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COMMENTARY

GSK-3 – at the crossroads of cell death and survival

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ABSTRACT

Glycogen synthase kinase 3 (GSK-3) is involved in various signaling pathways controlling metabolism, differentiation and immunity, as well as cell death and survival. GSK-3 targets transcription factors, regulates the activity of metabolic and signaling enzymes, and controls the half-life of proteins by earmarking them for degradation. GSK-3 is unique in its mode of substrate recognition and the regulation of its kinase activity, which is repressed by pro-survival phosphoinositide 3-kinase (PI3K)–AKT signaling. In turn, GSK-3 exhibits pro-apoptotic functions when the PI3K–AKT pathway is inactive. Nevertheless, as GSK-3 is crucially involved in many signaling pathways, its role in cell death regulation is not uniform, and in some situations it promotes cell survival. In this Commentary, we focus on the various aspects of GSK-3 in the regulation of cell death and survival. We discuss the effects of GSK-3 on the regulation of proteins of the BCL-2 family, through which GSK-3 exhibits pro-apoptotic activity. We also highlight the pro-survival activities of GSK-3, which are observed in the context of nuclear factor κB (NFκB) signaling, and we discuss how GSK-3, by impacting on cell death and survival, might play a role in diseases such as cancer.

KEY WORDS: Glycogen synthase kinase 3, GSK-3, Phosphoinositide 3-kinase, PI3K, Apoptosis

Introduction

Glycogen synthase kinase 3 (GSK-3) was initially characterized as a kinase involved in the regulation of metabolism; early studies demonstrated that GSK-3 negatively regulates glycogen synthesis by phosphorylating and inactivating glycogen synthase (Embi et al., 1980). A few years later, insulin was described to have the opposite effect on glycogen synthase; by inducing the dephosphorylation of the sites that are targeted by GSK-3, it mediates the activation of glycogen synthase (Parker et al., 1983).

Almost 10 years later it became clear that insulin also mediates the inactivation of GSK-3 by phosphorylation (Welsh and Proud, 1993), providing a possible mechanism by which insulin mediates the dephosphorylation and activation of glycogen synthase. Soon after, it was shown that the phosphoinositide 3-kinase (PI3K) signaling pathway, which is also induced by insulin, is responsible for the inhibition of GSK-3 by mediating its phosphorylation (Cross et al., 1994). The general scenario that receptor tyrosine kinase signaling negatively regulates GSK-3 by mediating its N-terminal phosphorylation, thereby preventing GSK-3 from negatively regulating its substrate, also applies to other pathways, as we will describe below.

GSK-3 exists as two isoforms, GSK-3α (51 kDa) and GSK-3β (47 kDa), which are encoded by distinct genes. These isoforms often have overlapping functions, but they do not always compensate for each other. Nevertheless, for simplicity, we will refer in this Commentary to both isoforms as GSK-3, where appropriate.

GSK-3 has a large number of phosphorylation targets and thereby regulates a variety of biological processes (Fig. 1). For instance, GSK-3 regulates transcription by directly modulating the activity of various transcription factors. The first-described examples include Jun family members and nuclear factor of activated T-cells (NF-AT), which are negatively regulated by GSK-3 (Beals et al., 1997; Nikolakaki et al., 1993). Protein translation is also repressed by GSK-3 through an inhibition of the activity of the eukaryotic initiation factor eIF2B (Welsh et al., 1998). Moreover, phosphorylation by GSK-3 is an important signal for degradation of a large number of proteins, the best-known example being β-catenin in the Wnt pathway (Aberle et al., 1997). Besides its early-identified role in metabolism, GSK-3 has a number of additional biological functions, including in stem cell renewal (Ying et al., 2008) and embryonic development (Dominguez et al., 1995), as well as different functions in neurons (for an overview, see Kaidanovich-Beilin et al., 2012) and in immunity (reviewed in Beurel et al., 2010). GSK-3 also plays a role in diseases such as diabetes (Kaidanovich and Eldar-Finkelman, 2002), Alzheimer's disease (Takashima, 2006) and psychiatric diseases, including bipolar disorder and schizophrenia (Jope and Roh, 2006), making this kinase an attractive drug target.

In this Commentary, we focus on the role of GSK-3 in regulating cell death and survival. We illustrate its pro-apoptotic role in the intrinsic apoptosis pathway, where GSK-3 regulates the activity of BCL-2 family proteins. We also discuss the anti-apoptotic activity of GSK-3, mainly in the context of nuclear factor κB (NFκB) signaling, by which the kinase promotes cell survival.

GSK-3 is inactivated by kinase signaling pathways

Upon interaction of insulin and various growth factors with their cognate tyrosine receptor kinases, the lipid kinase PI3K is recruited to the growth factor or insulin receptor complex at the plasma membrane. Here, PI3K phosphorylates phosphatidylinositol (3,4)-bisphosphate (PtdInsP_2), a membrane lipid, which generates phosphatidylinositol (3,4,5)-trisphosphate (PtdInsP_3). The presence of PtdInsP_3 in the inner membrane layer promotes the recruitment of kinases that contain a PtdInsP_3-interacting pleckstrin homology (PH) domain to the plasma membrane. Thus, the pro-survival kinase AKT is recruited to the

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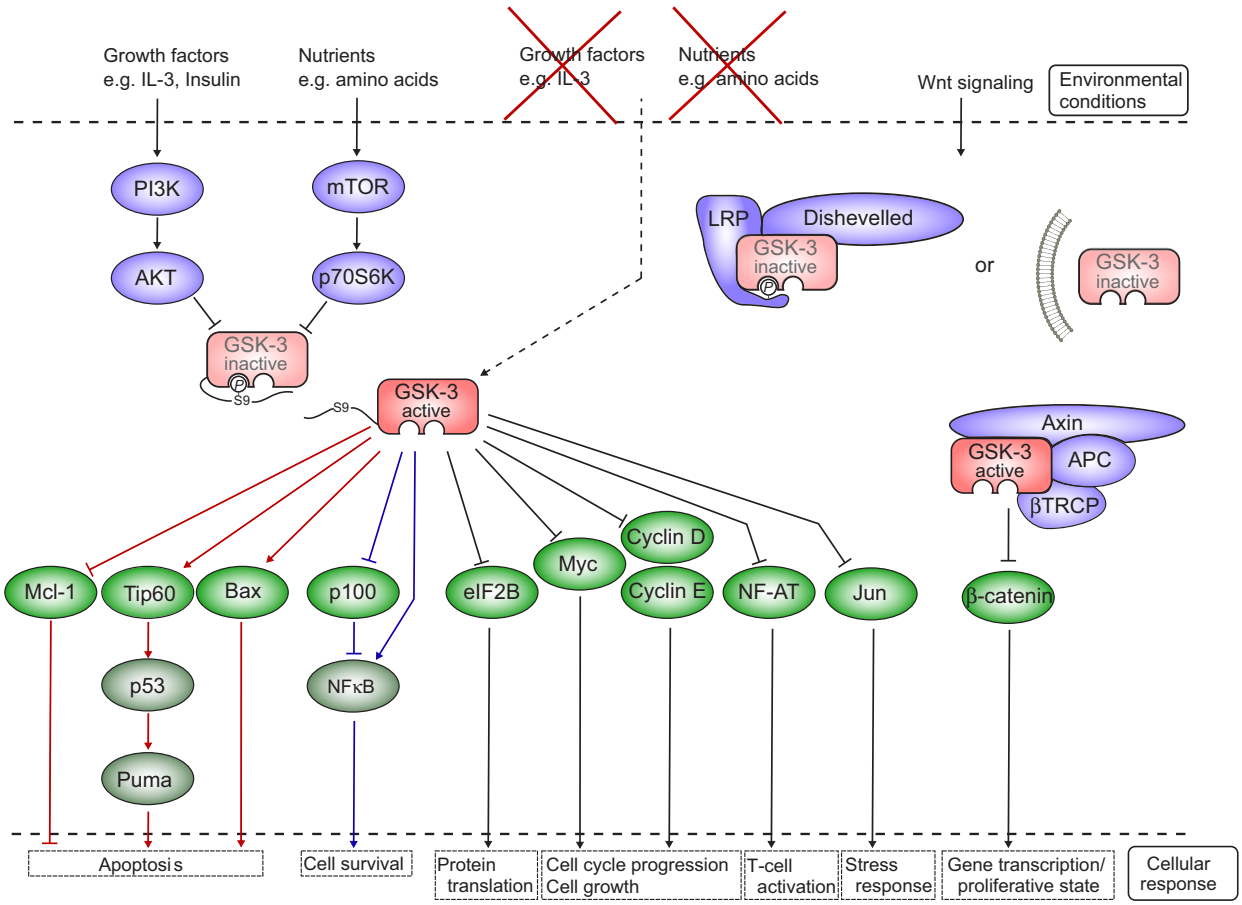

Fig. 1. Overview of signaling pathways involving GSK-3. GSK-3 is inactivated by PI3K or mTOR signaling (left) or by Wnt signaling (right). Targets of GSK-3 include BCL-2 proteins or their regulators, determining the onset of cell death. GSK-3 also regulates NFκB signaling, promoting cell survival. The regulation of protein translation by GSK-3 is mediated by its target eIF2b. GSK-3 also negatively regulates cell cycle proteins, targeting Myc and cyclin D and E for degradation. T-cell activation is negatively regulated by GSK-3 through phosphorylation of NF-AT. Impacting on the cell stress response, GSK-3 also inactivates Jun proteins. In the Wnt signaling pathway, β-catenin is degraded upon GSK-3 mediated phosphorylation in the absence on Wnt. Wnt stimulation inactivates GSK-3, stabilizing β-catenin (by one of the two indicated mechanisms), which along with TCF/LEF transcription factors induces Wnt target genes.

membrane, which brings it into close proximity to the kinases phosphoinositide-dependent kinase-1 (PDK1, also known as PDPK1) and mammalian target of rapamycin (mTOR) complex 2 (mTORC2); this results in phosphorylation of AKT on amino acid residues T308 and S473, respectively, and, consequently, its activation (Franke et al., 1997; Franke et al., 1995). When AKT is activated, it phosphorylates the two isoforms of GSK-3 (Cross et al., 1995) on their N-terminus; in the case of GSK-3α, position S21 is targeted, whereas GSK-3β is phosphorylated at position S9. The phosphorylation of the N-terminus results in the inactivation of both GSK-3 isoforms (Box 1). Because it is a major target of AKT, GSK-3β S9 phosphorylation is widely used to probe for activation of the PI3K–AKT signaling pathway. Treatment with growth factors that activate the PI3K–AKT pathway reduces the cellular activity of GSK-3 by 40–50%, whereas inhibition of PI3K has been shown to prevent growth-factor-mediated repression of GSK-3 (Cross et al., 1994).

Because PI3K signaling is known to promote the metabolic activity downstream of insulin, but also promotes cell survival upon growth factor signaling, there was the possibility that the suppression of GSK-3 activity by the PI3K–AKT pathway had not only a role in metabolism but also in the prevention of cell death. This indeed turned out to be the case, as we will elaborate below.

In addition to AKT, other kinases were found to phosphorylate and inactivate GSK-3. Downstream of mTOR, p70S6K (also known as RPS6KB) has been shown to phosphorylate the N-terminus of GSK-3 in the presence of amino acids (Armstrong et al., 2001). Consistently, elevated phosphorylation of GSK-3, mediated by p70S6K, was observed in cells that are deficient for the negative mTORC1 regulator TSC2 (Zhang et al., 2006). Thus, mTOR signaling mediates GSK-3 suppression upon availability of nutrients. Although less investigated in the context of GSK-3 signaling, both p90RSK (also known as RPS6KA1) (Cross et al., 1994) and PKA (Li et al., 2000), have also been reported to phosphorylate GSK-3 at the same N-terminal site as AKT. Apart from phosphorylation of the N-terminal serine and of the activation-loop tyrosine residues (Box 1), T390, which is located in the GSK-3 C-terminus, has been shown to be phosphorylated by the p38 mitogen-activated protein kinase (MAPK) (Thornton et al., 2008), and thus represents yet another way of regulating the activity of GSK-3. Therefore, many different signaling pathways converge on GSK-3, and inactivate it by phosphorylation.

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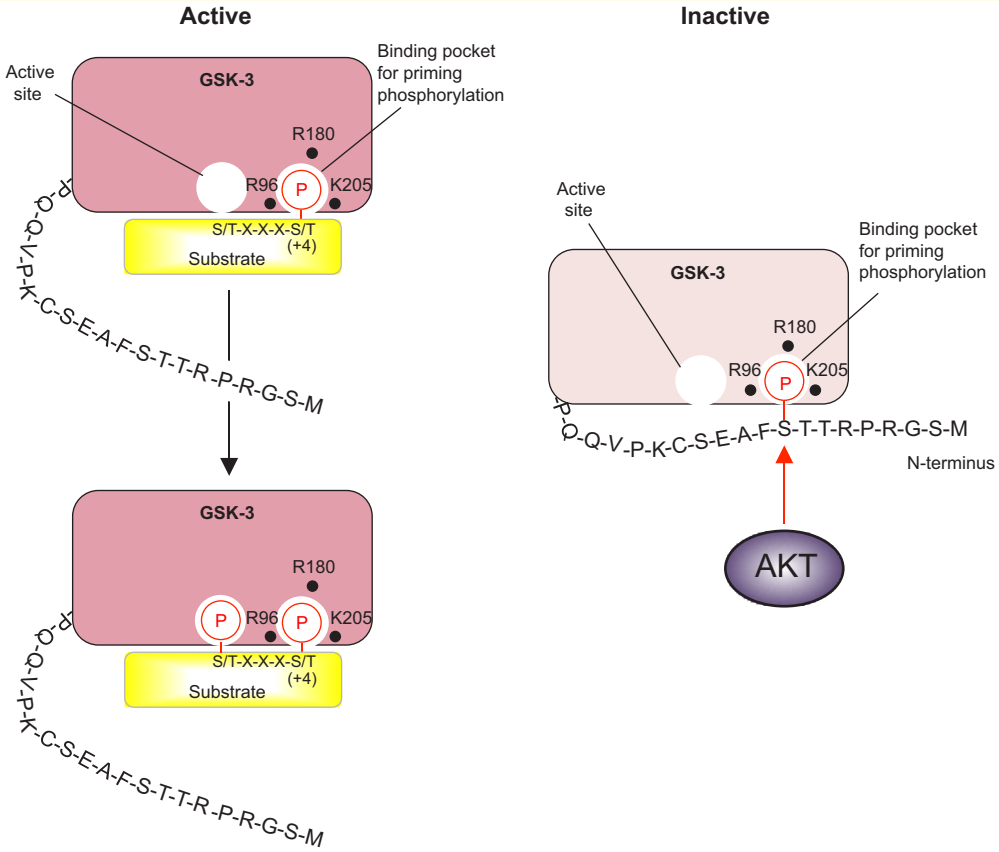
Box 1. Regulation of GSK-3

In order to be phosphorylated by GSK-3, its substrates need to be already phosphorylated, which is referred to as priming phosphorylation. This mode of regulation is unique among protein kinases. In fact, almost all GSK-3 substrates described so far contain the motif S/T-x-x-x-S/T, with the amino acid in the +4 position being the pre-phosphorylated priming site. This implies that GSK-3 substrates are always regulated by a mechanism that involves two kinases, with the priming kinase adding an additional layer of regulation.

A pocket that is formed by three positively charged amino acids (R96, R180 and K205) can interact with the phosphate group that comes with the substrate (ter Haar et al., 2001) (see figure, illustrated on the left). This is in contrast to most other serine/threonine kinases (such as MAPKs, which are structurally closely related to GSK-3) where this pocket binds the phospho-group of the activation loop, which is in this case delivered by an activating kinase. Similar to an activating phosphorylation in other kinases, the phospho-group of the GSK-3 substrate mediates the alignment of the N-terminal β-strand domain and the C-terminal α-helix domain (Frame et al., 2001).

The requirement for a phosphorylated substrate is the underlying basis for the mechanism by which GSK-3 itself is inactivated by phosphorylation. When the N-terminal S21, in case of GSK-3α, or S9, in case of GSK-3β, are phosphorylated by an inactivating kinase (e.g. AKT) (see figure, illustrated on the right), the N-terminus of GSK-3 can fold into the substrate-binding site, and the N-terminal phosphate group will occupy the basic pocket generated by R96, R180 and K205, thus blocking it from binding potential (pre-phosphorylated) substrates. Thus, the phosphorylation of the N-terminus of GSK-3 creates a pseudo-substrate, resulting in inactivation of GSK-3 by auto-inhibition (Frame et al., 2001).

Similar to MAPKs, tyrosine phosphorylation of the activation loop of GSK-3 has been shown to increase its activity ~100-fold (Hughes et al., 1993). Thus, full activity of GSK-3 requires not only the absence of the N-terminal phosphorylation, but also the presence of a phosphorylation at position Y279 in GSK-3α and Y216 in GSK-3β. However, in contrast to MAPKs, the activating tyrosine phosphorylation of GSK-3 occurs by auto-phosphorylation (Cole et al., 2004).



Inactivation of GSK-3 in protein complexes

The Wnt signaling pathway has emerged as another principal signaling pathway that includes GSK-3 as a central player. Activation of the Wnt pathway, which can occur in stem and progenitor cells, results in transcriptional activation of the genes encoding cyclin D1 and Myc, thereby driving cell proliferation and tissue renewal (Clevers, 2006). Wnt signaling represents an entirely different mode of GSK-3 regulation. In the absence of a Wnt signal, GSK-3 exerts its activity and is in a protein complex containing axin, adenomatous polyposis coli (APC) and the ubiquitin ligase βTRCP (also known as BTRC), which mediates phosphorylation and degradation of β-catenin (Aberle et al., 1997). In this context, phosphorylation of the N-terminus of GSK-3 does not appear to play a role in the regulation of its kinase activity (Ng et al., 2009). Instead, two models have been proposed for how GSK-3 might be inactivated in the Wnt signaling pathway. According to the first model, the protein Disheveled is recruited to the Wnt receptors [Frizzled and low

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density lipoprotein receptor-related protein 6 (LRP6)] upon Wnt binding, which, in turn, recruit axin together with GSK-3 to the membrane. Here, phosphorylation of the cytoplasmic portion of LRP6 by GSK-3 and casein kinase 1 (CK-1, also known as CSNK1A1) might form a type of GSK-3 pseudo-substrate (Box 1) that inhibits GSK-3, thereby preventing it from phosphorylating β-catenin (MacDonald et al., 2009). An alternative model proposes that upon Wnt binding, GSK-3 is sequestered in vesicles, sequestering it from β-catenin (Taelman et al., 2010). It is important to note that, although the majority of the literature refers to GSK-3β as being a constituent of the β-catenin degradation complex, GSK-3α and GSK-3β have been shown to be functionally redundant in this context (Doble et al., 2007). As is the case for most other, if not all, GSK-3 substrates (Box 1), phosphorylation of β-catenin by GSK-3 in this complex requires a priming phosphorylation (Liu et al., 2002). As a consequence, when Wnt signaling is activated, GSK-3 is prevented from mediating degradation of β-catenin, and downstream targets, such as Myc, are transcriptionally induced through T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors that maintain the proliferative state of progenitor cells, as for example in the colonic crypt (Clevers, 2013).

Taken together, GSK-3 integrates many different signals from different pathways, and it is conceivable that, at any given time, different pools of GSK-3 with a different state of activity, location in the cell or phosphorylation status are involved in signaling through these pathways.

GSK-3 and the regulation of cell death

Growth factors protect cells from cell death by activating pro-survival kinase signaling pathways. A key signaling pathway that is induced by growth factor receptor signaling is the PI3K pathway, which potently promotes cell survival (Yao and Cooper, 1995). PI3K signaling, as well as its downstream kinase AKT, has been shown to suppress Myc-induced apoptosis (Kauffmann-Zeh et al., 1997; Kennedy et al., 1997). Consistent with this, PI3K has been found to be necessary for growth-factor-induced survival of cerebellar neurons, an effect that is compromised when inactive AKT mutants are expressed in these cells (Dudek et al., 1997). Furthermore, a pro-survival role of AKT in hematopoietic interleukin (IL)-3-dependent 32D cells has been demonstrated, as these cells are protected from cell death upon growth factor withdrawal when they express oncogenic v-AKT. 32D cells expressing v-AKT are also protected against the effects of the DNA-damaging agent etoposide (Songyang et al., 1997).

Because it delivers a strong pro-survival signal, it is not surprising that the PI3K–AKT pathway is constitutively active in many cancers, which is achieved through mutations in PI3K, the loss of the PtdInsP_3 phosphatase PTEN or gain-of-function in upstream activators such as mutated K-Ras (Yuan and Cantley, 2008). Importantly, PI3K–AKT signaling inhibits the permeabilization of the mitochondrial outer membrane, an event, which represents the decisive step in the intrinsic apoptosis pathway (Kennedy et al., 1999). The release of cytochrome *c* and other proteins that are located in the mitochondrial intermembrane space is regulated by members of the Bcl-2 protein family (Chipuk et al., 2010). Early findings (Kennedy et al., 1999) suggested that Bcl-2 family members are involved in PI3K–AKT-mediated cell survival signaling. Indeed, the characterization of the BH3-only protein BAD as an AKT substrate, being inactivated by AKT, provided one of the first examples for how a pro-survival kinase signaling pathway can directly regulate the activity of a Bcl-2 family protein (Datta et al., 1997; del Peso et al., 1997).

The fact that PI3K both represses cell death and inhibits GSK-3 activity suggests that GSK-3 itself might have a role in the regulation of cell death. The first clear demonstration of a role of GSK-3 in the regulation of apoptosis, downstream of PI3K signaling, was the finding that overexpression of GSK-3β in Rat1 fibroblasts and neuronal PC12 cells results in apoptosis. Conversely, cell death that is induced by PI3K inhibition could be prevented by expression of a dominant-negative GSK-3β-K85R mutant (Pap and Cooper, 1998). This study showed the importance of the suppression of GSK-3 activity for the pro-survival effects of PI3K. Likewise, GSK-3 has been shown to promote apoptosis of neuronal SH-SY5Y cells upon staurosporine treatment and heat shock (Bijur et al., 1999). In another study, inhibition of GSK-3, or expression of the inactive GSK-3β-K85R mutant, has been shown to reduce the number of sympathetic neurons undergoing cell death mediated by loss of PI3K signaling (Crowder and Freeman, 1998). The use of small-molecule GSK-3 inhibitors SB-216763 and SB-415286 has also supported the concept that GSK-3 is required for neuronal cell death, as these inhibitors protect cerebellar granule neurons from death upon inhibition of PI3K signaling or withdrawal of K^+ (Cross et al., 2001). Taken together, these findings suggest a pro-apoptotic role for GSK-3 in neuronal cells and, moreover, that inhibition of GSK-3 in these cells is part of the pro-survival PI3K signaling pathway.

GSK-3 controls the activity of Bcl-2 family proteins

The pro-apoptotic role of GSK-3 raised the question what the underlying mechanisms are by which it promotes cell death. The intrinsic apoptosis pathway (Box 2) is regulated by the Bcl-2 proteins, which are thus promising target candidates for GSK-3 to regulate apoptosis. The first study addressing this possibility found that the activity of the pro-apoptotic Bcl-2 protein Bax depended on GSK-3 (Linseman et al., 2004). To mediate cytochrome *c* release, Bax has to translocate to the mitochondria (Hsu et al., 1997) and change its conformation to an active state (Hsu and Youle, 1997). Both these effects are observed upon trophic growth factor depletion from cerebellar granule neurons, but they were found to be prevented upon inhibition of GSK-3 (Linseman et al., 2004). That study suggested that Bax activation requires a direct phosphorylation by GSK-3 because GSK-3 could phosphorylate Bax in an *in vitro* kinase assay, and mutation of the GSK-3 phosphorylation site in Bax abolished its mitochondrial translocation (Linseman et al., 2004). Although a direct regulation of Bax by GSK-3 is an intriguing possibility, how exactly GSK-3-induced phosphorylation of Bax could mediate its mitochondrial translocation activation has not been further addressed to date.

GSK-3 has also been described to act in concert with the JNK pathway during neuronal cell death by regulating Bim expression and apoptosis (Hongisto et al., 2003). However, in that study, no direct phosphorylation target of GSK-3 that would promote the observed induction of Bim was identified. Interestingly, the same group later showed that neurons from GSK-3-S21A/S9A knock-in mice (which cannot be repressed by PI3K–AKT signaling) are viable. Furthermore, on the basis of their finding that cell death induced by trophic factor deprivation could nevertheless be prevented by an inhibition of GSK-3, the authors suggested that a Wnt-regulated pool of GSK-3

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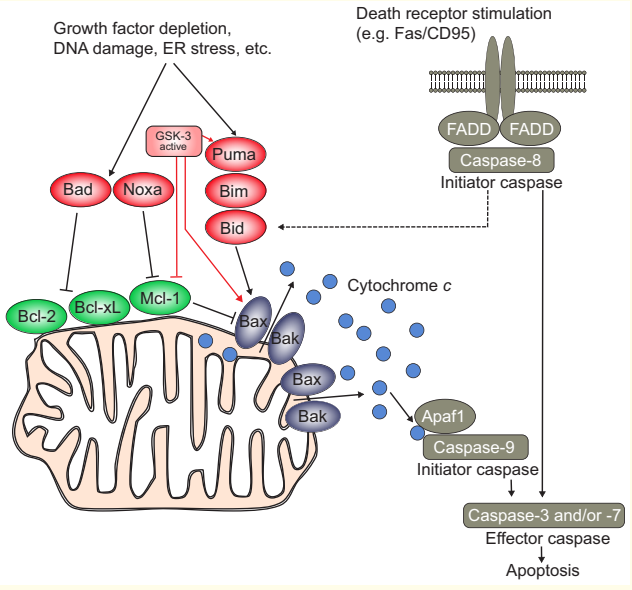
Box 2. Intrinsic and extrinsic apoptosis pathways

Apoptosis is characterized by the activity of caspases, which are activated by two main pathways. First, in the mitochondrial pathway, the permeabilization of the outer mitochondrial membranes is the key event and the point of no return for cell death. Mitochondrial membrane permeabilization requires the Bcl-2 proteins Bax and/or Bak, which are activated by the BH3-only proteins Bim, Bid and possibly also Puma (see figure). This is opposed by anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL or Mcl-1, which either sequester BH3-only proteins or activated Bax or Bak. A second group of BH3-only proteins, such and Bad and Noxa, displace the Bax/Bak-activating BH3 proteins, or prevent activated Bax/Bak from binding to the anti-apoptotic Bcl-2 family members, thereby also promoting mitochondrial outer membrane permeabilization (Chipuk et al., 2010; Llambi et al., 2011). Upon mitochondrial outer membrane permeabilization, a number of proteins are released from the mitochondrial inter-membrane space. Among these, cytochrome *c* associates with Apaf-1 and the initiator caspase-9 (and ATP) to form a protein complex, the apoptosome, which brings pairs of caspase-9 closely together to induce their protease activity. Caspase-9 undergoes auto-processing and then cleaves the effector caspase-3 and caspase-7, resulting in their activation. Caspase-3 (and caspase-7) activity mediates all the hallmarks of apoptosis, such as nuclear condensation, oligonucleosomal DNA cleavage, phosphatidylserine exposure and the shut-off of respiration, resulting in cell death and immediate uptake of apoptotic cells by phagocytes, in general without triggering inflammation (Martin et al., 2012; Riedl and Salvesen, 2007) .

The second pathway that activates caspases is the extrinsic apoptosis pathway (shown on the top right); here, death ligands, such as FasL or TNF, directly instruct the cell to undergo cell death. The stimulation of their receptors (e.g. Fas/CD95 or TNFR1) creates protein platforms (by recruitment of the adaptor Fas-associated protein with death domain; FADD) that activate the initiator caspase-8 by induced proximity. Caspase-8, after autoprocessing, cleaves and activates caspase-3 and caspase-7, resulting in cell demise by apoptosis. Caspase-8 also cleaves and activates the BH3-only protein Bid, thereby activating the mitochondrial apoptosis pathway (Dickens et al., 2012).



contributes to cell death (Hongisto et al., 2008). Although this is an interesting possibility, the majority of the available data suggest that suppression of GSK-3 activity by PI3K–AKT signaling is the major pathway through which its pro-apoptotic roles of are prevented.

GSK-3 not only contributes to apoptosis of neural cells, as its inhibition has also been shown to be rate-limiting for apoptosis of hematopoietic cells when they are deprived of growth factors. A study on erythroid progenitor cells has demonstrated a connection between growth-factor-mediated cell survival signaling by PI3K–AKT and inhibition of GSK-3 (Somervaille et al., 2001). Those authors found that pro-survival signaling by Epo and stem cell factor (SCF) requires PI3K activity and suppresses GSK-3 activity. Inhibition of GSK-3 conferred survival of these cells when they were depleted from the growth factors, and also prevented the conformational change of pro-apoptotic Bax, which is required for GSK-3 activation. This suggested that upon loss of growth factor and PI3K signaling, GSK-3 promotes the activation of Bax. In turn, these findings also suggested that PI3K–AKT-mediated inactivation of GSK-3 is a substantial component of the pro-survival signaling in hematopoietic cells.

Our own experiments, employing IL-3-dependent BAF-3 or FL5.12 pro-B-cells demonstrated that these cells undergo rapid GSK-3-dependent apoptosis when they are depleted of IL-3 (Maurer et al., 2006). We found that the rate of cell death owing to IL-3 withdrawal is profoundly reduced upon inhibition of GSK-3 with the small-molecule inhibitors CT98014 or CT99021, inhibitors that exhibit high potency and specificity in the nanomolar range (Bain et al., 2007; Ring et al., 2003). Again, this indicates that inhibition of GSK-3 is an important contribution to the effect of a pro-survival cytokine. Similarly, we found that GSK-3 is rate-limiting for apoptosis of activated lymphocytes, which depend on the cytokine IL-2 for survival; upon withdrawal of IL-2 (after lymphocyte activation *in vitro*), apoptosis occurred with a substantially reduced rate in the presence of GSK-3 inhibitors (C.C. and U.M., unpublished data). The transcription factor NF-AT is crucially involved in lymphocyte activation, and GSK-3 had been shown to prevent the nuclear translocation of NF-AT (Beals et al., 1997). However, when we conducted experiments in IL-3-dependent cell lines, we did not find evidence that NF-AT has a role in the induction of GSK-3-dependent apoptosis upon cytokine withdrawal, although we found that nuclear translocation of NF-AT was regulated by GSK-3 (C.C. and U.M., unpublished data). Monitoring the release of cytochrome *c* in FL5.12 cells has shown that inhibition of GSK-3 prevents mitochondrial outer membrane permeabilization upon IL-3 deprivation (Maurer et al., 2006), suggesting that GSK-3 affects the activity of Bcl-2 proteins.

The Bcl-2 family member Mcl-1 has a short half-life of only ~40 min, which allows the cell to adapt the levels of Mcl-1 more quickly in response to environmental cues than for instance those of the anti-apoptotic factors Bcl-2 and Bcl-xL (the long isoform of Bcl-x, encoded by *BCL2L1*). Mcl-1 contains an evolutionarily conserved GSK-3 phosphorylation site, which we, and subsequently other groups, have shown is phosphorylated by GSK-3 (Ding et al., 2007; Inuzuka et al., 2011; Maurer et al., 2006; Morel et al., 2009; Zhao et al., 2007). Phosphorylation of a protein by GSK-3 often results in its destabilization. As described above, this is the case for β-catenin (Aberle et al., 1997), which is degraded after GSK-3-mediated phosphorylation owing to ubiquitylation by the E3 ligase $SCF^{\beta\text{-TRCP}}$ (an SCF complex with β-TRCP as the F-box protein). Similarly,

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Snail, a transcription factor involved in epithelial–mesenchymal transition (EMT) is also marked for degradation by $SCF^{\beta\text{-}TRCP}$ upon phosphorylation by GSK-3 (Zhou et al., 2004). Indeed, we and others observed a similar mechanism by which Mcl-1 is destabilized upon phosphorylation by GSK-3 (Ding et al., 2007; Inuzuka et al., 2011; Maurer et al., 2006; Morel et al., 2009; Zhao et al., 2007).

Although all these studies show that there is GSK-3-mediated Mcl-1 phosphorylation and degradation, GSK-3 is activated in different ways in these systems. In our study, GSK-3 activity was triggered by growth factor withdrawal, but direct inhibition of PI3K also resulted in the loss of Mcl-1 (Maurer et al., 2006), as also reported by Hung and colleagues (Ding et al., 2007). However, GSK-3-dependent loss of Mcl-1 can also be induced by low glucose (Zhao et al., 2007) or UV irradiation (Ding et al., 2007; Morel et al., 2009). Consistent with a role for $SCF^{\beta\text{-}TRCP}$ in mediating degradation of Mcl-1, knockdown of $SCF^{\beta\text{-}TRCP}$ has been shown to elevate Mcl-1 levels, and $SCF^{\beta\text{-}TRCP}$ can also promote degradation of wild-type Mcl-1, but not of an Mcl-1 mutant that is unable to be phosphorylated by GSK-3 (Ding et al., 2007).

GSK-3 has also been shown to regulate many of its substrates by creating a phosphodegron which is recognized by the SCF^{Fbw7} ubiquitin ligase. For instance, the phosphorylation of Myc by GSK-3 earmarks it for ubiquitylation by SCF^{Fbw7} and subsequent degradation (Welcker et al., 2004). It has been suggested that a GSK-3-induced phosphodegron might actually be common to all SCF^{Fbw7} substrates (Welcker and Clurman, 2008). Accordingly, SCF^{Fbw7} has been found to be another GSK-3-dependent Mcl-1 ubiquitin ligase, which destabilizes Mcl-1 upon phosphorylation at its S159 residue (Inuzuka et al., 2011).

The phosphorylation of Mcl-1 by GSK-3 requires a priming phosphorylation, which has been shown to be delivered by MAPKs. Upon UV treatment, which activates JNK, Mcl-1 was found to be rapidly degraded. However, Mcl-1 exhibits increased stability in JNK1/JNK2 double knockout mouse embryonic fibroblasts (MEFs), suggesting that JNK phosphorylation (on T144 in mouse Mcl-1) primes Mcl-1 for a destabilizing phosphorylation (on S140) that is mediated by GSK-3 (Morel et al., 2009). Intriguingly, ERK-mediated phosphorylation of T163 (the GSK-3-priming site in human Mcl-1) results in Mcl-1 stabilization (Domina et al., 2004) in a way that is reminiscent of the regulation of Myc protein stability. The N-terminus of Myc contains two phosphorylation sites; S62 represents the GSK-3-priming site that is targeted by ERK, which then allows the phosphorylation of residue T58 by GSK-3. Although phosphorylation of T58 by GSK-3 has been shown to promote the degradation of Myc (which is inhibited by Ras), phosphorylation of S62 (by ERK) alone results in its stabilization (Sears et al., 1999; Sears et al., 2000).

Although an accelerated degradation of Mcl-1 is sufficient to explain the inhibitory effect GSK-3 has on Mcl-1, it remains to be investigated whether GSK-3-mediated Mcl-1 phosphorylation also affects Mcl-1 activity per se, such as its capacity to sequester BH3-only proteins and/or activated Bak (Llambi et al., 2011; Willis et al., 2005). We recently investigated the *in vivo* significance of GSK-3-mediated Mcl-1 phosphorylation in a mouse model by employing adoptive bone marrow transfer (Lindner et al., 2013). In experiments where a GSK-3-phosphorylation-deficient Mcl-1 mutant, Mcl-1-S159A, or wild-type Mcl-1 was expressed in mouse bone marrow cells, followed by adoptive transfer to irradiated recipient mice, mice that had received bone marrow expressing Mcl-1-S159A exhibit an elevated leukocyte count compared to mice that received bone marrow cells infected with wild-type Mcl-1 (Lindner et al., 2013). These data suggest that the absence of Mcl-1 S159 phosphorylation, which is mediated exclusively by GSK-3 (our own unpublished data), confers a survival advantage to hematopoietic cells.

Moreover, we found that the Mcl-1-S159A mutant, when introduced into bone marrow from Eμ-MYC transgenic donor mice, confers increased leukocytosis in recipient mice, as compared to wild-type Mcl-1. This indicates that the absence of Mcl-1-S159 phosphorylation, i.e. in the presence of elevated PI3K signaling, promotes an accelerated onset of lymphoma (Lindner et al., 2013). Furthermore, as Mcl-1 represents the main barrier for the response to the BH3 mimetic ABT-737 (van Delft et al., 2006), these data suggest that drugs interfering with PI3K signaling and thereby reducing Mcl-1, might well cooperate with BH3-mimetic drugs such as ABT-263 *in vivo*.

An increase in hematopoietic cell survival upon loss of GSK-3-mediated Mcl-1 phosphorylation is compatible with an earlier report of an improved bone marrow engraftment upon *in vivo* administration of GSK-3 inhibitors (Trowbridge et al., 2006). Although that study demonstrated effects of GSK-3 on the Wnt, Notch and Hedgehog pathways, which likely do not directly affect cell survival, it is nevertheless possible that, in this context, an inhibition of GSK-3 also promotes cell survival, for instance by acting on Mcl-1. Similarly, the treatment of embryonic stem cells with the GSK-3 inhibitor CT99021 promotes their propagation (Ying et al., 2008). Because Mcl-1 was found be required for self-renewal of primitive stem cells (Campbell et al., 2010), its GSK-3 mediated phosphorylation might also have a role in this context.

GSK-3 promotes p53-mediated apoptosis

GSK-3 also influences mitochondrial apoptosis signaling that is mediated by p53. It has been known for a long time that growth factors prevent p53-mediated apoptosis. For example, IL-6 has been shown to protect myeloid cells from apoptosis that is induced by a temperature-sensitive mutant of p53 (Yonish-Rouach et al., 1991). Similarly, in BAF-3 cells, IL-3 stimulation has been shown to prevent p53-induced apoptosis but not cell cycle arrest upon γ-irradiation (Canman et al., 1995; Collins et al., 1992). Furthermore, constitutively active PI3K or AKT have been shown to slow down the rate of p53-mediated apoptosis (Sabbatini and McCormick, 1999). Likewise, MEFs that are deficient in the PtdInsP_3 phosphatase PTEN are protected from p53-mediated apoptosis (Stambolic et al., 2001). Increasing evidence now suggests that the prevention of p53-mediated cell death by PI3K–AKT signaling is, at least in part, mediated by a suppression of GSK-3.

The first hint for a functional link between GSK-3 and p53 in the induction of apoptosis came from a study that found that dominant-negative p53 prevents the promotion of apoptosis by GSK-3 (Pap and Cooper, 1998). An effect of GSK-3 on p53 was also suggested based on a study that showed a reduction of p53 levels upon treatment of neuroblastoma with lithium (an albeit relatively low-potency GSK-3 inhibitor, active in the millimolar range) (Lu et al., 1999). The same group later described an increased nuclear activity of GSK-3 upon DNA damage that is induced by treatment with camptothecin, which results in a direct interaction between GSK-3 and p53 (Watcharasit et al., 2002). Furthermore, inhibition of GSK-3

with lithium has been suggested to prevent the induction of p21 (also known as CDKN1A, CIP1 and WAF1), a p53 target, and Bax, as well as cytochome *c* release and caspase activation (Watcharasit et al., 2003). Although that study showed an increased activity of GSK-3 upon DNA damage (Watcharasit et al., 2002), another study showed that γ-irradiation decreases GSK-3 activity (Kulikov et al., 2005). In that report, GSK-3 was shown to directly phosphorylate and activate the p53-degrading E3 ligase MDM2, so that γ-irradiation, and thus GSK-3 inactivation, resulted in the stabilization of p53 (Kulikov et al., 2005). Although we did not observe any effect of DNA damage on GSK-3 activity (Charvet et al., 2011), further investigation is clearly required to clarify this issue.

Apoptosis that is mediated by p53 requires the transcriptional regulation of the pro-apoptotic BH3 protein Puma (Villunger et al., 2003). Our own experiments have shown that GSK-3 is required for the induction of Puma by p53. IL-3-dependent (and p53 proficient) BAF-3 cells, which we maintained in the presence of reduced amounts of growth factors, to permit high GSK-3 activity, exhibited the induction of Puma and apoptosis upon γ-irradiation. Strikingly, we found that Puma induction was completely abrogated when we inhibited GSK-3 pharmacologically using the compounds CT99021 and CT98014. Importantly, unlike expression of Puma, the expression of p21 was not affected by GSK-3 activity (Charvet et al., 2011).

The p53-mediated transcriptional induction of Puma, but not of p21, has been shown previously to require lysine acetylation of p53 at position K120, mediated by the acetyltransferase Tip60 (also known as KAT5) (Sykes et al., 2006; Tang et al., 2006). Moreover, recruitment of Tip60 to the Puma promoter by K120-acetylated p53 induces acetylation of histone H4 proximal to the p53-binding site (Tang et al., 2006). These data, together with the requirement of GSK-3 for the induction of Puma suggest that there is a connection between p53-K120 acetylation and GSK-3 activity. Indeed, we found the acetyltransferase Tip60, which has been demonstrated to mediate K120-acetylation of p53, to be a bona fide GSK-3 substrate. Phosphorylation of Tip60 by GSK-3 at its position S86 was required for p53-K120 acetylation and γ-irradiation-mediated induction of Puma (Charvet et al., 2011). Therefore, our data establish a direct link between the PI3K signaling pathway and p53-K120 acetylation, which promotes the expression of Puma (Charvet et al., 2011). Interestingly, it has been suggested that Tip60 forms a complex with axin, along with HIPK2 and p53, to induce apoptosis upon DNA damage (Li et al., 2009), and it will be interesting to investigate how these findings relate to each other.

Puma has been shown to be largely responsible for radiation-induced damage of healthy tissue, which is a major side effect of radiation therapy during cancer treatment (Qiu et al., 2008). This damage could be prevented in mice with the administration of GSK-3 inhibitors, and rescued hippocampal neurons as well as intestinal crypt cells when the animals were treated with γ-irradiation (Thotala et al., 2010; Thotala et al., 2008). These data support the role of GSK-3 in the induction of Puma and in apoptosis upon DNA damage *in vivo*, and might open a therapeutic avenue for protection of healthy tissue from chemotherapy-induced damage, a major side effect during cancer therapy. It will also be interesting to see whether induction of Puma by p53 homologues, such as p63 (Kerr et al., 2012) or p73 (Qiu et al., 2008), also depends on GSK-3, and possibly also on Tip60, as both p63 and p73 contain a site with a high sequence similarity to site in p53 that is acetylated by Tip60.

Other transcription factors have also been suggested to induce Puma in a GSK-3-dependent manner: Puma induction upon K^+ withdrawal from cerebellar granule neurons required GSK-3 and JNK, and has been suggested to depend on Foxo3a (Ambacher et al., 2012). Although Foxo3a is not a direct phosphorylation target of GSK-3, it is possible that GSK-3 promotes Puma transcription, through Tip60 phosphorylation, by transcription factors different from p53. Another study has shown that the p65 subunit of NFκB induced Puma in a GSK-3-dependent manner – and independently from IκBα degradation – upon treatment of HCT116 cells with the kinase inhibitor Sorafenib (Dudgeon et al., 2012). It remains to be investigated how this mechanism, by which GSK-3-dependent NFκB activity is suggested to promote apoptosis, relates to anti-apoptotic, GSK-3-dependent NFκB functions as discussed below.

GSK-3 as a mediator of cell survival

Contrasting with the pro-apoptotic roles of GSK-3, the kinase also has another identity as it has been found to function as a pro-survival enzyme. Mice with a homozygous deletion of GSK-3β revealed the pro-survival role of this kinase in the context of NFκB signaling. NFκB signaling, driven, for instance, by inflammatory cytokines such as tumor necrosis factor (TNF), which activates NFκB through the assembly of the TNF receptor (TNFR) complex I, represents an important pro-survival pathway. The transcriptional targets of NFκB include the inhibitor of apoptosis (IAP) proteins and c-FLIP (also known as CFLAR) (Micheau et al., 2001; Wang et al., 1998). cIAP proteins are E3 ubiquitin ligases that promote survival through K63-linked ubiquitylation of constituents of TNFR complex I, such as RIPK1 (Gyrd-Hansen and Meier, 2010). c-FLIP, a close relative of caspase-8 without enzymatic activity, prevents pro-apoptotic homodimeric activation of caspase-8 by heterodimerizing with caspase-8 (Dickens et al., 2012). In the absence of these proteins, the activation of TNFR1 by TNF turns into a cell death signal, through the assembly of the TNFR complex II, which is a protein platform activating caspase-8 and thereby apoptosis.

Mice with a homozygous deletion of GSK-3β exhibit liver degeneration owing to apoptosis, which results in embryonic death by E13.5 (Hoeflich et al., 2000). The phenotype of GSK-3β-null mice very much resembles mice that lack the p65 NFκB subunit (also known as RelA) (Beg et al., 1995); these also die of liver damage owing to pro-apoptotic TNF signaling, which is not counteracted by NFκB in these animals. Liver apoptosis of the GSK-3β-null embryos can be rescued by injection of pregnant females with neutralizing anti-TNF antibodies, indicating that GSK-3β represses a TNF-dependent, extrinsic apoptosis pathway. Consistently, in GSK-3β-null MEFs derived from these embryos, pro-survival NFκB signaling is attenuated. These effects are specific for the loss of GSK-3β, as the presence of the prevailing GSK-3α does not compensate for GSK-3β (Hoeflich et al., 2000), and mice that lack GSK-3α exhibit normal NFκB signaling (MacAulay et al., 2007).

The pro-survival role of GSK-3 through NFκB also has a role in cancer, as shown for chronic lymphocytic leukemia (CLL). Here, inhibition of GSK-3 results in increased apoptosis of primary CLL B-cells, and decreased binding of p65 to the promoters of the genes encoding Bcl-2 and XIAP, which were found to be downregulated (Ougolkov et al., 2007). GSK-3 activity also contributes to pro-survival NFκB signaling by phosphorylation of the NFκB inhibitory protein p100 (also known

as NFKB2). This creates a phosphodegron that is recognized by the ubiquitin ligase SCF^{Fbw7}, resulting in p100 degradation and thus preventing its inhibitory IκB-like function towards NFκB, resulting in NFκB activation and thereby the survival of multiple myeloma cells (Busino et al., 2012; Fukushima et al., 2012). Although these findings explain some of the pro-survival effects of GSK-3, the role of GSK-3 for the regulation of NFκB is likely to be more complex, and, in particular in the context of TNF signaling, is only incompletely understood.

The involvement of GSK-3 in mixed lineage leukemia (MLL) is another indication that GSK-3 activity does not always promote cell death. In this context, GSK-3 has been shown to be required for maintaining the transformation state of MLL, which could be prevented by loss or inhibition of GSK-3, and, interestingly, also by activation of AKT (Wang et al., 2008). Thus, GSK-3 clearly has functions for cell survival, which are, at least in part, mediated by NFκB.

Conclusions

GSK-3 is involved in a number of signaling pathways and has pleiotropic effects in various cellular processes. Likewise, the effect of GSK-3 on the regulation on cell death is heterogeneous, and its role in apoptosis depends on the cellular or signaling context. In the context of p53 signaling, as well as loss of growth factor signals, which activates the intrinsic apoptosis pathway, there is accumulating evidence that GSK-3 promotes cell death. Here, the inhibition of GSK-3 activity, owing to its N-terminal phosphorylation, is an important contribution to the function of the PI3K–AKT pathway in mediating cell survival, as GSK-3 activity directly or indirectly regulates the activity of different Bcl-2 family proteins, such as Puma, Mcl-1 and Bax. Thus, limiting amounts of growth factor, or loss of integrin signaling upon cell detachment, might promote, or at least sensitize, cells towards death by unleashing the pro-apoptotic activities of GSK-3. However, although the majority of studies have focused on the pro-apoptotic role of GSK-3, the observed TNF-induced embryonic lethality in GSK-3β-knockout mice points to a protective role of GSK-3, and accumulating data demonstrate that GSK-3 promotes pro-survival NFκB signaling. In light of these findings, the use of GSK-3 inhibitors for therapeutic approaches requires careful consideration of all their effects on cell death and survival.

Competing interests

The authors declare no competing interests.

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