrine fashion, Akt may also inhibit the expression of genes whose products act within the cell to control the apoptotic machinery. Indeed, a recent study indicates that FOXOs induce the expression of the pro-apoptotic Bcl-2 family member BIM-1 [45]. However, it is still unclear whether the BIM-1 promoter contains FOXO-binding sites or whether FOXO-mediated apoptosis requires the expression of BIM-1. Nevertheless, as BIM-1 overexpression elicits apoptosis and BIM-1 is present in neuronal cells [46] (see also Update), FOXO-induced BIM-1 expression may represent a relevant mechanism that at least partly accounts for FOXO-induced cell death in neurons.

Finally, the cell-cycle inhibitor p27KIP1 is another recently identified FOXO target gene that may participate in the regulation of apoptosis [43,44,47]. The p27KIP1 gene is strongly upregulated by FOXO and the p27KIP1 promoter contains several FOXO-binding sites. In addition, p27KIP1 seems to be necessary for apoptosis to occur in response to survival factor withdrawal, at least in hematopoietic cells [43]. The mechanism by which p27KIP1 may promote apoptosis is still unclear, although the effect of p27KIP1 on apoptosis appear to be independent from the effects p27KIP1 on cell-cycle progression [48].

p53: another transcriptional target of Akt?

A recent study by Yamaguchi *et al.* [49•] indicates that Akt, in addition to regulating FOXO-dependent transcription, also promotes survival in hippocampal neurons by inhibiting the activity of the tumor suppressor p53. Active p53 is known to induce the expression of death genes, including the pro-apoptotic Bcl-2 family member BAX [50]. p53 activity was recently shown to be critical in controlling sympathetic neuronal death following deprivation of NGF [6]. In these neurons, withdrawal of NGF leads to BAX transcription [6], and BAX mutant mice display a large excess of neurons that are resistant to p53-induced apoptosis [51].

These findings raise the possibility that Akt may promote survival partly by repressing p53 activity, thereby inhibiting BAX transcription. But BAX may not be the only p53 target that mediates the apoptotic effects of p53. Zhou *et al.* [52•] have shown that BAX levels are not affected by Akt activity in a motor neuron cell line. In this case, other recently identified p53 target death genes, including the pro-apoptotic Bcl-2 family member Noxa [53] and the mitochondrial protein p53AIP1 [54], may participate in p53-mediated neuronal death. At present, the mechanism by which Akt inhibits the activity of p53 remains unclear as Akt does not appear to phosphorylate p53 directly [49]. A possible Akt substrate that may explain the ability of Akt to inhibit p53 is the p53 regulator Mdm2, which contains two potential Akt phosphorylation sites.

Akt may induce the expression of survival genes by activating CREB or NF-κB

In addition to its function as a suppressor of critical death genes, under some circumstances activation of the PI3K–Akt survival pathway also triggers the expression of survival genes. Recent evidence suggests that the two transcription factors cAMP-responsive element binding protein (CREB) and nuclear factor κB (NF-κB), which induce the expression of survival genes, may be regulated by Akt [35,36,55]. Although the regulation of CREB and NF-κB by Akt is still controversial and has not been reported in neurons, three research groups [56–58] have shown recently that CREB and NF-κB play an important role in neuronal survival.

Several survival target genes of these transcription factors have been identified, which may account in part for the survival effect of the PI3K–Akt pathway. For example, the genes encoding the pro-survival Bcl-2 family members Bcl-x_L and A1 [59,60] and several IAPs [61] are upregulated by NF-κB (Figure 2), whereas the genes encoding Bcl-2 [57] and the pro-survival neurotrophin BDNF [62] are induced by CREB.

Akt directly inhibits members of the apoptotic machinery

In addition to its effects on transcription, the PI3K–Akt pathway also directly regulates the cytoplasmic apoptotic machinery. Akt has been proposed to act both prior to the release of cytochrome *c*, by regulating Bcl-2 family member activity and mitochondrial function, and subsequent to the release of cytochrome *c*, by regulating components of the apoptosome.

Akt phosphorylates the pro-apoptotic Bcl-2 family member BAD, thereby inhibiting BAD pro-apoptotic functions [63,64]. In the absence of survival factors, BAD is complexed with the pro-survival Bcl-2 family member, Bcl-x_L, thereby preventing Bcl-x_L from promoting cell survival. Upon addition of survival factors, Akt is activated and phosphorylates BAD at a specific amino acid residue, serine 136, which creates a binding motif for the chaperone molecule 14-3-3 (Figure 2).

The binding of BAD to 14-3-3 allows survival factors to elicit a second phosphorylation event at serine 155 [65•]. The kinase responsible for BAD phosphorylation at serine 155 *in vivo* is not yet known but may be PKA or even Akt itself. Phosphorylation of BAD at serine 155 is necessary to promote the complete release of BAD from Bcl-x_L, as the phosphorylation of serine 155, which is located within the BH3 (Bcl2-homology 3) domain of BAD, interferes with the interaction of BAD with Bcl-x_L [65•]. Once phosphorylated at serine 155, BAD is released from Bcl-x_L, which then promotes cell survival by inhibiting the release of cytochrome *c*.

In a motor neuron cell line, Akt is also capable of blocking apoptosis by acting at a step subsequent to cytochrome *c* release [52•]. Akt substrates that function after to cytochrome *c* release are not yet defined, but may include components of the apoptosome and the IAPs. Human caspase 9 itself is a substrate of Akt [66], but the importance of caspase 9 phosphorylation as a general mechanism for survival is the subject of some controversy, as the Akt phosphorylation site is not present in rodent caspase 9 [67]. Alternative post-mitochondrial targets for Akt include APAF-1 and neuronal IAPs, both of which contain Akt phosphorylation sites that are conserved across species. An appealing speculation is that Akt's phosphorylation of APAF1 may inhibit its pro-apoptotic function, whereas Akt's phosphorylation of IAPs may enhance their pro-survival function. But it remains to be determined whether APAF1 or IAP are *bona fide* targets of Akt. It is worth noting that, because the complete loss of cytochrome *c* from the mitochondria is fatal even in the presence of survival factors, post-mitochondrial mechanisms used by Akt to promote survival probably represent fail-safe mechanisms against transient or low levels of cytochrome *c* release.

Other effects of Akt: the metabolism hypothesis

Akt may also promote survival in an indirect fashion by regulating cellular metabolism. Recent studies by Crowder and Freeman [68•] and Xia and co-workers [69•] suggest that Akt mediates neuronal survival by repressing the activity of GSK-3. However, the mechanism by which active GSK-3 induces apoptosis is not yet clear. GSK-3, by phosphorylating glycogen synthase, may induce apoptosis through the regulation of glucose metabolism.

Recent studies indicate that the PI3K–Akt pathway may affect cell survival through the general control of metabolism [70••,71••]. Survival factor withdrawal triggers a depletion of ATP and glucose-derived metabolic substrates. Thompson and colleagues [70••,71••] have proposed that the control of mitochondrial function and ATP production by survival factors may be critical for the ability of these trophic factors to suppress apoptosis.

Consistent with this theory, Akt promotes survival by acting upstream of cytochrome *c* release [72•], perhaps in part through control of ATP production by the mitochondria. Although this finding was observed in a non-neuronal cell type and is in apparent contradiction with the report by Zhou and colleagues [52•], it suggests that under some circumstances, Akt may act by regulating ATP production. If such a view is correct, Akt may prevent the depletion of metabolites by increasing ATP or glucose levels, allowing the energy balance to tip towards cell survival. In this respect, it is also worth noting that Akt can increase glucose transport by promoting the translocation of the glucose transporter Glut-4 to the membrane [73]. In addition, the PI3K–Akt pathway inhibits the expression of genes encoding enzymes that decrease glucose metabolism, such as phosphoenolpyruvatecarboxykinase, or glucose 6 phosphatase [74,75], whereas the PI3K–Akt pathway induces the expression of the glucose transporter Glut-1 [76], which may also contribute to an Akt-dependent increase in glucose levels.

Conclusions

Is the PI3K–Akt pathway interconnected with other signaling cascades to promote neuronal survival? Certainly other signaling pathways that are critical to cell survival such as the Ras–MAPK–RSK or the cAMP/PKA pathway may also act on substrates that are regulated by Akt, as the terminal kinases in these pathways phosphorylate similar and overlapping (although not identical) consensus sequences. For example, BAD can be phosphorylated at serine 136 by both PKA and Akt [77], and can also be phosphorylated by RSK at serine 112 — another site critical for the suppression of BAD's pro-apoptotic function [56,78]. The overlapping substrate specificity of different survival signaling cascades may explain the frequently observed synergy between different trophic factors in neuronal culture systems [79]. In addition, the PI3K–Akt pathway may use other signaling pathways to promote cell survival. For example, Akt has been shown to potentiate calcium influx through L-type calcium channels [80], which may in turn promote survival of neurons by calcium-dependent pathways [81,82].

The continuing characterization of novel components of the apoptotic machinery has raised the possibility for new connections between survival pathways and the execution of cell death. For example, Akt may regulate the recently discovered Diablo/Smac either in a transcriptional or post-translational manner.

It is not known what role Akt plays in survival *in vivo*. Does Akt, when expressed at physiological levels, promote survival by regulating a single substrate, thereby acting at a unique step in the apoptotic program? Or do the many Akt substrates implicated in apoptosis through overexpression studies imply that endogenous Akt promotes survival by phosphorylating several components that each play a role in apoptosis execution? Available evidence supports a model in which Akt acts through multiple substrates. Such pleiotropic activity may represent a safety mechanism that allows a tight repression of apoptosis, in both acute or sustained conditions of survival factor withdrawal. In addition, all mechanisms of control by Akt may not be used under all circumstances; and some targets of Akt may be more critical than others, depending on the neuron type and the extent and type of stimulation.

The methodologies that are currently used to identify potential Akt targets unfortunately rely on overexpression paradigms to analyze the importance of particular phosphorylation events. These approaches do not prove that Akt targets are necessarily relevant *in vivo*. Indeed, the future of the field will rely both on the identification of new Akt substrates and the *in vivo* analysis of the importance of various Akt substrates. In particular, the generation of mouse models in which potential Akt targets are rendered unregulatable through the targeted introduction of mutations in the relevant Akt phospho-acceptor sites should help to dissect the relative importance of the various proposed phosphorylation events in mediating the Akt survival signal. Because such 'knock-in' experiments are technically challenging and the list of Akt targets continually grows longer, it is not clear when we will have a definitive resolution to the central question: how does the PI3K–Akt cascade promote survival? The critical importance of PI3K, Akt and related kinases in promoting neuronal survival, and the consequent potential for these molecules as targets for drug discovery suggests, however, that work towards understanding the mysteries of survival signaling will continue for some time to come.

Update

Two groups [83•,84•] recently reported that the proapoptotic Bcl-2 family member Bim is strongly induced in sympathetic neurons in response to NGF withdrawal. They showed that sympathetic neurons that are derived from Bim-deficient mice display a delayed apoptosis upon NGF deprivation, indicating that Bim induction is critical for neuronal death upon survival factor withdrawal.

The signaling pathways and transcription factors that regulate Bim induction is currently unclear, but c-Jun [84•] and the FOXO transcription factors [45] have been proposed to participate in the regulation of Bim expression.

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- •• of outstanding interest

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Despite the wealth of evidence suggesting that PI3K activity is critical to cell survival, there has been little investigation of how PI3K activity contributes to survival in circumstances that mimic *in vivo* situations. Using Campenot chambers that isolate the cell bodies and axons of cultured sympathetic neurons, the authors demonstrate that the PI3K pathway is activated locally at distal axons, and that this signal is transported retrogradely into the cell body. Through the use of pharmacological inhibitors, they also show that, when NGF is acting only on axons, the PI3K activity in both distal axons and the cell body is critical to cell survival. In contrast, PI3K activity is less important for cell survival when NGF acts on the cell body.

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