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Review

Glycogen synthase kinase 3: A key regulator of cellular fate

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Abstract. The serine/threonine kinase glycogen synthase kinase-3 (GSK-3) was initially identified as a key regulator of insulin-dependent glycogen synthesis. GSK-3 was subsequently shown to function in a wide range of cellular processes including differentiation, growth, motility and apoptosis. Aberrant regulation of GSK-3 has been implicated in a range of human pathologies including Alzheimer's disease, non-insulin-dependent diabetes mellitus (NIDDM) and cancer. As a consequence, the regulation of GSK-3 and the therapeutic potential of GSK-3 inhibitors have become key areas of investigation. This review will focus on the mechanisms of GSK-3 regulation, with emphasis on modulation by upstream signals, control of substrate specificity and GSK-3 localisation. The details of these mechanisms will be discussed in the context of specific signalling pathways.

Keywords. Glycogen synthase kinase-3 (GSK-3), mechanism of action, inhibitors, signalling, disease, kinase, regulation.

Introduction

GSK-3 is a multi-functional kinase that performs a role in several signalling pathways involved in the regulation of cell fate, including Wnt and Hedgehog signal transduction, protein synthesis, glycogen metabolism, mitosis and apoptosis. GSK-3 was originally identified in mammals [1, 2], and homologues have been found in all eukaryotes. These homologues share a significant degree of sequence homology; species as distant as humans and *Dictyostelium* share over 80% similarity within their kinase domains [3] (Fig. 1). Protein function is also well conserved between GSK-3 homologues: mammalian *GSK-3β* under the control of the GAPDH promoter can rescue a temperature sensitivity defect in *Saccharomyces cerevisiae* cells in which all four GSK-3 homologues have been deleted [4].

Two GSK-3 isoforms encoded by distinct genes have been identified in mammals: GSK-3α (51 kDa) and GSK-3β (47 kDa). Both proteins are widely expressed, with particularly high levels in the brain [5]. The α and β isoforms share over 98% identity within their kinase domains but differ in their N-termini [6]. Despite a high degree of similarity and functional overlap, these isoforms may not be redundant *in vivo*; *GSK-3β*-null mice reportedly die during embryogenesis (between E13.5 and E14.5) as a result of liver degeneration caused by widespread hepatocyte apoptosis [7]. This phenotype is similar to that observed in *Rel-A*- [8] and *IkB kinase-2*- [9] null mice and was thought to be indicative of a role for GSK-3β in the NFκB signalling pathway. Recently, however, *GSK-3β*-null mice were generated in a different genetic background, and while the authors reported that these mice died perinatally, they did not exhibit

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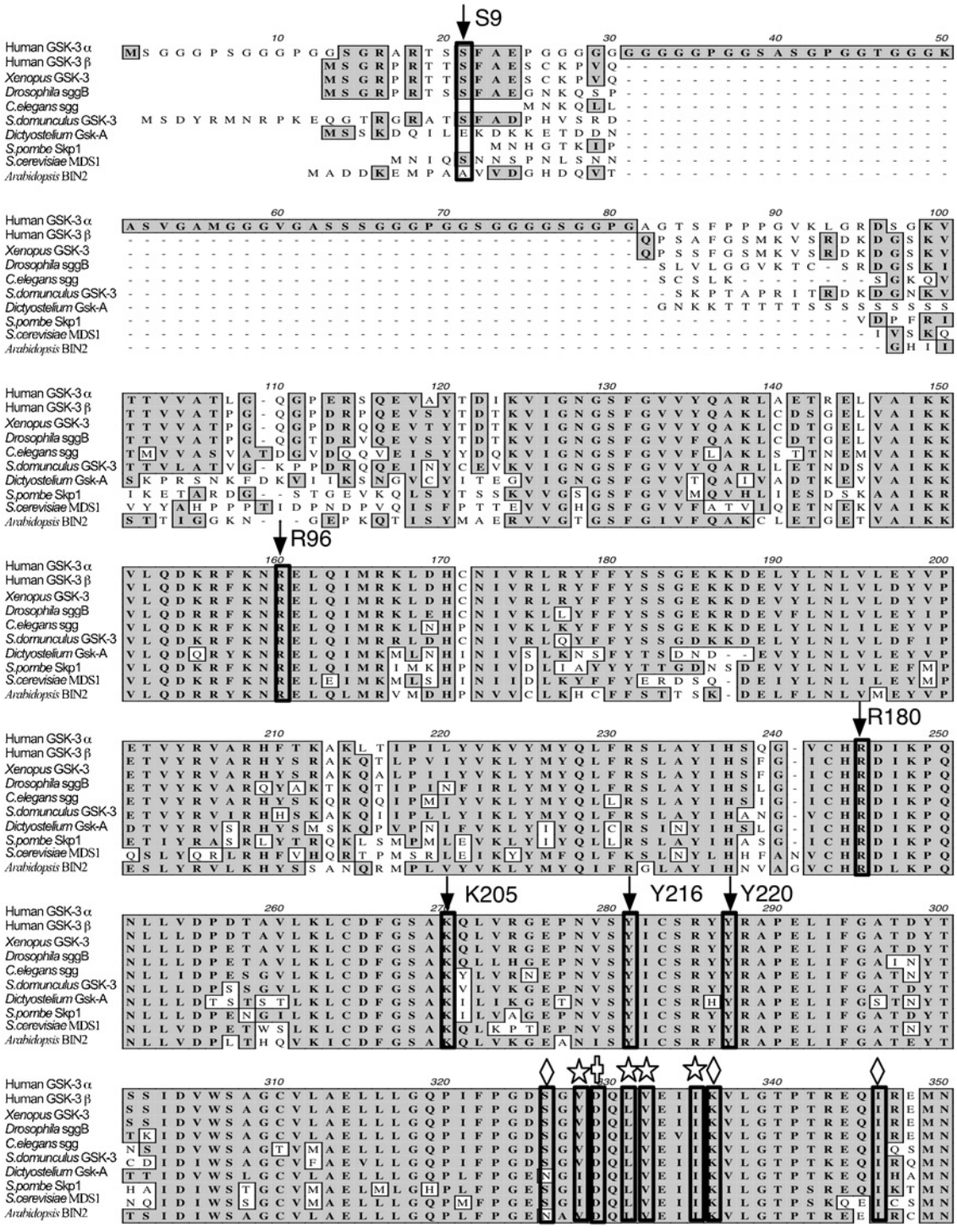
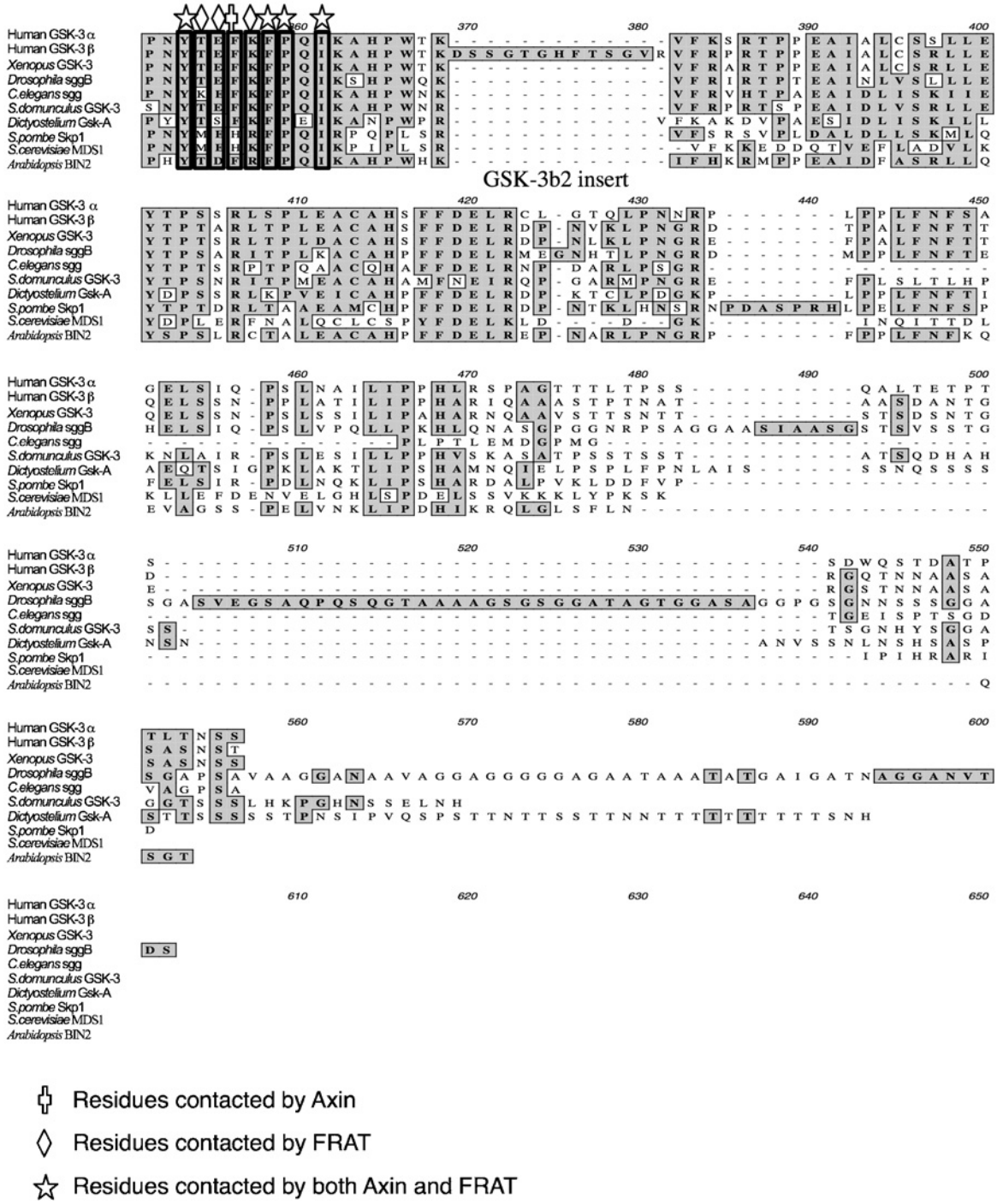


Figure 1. Alignment of GSK-3 proteins. All sequences were obtained from the NCBI database.

liver degeneration [10]. A phenotype for *GSK-3α*-null mice has yet to be reported.

GSK-3β can be differentially spliced; Mukai et al. [11] identified a variant containing a 13-amino acid insert within the kinase domain (GSK-3β2) (Figs. 1 and 2) that was preferentially expressed in the brain and had decreased activity towards the microtubule-binding protein Tau. The mechanism by which this insertion alters the biochemical properties of the protein remains unclear. Very few kinases exhibit differential splicing within their catalytic domain. Exceptions to this rule include JNK [12] and MEK5 [13]; splice



variants of these proteins both contain additional amino acids between subdomains IX and X of their catalytic domains [14]. Alternatively spliced JNK [12] exhibits altered activity towards known substrates, while an insert within the same region of MEK5 alters the intracellular localisation of the kinase [13]. The site of the 13-amino acid insertion in GSK-3β lies between subdomains X and XI; GSK-3β is the only known protein to exhibit splicing between these subdomains. This site is remote from the substrate-binding groove, making it unlikely that it would directly interfere with substrate access (Fig. 2). Instead, the insertion may promote or interfere with GSK-3-scaffold interactions (as discussed below) or indirectly alter substrate binding through induced conformational changes.

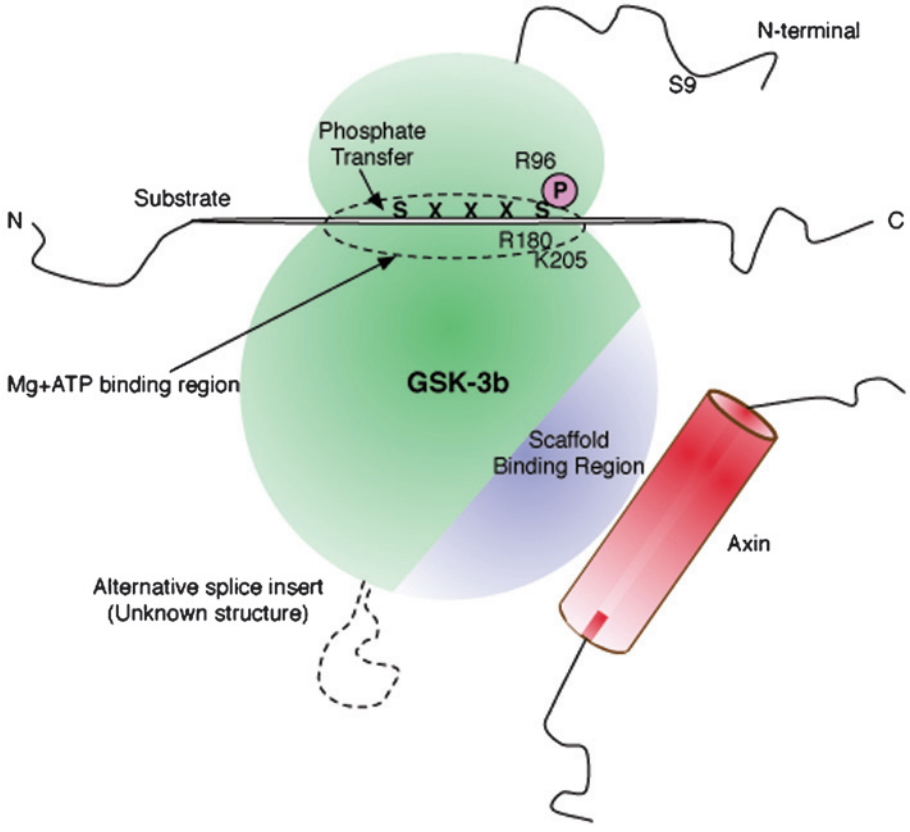


Figure 2. Schematic representation of GSK-3β highlighting key residues involved in primed substrate recognition (R96, R180 and K205) and auto inhibition (S9). The scaffold-binding region (SBR) is highlighted in blue; this region interacts with scaffolding proteins including Axin (shown in red) and FRAT.

A second splice variant of *GSK-3β* lacks exon 10 and is expressed most prominently in the CNS [15]. Exon 10 of *GSK-3β* encodes a region outside the catalytic domain and is poorly conserved between species and between α and β isoforms. The activity of this variant remains to be characterised.

GSK-3 substrates

GSK-3 demonstrates a preference for pre-phosphorylated (primed) substrates, recognizing the consensus sequence S/T-X-X-X-Phospho-S/T, where the first S/T residue is the target for GSK-3 phosphorylation, 'X' represents any amino acid and the S/T C-terminal to the GSK-3 target residue has undergone priming [16]. The relative activity of GSK-3 towards individual substrates differs considerably. However, the requirement for priming of substrates is observed across a range of substrates. Priming decreases the K_m of the substrate. For example, prior phosphorylation of the GSK-3 substrate β-catenin by casein kinase 1 at S45 decreased the K_m 30-fold [17].

The crystal structure of GSK-3β has been elucidated [18–20] and provides insight into the mechanism by which phospho-primed substrates are recognised. The residues R96, R180 and K205 are located within close proximity of each other on the surface of the protein and create a region of positive charge. This region was proposed to interact with the primed phospho-S/T residue and to align the substrate within the substrate-binding groove such that the kinase activation loop and catalytic residues are optimally orientated [19]. Consistent with this proposal, mutation of substrate-priming sites dramatically reduced GSK-3 phosphorylation [21, 22], while a corresponding mutation in GSK-3β that substituted the positive charge of R96 for an alanine residue dramatically reduced the ability of the protein to phosphorylate its primed targets [22, 23]. The importance of priming phosphorylation for GSK-3 function is supported by the observation that the key residues (R96, R180 and K205) are found in all GSK-3 homologues examined (Fig. 1).

Specific substrates are primed for GSK-3 phosphorylation by different priming kinases, whose activity can be regulated in a pathway-specific manner. Consequently, the affinity of GSK-3 towards specific substrates can be controlled by the availability and activity of the priming kinase for a given substrate. For example, the collapsin response mediator proteins CRMP2 and CRMP4 were both identified as substrates for GSK-3 (as discussed later). CRMP2 was found to be primed by Cdk5 alone, whereas either Cdk5 or DYRK2 was able to prime CRMP4 [24].

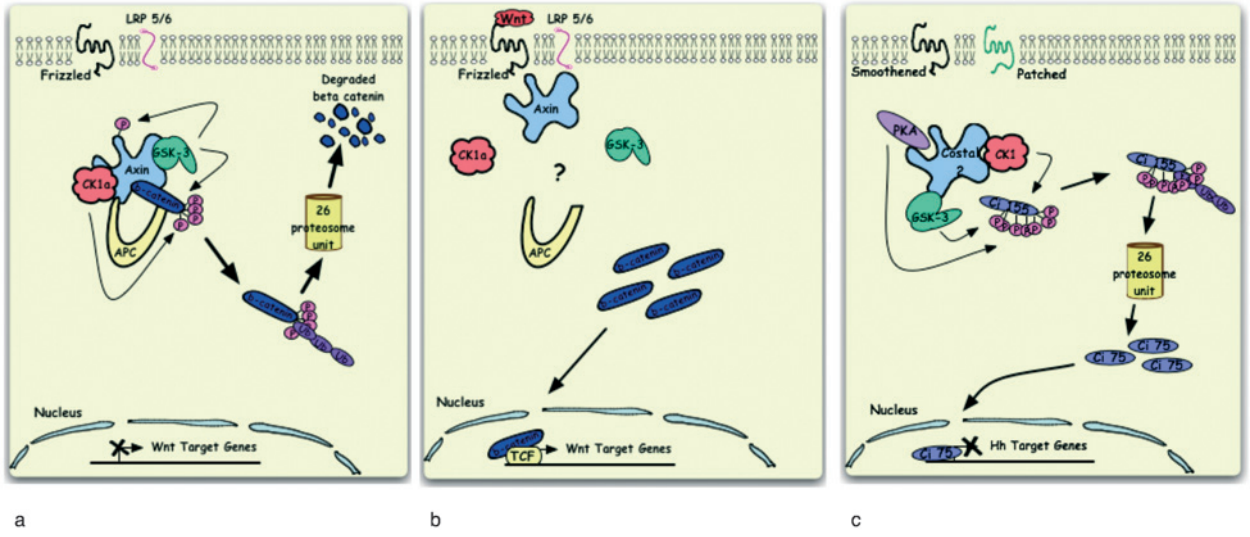


Figure 3. Schematic representation of Wnt and Hedgehog signal transduction. (*a*) In the absence of a Wnt ligand, the destruction complex (consisting minimally of Axin, GSK-3, APC and CK1α) mediates the phosphorylation of β-catenin. Phospho-β-catenin is then ubiquitinated and undergoes proteolysis. (*b*) In the presence of a Wnt ligand, the destruction complex fails to target β-catenin for degradation, although it is not clear whether this requires physical dissociation of the complex. (*c*) In the absence of a Hedgehog (Hh) ligand, it has been proposed that a multi-protein complex containing Costal 2, CK1, PKA and GSK-3 may form to phosphorylate cubitus interruptus (Ci155) [86]. Phospho-Ci155 is subsequently targeted for ubiquitination and partial proteolysis. Truncated Ci75 acts as a transcriptional repressor of Hh target genes.

Inhibition of both Cdk5 and DYRK2 prevented phosphorylation of both CRMP isoforms, whereas only CRMP2 phosphorylation was affected in *Cdk5*$^{-/-}$ cells [24], indicating that GSK-3 phosphorylation of these isoforms can be differentially regulated in response to cellular cues.

A number of proteins have been suggested to be phosphorylated by GSK-3 without prior priming phosphorylation. However, stoichiometric phosphorylation of an un-primed substrate has only been demonstrated for the microtubule-binding protein Tau [25]: recombinant Tau, expressed and purified from *E.coli*, was stoichiometrically phosphorylated at S396 by GSK-3β. Substoichiometric phosphorylation at S46, T50, S202, T205 and S404 was also reported [25]. In the absence of phospho-substrate contacts with R96, R180 and K205, the interactions required to facilitate Tau phosphorylation must be provided through alternative means. As Tau is itself a large protein (ranging from 50–67 kDa in weight), it is conceivable that Tau and GSK-3 may interact within regions other than the catalytic domain and that these interactions promote the phosphorylation of S396.

Inhibitory phosphorylation of GSK-3

The kinase activity of GSK-3 is inhibited through phosphorylation of a serine residue located at the N-terminus of the protein (S21 and S9 of GSK-3α and GSK-3β, respectively) [26, 27]. Dajani et al. [19] demonstrated that a phospho-peptide based on the N-terminus of GSK-3β acted as a competitive inhibitor towards primed GSK-3 substrates. This led to the suggestion that the phosphorylated N-terminus of GSK-3 auto-inhibits its activity by looping back into its own active site. The phospho-serine would interact with the region of positive charge generated by R96, R180 and K205, mimicking the primed residue of a GSK-3 substrate. Consistent with this mechanism of inhibition, mutation of R96 to an alanine residue abolished the ability of the phosphorylated N-terminal peptide to inhibit GSK-3β activity [23].

A number of kinases have been found to phosphorylate S21/9 of GSK-3α and GSK-3β in the context of specific signalling pathways: PKB targets GSK-3 in response to insulin signalling [28], PKA targets GSK-3 in response to increases in cAMP levels [29], p90^{RSK}/MAPKAP kinase-1 phosphorylates GSK-3 following activation by EGF or PDGF [27, 30] and p70 ribosomal S6 kinase (p70^{S6K}) phosphorylates GSK-3 in response to stimulation by insulin and other growth factors [26]. Phospho-S21/9 autoinhibition was also suggested to be involved in the regulation of GSK-3 activity during Wnt signalling [31, 32] and in the control of microtubule stability [33, 34] (see below for detailed discussions of these functions). It was therefore surprising that homozygous knock-in mice in which both S21 and S9 of GSK-3α and GSK-3β were replaced with alanine residues were viable and

healthy [35]. Glycogen metabolism was partially disrupted in these mice (discussed below). However, McManus et al. [35] reported no other obvious phenotypes and specifically demonstrated that Wnt signalling was not affected. Analogous studies in *Drosophila* showed that the equivalent S9A pseudo-substrate mutation did not interfere with insulin/PI3K-dependent growth regulation, although a S9E mutation showed signs of mild Wnt pathway deregulation [36]. It is therefore tempting to speculate that, with the exception of glycogen metabolism, S21/9 phosphorylation is a redundant form of GSK-3 regulation that can be compensated for by other mechanisms. Alternatively, S21/9 phosphorylation of GSK-3 may perform a subtle role in signalling pathways that has not yet been revealed by phenotypic analyses. The observation that S9 of GSK-3β is only conserved in metazoans (excluding *Caenorhabditis elegans*) further supports the idea that this is a recent adaptation and perhaps a less essential mechanism of GSK-3 regulation.

Activating phosphorylation of GSK-3

GSK-3 is a member of the CMGC family of protein kinases. This group consists of cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinases (GSKs) and CDK-like kinases (CLKs) [14, 37]. The kinase activity of several CMGC family members, including MAPK2 (also known as ERK2), is dependent upon the phosphorylation of tyrosine (Y185 in MAPK2) and threonine (T183 in MAPK2) residues located within the activation loop of the protein [38]. Phosphorylation by MEK was required to bring the activation loop into an active conformation for substrate binding and resulted in a 100- to 500-fold increase in activity [38]. Hughes et al. [39] investigated the possibility that GSK-3β activity is regulated in a similar fashion; Y216 was identified as being phosphorylated *in vivo*, and phosphorylation of this residue was later shown to be associated with a 5-fold increase in K_{cat} [19, 39]. In place of the phospho-T183 located in the activation loop of MAPK2, GSK-3β has a valine residue (V214). Structural comparisons suggest that the primed phosphate of GSK-3β's substrate would occupy a 3-dimensional location equivalent to the phosphate of MAPK2's T183. This observation further suggests that the primed substrate of GSK-3 may functionally substitute for the activating changes induced by MEK phosphorylation of T183 in MAPK2.

The conformational change associated with Y216 phosphorylation in GSK-3β was minimal, and the un-phosphorylated protein was active [19]. The requirement for tyrosine phosphorylation was not observed in *Xenopus* assays, where both wild-type and Y216F mutant GSK-3β were capable of inducing ectopic cement gland formation. Embryos injected with mRNA encoding kinase-dead, truncated forms of GSK-3β developed normally, indicating that the Y216F mutant was sufficiently active to target its substrates *in vivo* [40]. In contrast, expression of the equivalent Y216F mutation from a minigene in *Drosophila* embryos was unable to rescue endogenous shaggy (GSK-3) function [36].

The underlying mechanisms responsible for regulating tyrosine phosphorylation of GSK-3 remain controversial. Recombinant GSK-3β expressed and purified from *E. coli* was phosphorylated on Y216 and underwent further phosphorylation upon incubation with Mg^{2+}ATP [25, 39, 41]. However, this phosphorylation was not shown to be stoichiometric. Lochhead et al. [42] recently proposed that GSK-3 autophosphorylation represents a transient capacity, specific to a partially folded state, for GSK-3 to target its own tyrosine residues. However, it remains unclear whether GSK-3 autophosphorylation is an intramolecular rather than intermolecular event.

Mammalian GSK-3 has also been shown to be phosphorylated by distinct tyrosine kinases *in trans*. For example, in neuronal cells, the tyrosine kinase Pyk2 phosphorylated GSK-3β following stimulation with lysophosphatidic acid (LPA) [43]. The physiological phosphatase(s) that dephosphorylate tyrosine residues within GSK-3 remain to be identified.

In *Dictyostelium*, a role for GSK-3 tyrosine phosphorylation was defined in the regulation of cell differentiation and patterning during development. In the absence of an abundant food source, *Dictyostelium* were found to aggregate, using cAMP as a chemoattractant. The 7-transmembrane receptor cAR3 [44] responded to increased cAMP levels by activating the tyrosine kinase ZAK-1. ZAK-1, in turn, phosphorylated GskA (GSK-3) on Y214 (analogous to Y216 of GSK-3β) as well as Y220 [45, 46]. Mutation of either Y214 or Y220 in GskA reduced kinase activity [45]. The Y220 residue that was phosphorylated by ZAK-1 is conserved in mammalian GSK-3β (Y222) but was not found to be phosphorylated in preparations from insect cells that were catalytically active and contained phospho-Y216 [19].

GSK-3 inhibitors

Inappropriate regulation of GSK-3 has been implicated in the progression of multiple human conditions including Alzheimer's disease, bipolar disorder, non-insulin-dependent diabetes mellitus (NIDDM) and

Table 1. GSK-3 inhibitors.

Inhibitor	Inhibition potency	Mode of Inhibition	Notes	Reference
Beryllium	IC_{50} = 6 μM	Mg and ATP competitor	Also inhibits cdc2	[6]
Bivalent Zinc	IC_{50} = 15 μM	Unknown, does not compete for substrate		[126]
Lithium	Ki = 2 mM	Mg competitor		[6, 54]
Aldisine alkaloids (hymenialdisine)	IC_{50} = 10 nM	ATP competitor	Also inhibits MEKs, CK1 and CDKs	[127, 128]
Aloisines (*e.g.* aloisine A)	IC_{50} = 0.4–85 μM	ATP competitor	Also inhibits CDK1/cyclin B and CDK5	[53]
Anilino maleimides (SB216763, SB415286)	Ki = 10–30 nM	ATP competitor	Does not inhibit a range of other kinases	[129, 130]
Arylpyrazolopyridazines (*e.g.* 6-aryl pyrazole [3,4-b] byridine 4)	IC_{50} = 0.8–150 nM	ATP competitor	Also inhibits CDK2	[131]
Bisindole maleimides (*e.g.* Ro 31–8220, GF 109203x)	IC_{50} = 5–170 nM	ATP competitor	Also inhibits PKC	[132]
Halomethylarylketones (*e.g.* 2-chloroacetyl – 4,5-dichlorothiophene)	IC_{50} = 0.5–75 μM	Unknown, does not compete for ATP or substrate		[133]
Indirubins (6-bromoindirubin-3'-oxime, aka BIO)	IC_{50} = 5–50 nM	ATP competitor	Also inhibits CDKs	[52, 134]
Paullones (alsterpaullone)	IC_{50} = 4–80 nM	ATP competitor	Also inhibits CDKs and mMDH	[135, 136]
Pyrazoloquinoxalines (*e.g.* 3-amino-2-quinoxaline carbo-nitrile)	IC_{50} = 1 μM	ATP competitor	Potent inhibitor of CDKs (IC_{50} = 500 nM)	[137]
Thiadiazolidinones (*e.g.* 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione)	IC_{50} = 2 μM	Unknown, does not compete for ATP or substrate	Does not inhibit CDK1/cyclin B, CK2, PKA or PKC at 100 μM	[138]
Pseudosubstrate peptide	Ki = 0.7 mM	Substrate competitor	Specific	[20]

cancer. As a consequence, several molecular inhibitors have been developed (Table 1). In this review, the mechanisms of action of GSK-3 inhibitors will be discussed. Details concerning the potential medical applications of these inhibitors have been reported elsewhere [47–50].

Four distinct regions of GSK-3 have been targeted for inhibition: the Mg^{2+}ATP-binding domain, a separate magnesium (Mg^{2+})-binding region, the substrate-binding groove and the GSK-3 scaffold-binding region (Fig. 2).

Mg^{2+}ATP binds GSK-3 between the smaller N-terminal, β-sheet-rich lobe and a larger C-terminal lobe that is primarily composed of α-helices. Several inhibitors compete with Mg^{2+}ATP to occupy this site. However, the specificity of these inhibitors towards GSK-3 relative to other kinases varies significantly. For example, 6-bromoindirubin-3'-oxime has an IC_{50} of 5 nM for GSK-3 but an IC_{50} of 10 μM for MAPKK [51], while Aloisine A has an IC_{50} of 1.5 μM for GSK-3β but an IC_{50} of 0.15 μM for CDK1 [52]. A full inhibitor/kinase activity profile for putative GSK-3-specific inhibitors is not currently available.

The metal ions beryllium and lithium inhibit GSK-3 activity through mechanisms that are not fully understood at the structural level. Beryllium was shown to compete with both ATP and Mg^{2+}, while lithium competed only with Mg^{2+} [53, 54]. Further competition studies with these ions revealed that GSK-3 contains two distinct Mg^{2+}-binding sites that are sensitive to beryllium, only one of which is also sensitive to lithium. The lithium-insensitive Mg^{2+} binding site binds the Mg:ATP complex [54]. The site of the second Mg^{2+} binding domain has not been identified at the molecular level.

A third mechanism that has been used to inhibit GSK-3 activity in cultured cells involves cell-permeable, phosphorylated substrate-competitive peptides [55]. Rather like the phospho-S9 peptide (see above), these inhibitors are thought to interact with the phospho-recognition motif comprising R96, R180 and K205 to prevent substrate access to the active site.

The scaffold-binding region (SBR) is a region, distinct from the substrate-binding groove, where proteins bind directly to GSK-3 [56] and enhance activity towards specific substrates. For example, in the case of the canonical Wnt pathway (discussed below), the scaffolding protein Axin was found to bind GSK-3 within the SBR and directed GSK-3 activity towards β-catenin [57]. At least one other protein (FRAT; frequently rearranged in advanced T cell lymphocytes) binds to the SBR of GSK-3. Peptides containing GSK-3-interacting domains (GIDs) block the interaction between Axin and GSK-3 and prevent β-catenin phosphorylation [18, 58]. The inhibitory effect of GID peptides was thought to be substrate-selective, since FRATide (a peptide based on the GID region of FRAT) interfered with GSK-3-directed phosphorylation of Tau but did not affect glycogen synthase phosphorylation [58]. The SBR is highly conserved between species as distant as humans and Arabidopsis (Fig. 1); furthermore, mutation of the SBR in *Dictyostelium* GskA resulted in cell motility defects [56], suggesting that the region interacts with several yet-to-be-identified partners.

Regulation of glycogen synthesis

GSK-3 was originally identified for its role as a negative regulator of glycogen synthesis [1] (for a review see [59]). In the absence of insulin, the metabolic enzyme glycogen synthase (GS) was phosphorylated by casein kinase II (CK2) at S657 [59]. This primed GS for sequential phosphorylation at S653, S649, S645 and S641 by GSK-3 and inhibited GS activity [2, 16]. Increases in blood glucose led to the release of insulin, which in turn initiated a signalling cascade involving the insulin receptor (IR), the insulin receptor substrate (IRS) and the activation of both phosphoinositide 3 kinase (PI3K) and protein kinase B (PKB) [60]. Active PKB was found to inhibit GSK-3 by phosphorylating S21 and S9 of the GSK-3α and GSK-3β isoforms, respectively [28]. Inhibition of GSK-3 led to the accumulation of active GS and the subsequent assembly of UDP-glucose into glycogen. A recent study by McManus et al. [35] demonstrated that autoinhibition of GSK-3 activity is required for the activation of GS, since GS activity was dramatically lower in S21A GSK-3α/S9A GSK-3β double knock-in mice. Surprisingly, inhibition of GSK-3β alone was determined to be the principal method by which insulin activated GS. However, the mice generated in this study were not diabetic, and levels of muscle glycogen stores were comparable to those of wild-type mice, indicating that other pathways cooperate in the regulation of glycogen stores.

Regulation of the canonical Wnt pathway by GSK-3

The Wnt signal transduction pathway has been shown to direct many differentiation events during embryonic development and to regulate stem cell fate in adult organisms (for a review see [61]). In the 'canonical' Wnt signalling pathway, Wnt ligands were shown to stabilise levels of the transcriptional activator β-catenin. Stable β-catenin was shown to complex with TCF/LEF transcription factors and initiate transcription of Wnt target genes. GSK-3 has been shown to phosphorylate several intracellular components of the Wnt signal transduction pathway; the most well-characterized of these is β-catenin. In the absence of Wnt ligands, β-catenin was shown to be phosphorylated at S45 by casein kinase 1α (CK1α) [22, 62], which primed it for sequential GSK-3 phosphorylation at T41, S37 and S33 [63, 64]. Phosphorylated β-catenin was recognised by the F-box protein βTrCP, a component of the E3 ubiquitin ligase complex, and targeted for degradation via polyubiquitination and proteosomal degradation [65].

β-catenin alone was found to be a poor substrate for GSK-3 *in vitro*. Efficient phosphorylation of β-catenin required the formation of a multi-protein complex (also known as the 'destruction complex') that minimally comprised the scaffolding protein Axin, adenomatous polyposis coli (APC), CK1α and either GSK-3 isoform [57, 66, 67]. Axin directly bound and mediated interactions between CK1α, GSK-3 and their substrate β-catenin [40, 57, 66, 67]. In the presence of Axin, GSK-3-directed phosphorylation of β-catenin increased 600 000-fold relative to the unscaffolded reaction [17, 57]. Axin itself was a substrate for GSK-3, and phosphorylation decreased its proteolysis and increased β-catenin binding [57, 66, 68]. However, the GSK-3 target residues within Axin have not been directly identified. The tumour suppressor protein APC was shown to bind both Axin and β-catenin and was also found to be a substrate of GSK-3; APC was phosphorylated on a series of 20-amino acid repeats in a CK1- and GSK-3-dependent manner [69, 70]. APC phosphorylation increased its affinity for β-catenin by 300- to 500-fold [71]. Xing et al. [72] proposed that APC phosphorylation regulates the dynamics of β-catenin binding and release during a cycle of β-catenin degradation. In contrast, Ha et al. [73] proposed that the levels of phospho and non-phospho APC function as a rheostat to control the absolute levels of β-catenin in both the presence and absence of Wnt ligands.

In the presence of Wnt ligands, β-catenin turnover was inhibited through a mechanism that was suggested to involve the association of Axin with the low-density lipoprotein (LDL) receptor-related protein (LRP)

[74]. GSK-3 was recently suggested to be the kinase responsible for the phosphorylation of a series of conserved PPPSP motifs within LRP that are essential for Axin binding [75]. However, the PPPSP motifs do not constitute a typical GSK-3 substrate site, since they do not contain a priming residue. In contrast with its role in the turnover complex, this proposed function for GSK-3 would result in the stabilisation of β-catenin.

In *Xenopus* embryos, Wnt signalling was proposed to promote the dissociation of GSK-3 from Axin through the action of FRAT, also know as GBP (GSK-3-binding protein). FRAT/GBP contains a GSK-3 interaction domain (GID) and competes with Axin for binding to the SBR of GSK-3 [58]. Depletion of FRAT/GBP prevented β-catenin/TCF-dependent transcription and the formation of the *Xenopus* primary embryonic axis, while ectopic expression of FRAT/GBP induced axis duplication and the accumulation of β-catenin [76].

However, FRAT homologues were not found in *Drosophila* or *C. elegans*, suggesting that the protein is not a central component of a primitive Wnt pathway. Furthermore, Van Amerongen et al. [77] showed that the three murine FRAT homologues are dispensable for Wnt signal transduction during development. Nonetheless, the dissociation of GSK-3 from Axin may be involved in Wnt signalling in mammals, since GSK-3 was lost from immunoprecipitated Axin complexes within 3 min of Wnt ligand addition to mouse L929 cells through a mechanism involving G-alpha proteins [78].

Although Axin promotes GSK-3 activity toward β-catenin, it is not clear whether it prevents GSK-3 from targeting other substrates or whether it actively prevents β-catenin regulation by other signalling pathways. Axin is present at very low levels in the cell, suggesting that recognition of other substrates may be a minor issue. However, signals that modify GSK-3 (*e.g.* Phospho-S9) might in principle be expected to alter β-catenin regulation, particularly if the pool of GSK-3 associated with Axin is free to exchange with the larger, free cellular pool. Even if a proportion of Axin-associated GSK-3 were phosphorylated on S9, it is not clear that this would alter β-catenin phosphorylation, since both the phospho-S9 N-terminus of GSK-3 and the CK1-primed β-catenin would compete for binding to the substrate-binding groove in what is essentially an intramolecular interaction. Under these circumstances, the relative affinity of each peptide for the substrate-binding groove would determine the kinetics of β-catenin phosphorylation by mass action.

Regulation of the Hedgehog signalling by GSK-3

The Hedgehog (Hh) and Wnt signalling pathways frequently cooperate to direct cellular proliferation, differentiation and pattern formation during embryonic development. GSK-3 plays a role in regulating proteolysis in both the Wnt and Hh pathways, and it has been suggested that this role may reflect the evolution the two pathways from a common, ancestral signal transduction module (reviewed in [79, 80]).

Price et al. [81] demonstrated that in the absence of Hh ligands, the protein cubitus interuptus (Ci) was phosphorylated at S856 and S892 by PKA and subsequently at S852, S884 and S888 by GSK-3 [81, 82]. Phospho-Ci was further phosphorylated by members of the CK1 family, leading to its recognition by βTrCP and proteosomal processing [81, 83]. In contrast with Wnt signalling, in which β-catenin is fully degraded, full-length Ci (Ci155) was shown to be only partially processed, generating a truncated protein (Ci75) that acts as a transcriptional repressor of Hh target genes [84].

In response to an Hh ligand, Ci155 accumulated and functioned as a transcriptional activator [85]. Site-directed mutagenesis of PKA or GSK-3 target residues prevented Ci phosphorylation and processing and led to the accumulation of transcriptionally active full-length Ci [81, 82].

The underlying mechanism regulating GSK-3 activity in response to an Hh ligand remains to be determined. Zhang et al. [86] proposed that activity could be directed by the formation of a multi-protein complex similar to the one required for efficient phosphorylation of β-catenin in Wnt signalling. The protein Costal 2 (Cos2) was proposed to act as the scaffold around which this complex could form. Cos2 interacts with several components of the Hh signalling pathway, including GSK-3 [86], although the specifics of these interactions and their significance remain unclear at present.

Regulation of microtubule dynamics by GSK-3

Neurons are highly polarized cells consisting of a single axon and multiple dendrites that are structurally and functionally distinct [87]. Initially, a nerve cell body will generate lamellipodia at the cell surface, followed by the extension of multiple immature neurites. During this process, one neurite will undergo a transition and initiate a period of rapid growth to form an axon. The remaining immature neurites develop more slowly and become dendrites [88]. Multiple signalling pathways cooperate to bring about

these changes in cell structure, and GSK-3 performs roles in several of these pathways.

The extension of immature neurites requires the assembly of a stable microtubule infrastructure. Microtubules consist of α- and β-tubulin dimers and cycle between phases of growth and shrinkage (reviewed in [89]). Polymerization and stability of microtubules is regulated by a number of microtubule-associated proteins (MAPs). GSK-3 has been shown to phosphorylate several MAPs, including APC [90], Tau [91] and MAP2C [92]. Each of these MAPs influences stability through direct interactions with microtubules, but the mechanisms remain unknown. GSK-3-phosphorylated forms of APC, Tau and MAP2C all exhibit decreased affinity towards microtubules and fail to stabilize them [90–92]. Components of the Wnt signalling pathway may regulate the ability of GSK-3 to phosphorylate these targets; Dishevelled (Dvl) and Axin were both shown to localize to microtubules and influence their stability by inhibiting GSK-3 activity [93], although the mechanism is not known. Unlike the canonical Wnt pathway, this system does not influence gene transcription, indicating that these proteins function in a microtubule-specific pathway [93].

The establishment of the presumptive axon seemingly requires the inhibition of GSK-3 activity; Jiang et al. [94] demonstrated that S9-phosphorylated GSK-3β localized most strongly to the tip of the presumptive axon. Furthermore, treatment with GSK-3 inhibitors, shRNA against GSK-3β or peptides containing a GID led to the formation of multiple axons at the expense of dendrites [94]. Recently, Gärtner et al. [95] reported that the neurons of S21A GSK-3α/S9A GSK-3β double knock-in mice develop normally. The neurons of these mice were still sensitive to GSK-3 inhibitors, indicating that while regulation of GSK-3 activity is important, serine phosphorylation alone is not sufficient to determine the identity of the presumptive axon. It was interesting that peptides containing a GID caused the formation of multiple axons, suggesting that scaffolding proteins may interact with and direct GSK-3 activity within these cells. Neurite retraction also correlated with GSK-3 activity; the inhibitory guidance molecule Semaphorin-3A (Sema3A) was shown to influence the availability of phospho-S9 GSK-3β located at the leading edge of the axon and caused neurite retraction [96]. The GSK-3 substrates CRMP2 and CRMP4 were both identified as downstream targets of Sema3A signalling, and phosphorylation of these proteins reduced their ability to stabilise growth cones via the actin cytoskeleton [97, 98].

Careful regulation of microtubule dynamics is also required during mitosis to orient and assemble a spindle apparatus that accurately segregates chromosomes. Wakefield et al. [33] reported that GSK-3β localises specifically to mitotic microtubules and to the centrosome. Furthermore, inhibition of GSK-3 caused defects in astral microtubule length and in chromosome alignment [33], suggesting that GSK-3 activity is required during mitosis. Loss-of-function studies in a number of systems have shown that cell division can proceed in the absence of GSK-3 but report that the process is more prone to error and delay. In *S. cerevisiae,* deletion of the *GSK-3* homologue *MCK1* increased the rate of mitotic chromosome loss [99], while in *C. elegans,* RNAi depletion of *GSK-3* interfered with mitotic spindle orientation in 4 cell-stage embryos [100]. In *Drosophila* embryos, loss of *GSK-3* (*sgg*) interfered with interactions between spindle tubules and cortical actin [101]. Depletion of other canonical components of the Wnt signalling pathway induced related phenomena. For example, loss of *APC* in *Xenopus* extracts or expression of truncated APC in colorectal tumour cells was associated with defects in mitotic spindle assembly [102, 103]. Similarly, loss of *β-catenin* or expression of *AXIN2* was found to be associated with increases in mitotic errors [63, 104]. These findings, in conjunction with the characterisation of the roles of Dvl and Axin during neurite extension, may be indicative of a central role for components of the Wnt pathway in the regulation of microtubule dynamics.

Regulation of apoptosis by GSK-3

GSK-3 was shown to induce apoptosis in response to a range of stimuli including DNA damage [105], hypoxia [106], removal of NGF [107] or BDNF [108], exposure to staurosporine and heat shock [109]. GSK-3 was found to elicit a response to these stimuli through the regulation of transcription factors (discussed below) including p53 [105] and heat shock factor-1 [110]. Furthermore, it was demonstrated that as part of a pro-apoptotic signalling cascade, GSK-3 phosphorylates and inhibits the translation initiation factor eIF2B, providing a link between the global regulation of protein synthesis and cell survival [111, 112]. Conversely, complete loss of GSK-3β also resulted in apoptosis due to a failure to activate the TNFα/NF-κB survival signalling pathway [7]. These observations make it difficult to predict the outcome of inhibiting GSK-3 in particular systems due to its range of potentially conflicting roles.

Regulation of transcription factors by GSK-3

A plethora of putative GSK-3 substrates have been identified; a large proportion of these are transcription factors and include c-Jun [113, 114], c-Myc [115], CREB [116], NFATc [117, 118] and HSF-1 [110, 119]. GSK-3 negatively regulates the activity of these transcription factors via a number of different mechanisms. For example, N-terminal phosphorylation of NFATc by GSK-3 induces its nuclear export [117], while phosphorylation of c-Myc at S62 by GSK-3 targets the protein for ubiquitin-mediated proteolysis [115]. In contrast, c-Jun, CREB, HSF-1 and NFATc exhibit reduced binding affinity towards their target DNA sequences following GSK-3-directed phosphorylation [118–121].

The ability of GSK-3 to target transcription factors clearly requires multiple levels of regulation to ensure differential targeting of substrates. Specifically, localisation of GSK-3 is fundamental in regulating its activity towards transcription factors in the same way that localization of GSK-3 to mitotic spindles is key to regulating its role in spindle dynamics. Removal of growth factors or treatment with staurosporine causes nuclear accumulation of GSK-3 that presumably alters the access of GSK-3 to nuclear targets. Conversely, both FRAT- and Axin-bound GSK-3 were shown to be exported from the nucleus via a CRM-1-dependent mechanism [122, 123]. Mutations to the SBR of GSK-3 enhanced nuclear accumulation, suggesting that scaffolding proteins including Axin and FRAT are at least in part responsible for GSK-3 nuclear export.

Conclusions

GSK-3 is a highly conserved, multi-faceted protein required for the regulation of a diverse range of cellular processes. GSK-3 itself is subject to stringent regulation via several mechanisms:

- Splice variants of *GSK-3* exhibit differential activity towards specific substrates.
- Direct phosphorylation of an N-terminal serine residue (S9 of GSK-3β) within GSK-3 is associated with inhibition of activity resulting from conformational changes within the structure of the protein.
- Direct phosphorylation of a tyrosine residue (Y216 of GSK-3β) located within the catalytic domain is associated with an increase in kinase activity.
- GSK-3 has a penchant for pre-phosphorylated substrates. Priming kinases are subject to pathway-specific regulation.
- Differential localisation of GSK-3 affects the ability of the kinase to target specific substrates.
- Interactions between GSK-3 and scaffolding proteins direct or inhibit the activity of GSK-3 towards particular substrates.

Despite the identification of multiple mechanisms through which GSK-3 activity can be differentially controlled, it is not clear how GSK-3 reconciles its many roles. Is GSK-3 activity compartmentalized to ensure separation of every role? Or does a single stimulus coordinate multiple responses through regulation of a common pool of GSK-3? Compartmentalization of GSK-3 certainly occurs; insulin treatment did not influence β-catenin phosphorylation, while Wnt ligands did not influence GS activity [124]. There is certainly the potential for cross-talk at the level of GSK-3, since stimulation of skeletal muscle by insulin inhibited GSK-3-mediated phosphorylation of both GS and eIF2B [125]. What is not clear is whether a common pool of GSK-3 is subject to insulin-mediated inhibition or whether GSK-3 activity towards each substrate is independently compartmentalized through, for example, scaffolding.

Dozens of putative GSK-3 substrates have been identified, and more substrates await discovery. Many of the proteins that have been recognised as substrates remain to be fully characterised and demonstrated to be targets for stoichiometric phosphorylation by GSK-3 *in vivo*. The characterisation of GSK-3-substrate interactions may provide insight into how pathway specificity can be so efficiently maintained when a multi-functional protein is involved. Furthermore, binding partners may prove to be attractive targets for pharmacological inhibition of specific pools of GSK-3 activity.

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