REVIEW ARTICLE
GSK3 takes centre stage more than 20 years after its discovery

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Identified originally as a regulator of glycogen metabolism, glycogen synthase kinase-3 (GSK3) is now a well-established component of the Wnt signalling pathway, which is essential for setting up the entire body pattern during embryonic development. It may also play important roles in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics and cell motility by phosphorylating initiation factors, components of the cell-division cycle, transcription factors and proteins involved in microtubule function and cell adhesion. Generation of the mouse knockout of GSK3β, as well as studies in neurons, also suggest an important role in apoptosis. The substrate specificity of GSK3 is unusual in that efficient phosphorylation of many of its substrates requires the presence of another phosphorylated residue optimally located four amino acids C-terminal to the site of GSK3 phosphorylation. Recent experiments, including the elucidation of its three-dimensional structure, have enhanced our understanding of the molecular basis for the unique substrate specificity of GSK3. Insulin and growth factors inhibit GSK3 by triggering its phosphorylation, turning the N-terminus into a pseudosubstrate inhibitor that competes for binding with the 'priming phosphate' of substrates. In contrast, Wnt proteins inhibit GSK3 in a completely different way, by disrupting a multiprotein complex comprising GSK3 and its substrates in the Wnt signalling pathway, which do not appear to require a 'priming phosphate'. These latest findings have generated an enormous amount of interest in the development of drugs that inhibit GSK3 and which may have therapeutic potential for the treatment of diabetes, stroke and Alzheimer's disease.

Key words: cancer, diabetes, insulin, neurodegeneration, Wnt.

DISCOVERY AND CHARACTERIZATION OF GLYCOGEN SYNTHASE KINASE-3 (GSK3)

GSK3 was discovered over 20 years ago as one of several protein kinases that phosphorylated and inactivated glycogen synthase [1], the final enzyme in glycogen biosynthesis. It was subsequently purified to homogeneity from skeletal muscle [2], and molecular cloning revealed that there were two closely related isoforms, GSK3α and GSK3β, which are expressed ubiquitously in mammalian tissues [3,4]. The proteins share 97 % sequence similarity within their kinase catalytic domains, but differ significantly from one another outside this region, with GSK3α possessing an extended N-terminal glycine-rich tail.

REGULATION OF GSK3 BY INSULIN AND GROWTH FACTORS

It was suggested as early as 1978 that the inhibition of GSK3 might underlie the insulin-induced dephosphorylation and activation of glycogen synthase [5]. After 5 years it was shown that insulin stimulates the dephosphorylation of glycogen synthase at the sites phosphorylated by GSK3 [6], and subsequently that insulin inhibits GSK3 acutely [7,8]. We now know that the inhibition of GSK3 by insulin results from its phosphorylation at an N-terminal serine residue (Ser^{21} in GSK3α and Ser^9 in GSK3β) and that this is catalysed by protein kinase B (PKB; also called Akt) [9]. Subsequent work has elucidated the phosphatidylinositide (PI) 3-kinase-dependent pathway by which PKB is activated by insulin (Figure 1; for reviews, see [10,11]). Thus, in response to insulin, the inhibition of GSK3 promotes the dephosphorylation and activation of glycogen synthase, contributing to the stimulation of glycogen synthesis (Figure 1). It is also possible that insulin may stimulate the dephosphorylation of glycogen synthase by activating one of the glycogen-associated forms of protein phosphatase-1, in which the phosphatase catalytic subunit is complexed to one of four different glycogen-targeting subunits ([12]; reviewed in [13]). However, which form of protein phosphatase-1 is involved and the mechanism of activation is unknown [14].

GSK3 also catalyses the phosphorylation and inhibition of eukaryotic protein synthesis initiation factor 2B (eIF2B), thereby inhibiting protein synthesis. Hence insulin, by inhibiting GSK3, stimulates the dephosphorylation and activation of eIF2B, contributing to an increased rate of protein synthesis [7,8]. Insulin may also stimulate the protein phosphatase(s) that dephosphorylate(s) eIF2B. The catalytic subunits of protein phosphatases 1 and 2A can dephosphorylate the site on eIF2B that is targeted by GSK3 *in vitro* (C. Proud, L. E. Campbell and X. Wang, personal communication), but whether these or other phosphatases dephosphorylate eIF2B *in vivo* is unknown.
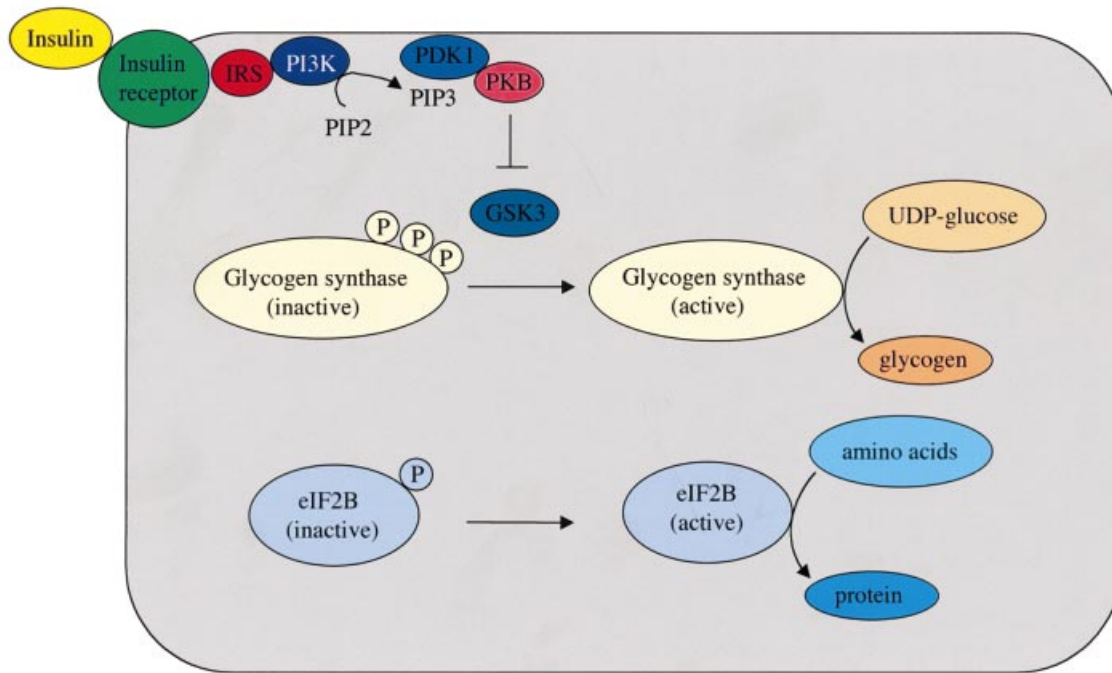
Figure 1   Insulin stimulates glycogen and protein synthesis via the inhibition of GSK3

The binding of insulin (yellow) to its receptor (green) in liver, adipose tissue and muscle, triggers the phosphorylation of IRS proteins (red) and their recruitment to the plasma membrane. The IRS proteins in turn become tyrosine-phosphorylated, recruiting PI 3-kinase (PI3K, purple) to the membrane, where it produces the second messenger PtdIns(3,4,5)P_3 (PIP3). This molecule binds to PDK1 (dark blue) and PKB (pink), co-localizing them at the plasma membrane and allowing the former to activate the latter. Active PKB then inhibits GSK3 (dull blue) by phosphorylating Ser[21] (GSK3α) and Ser[9] (GSK3β). As a result, the residues on glycogen synthase (pale yellow) and the ϵ-subunit of eukaryotic initiation factor 2B (eIF2B, lilac) that are targeted by GSK3 undergo a partial dephosphorylation, thereby increasing their activity and hence stimulating glycogen (orange) and protein (blue, bottom right) synthesis.

GSK3 can also be phosphorylated at Ser[9]/Ser[21] by the most downstream kinase of the classical mitogen-activated protein kinase (MAPK) cascade, called MAPK-activated protein kinase-1 (MAPKAP-K1, also called RSK). This provides a route for the inhibition of GSK3 by growth factors and other signals that activate this pathway. However, while insulin inhibits GSK3 via the PI 3-kinase-dependent/PKB pathway, and signals such as tumour-promoting phorbol esters inhibit GSK3 via the classical MAPK cascade, other growth factors, such as epidermal growth factor (EGF), can inhibit GSK3 by both pathways (Figure 2) [15].

Another protein kinase that phosphorylates GSK3 at Ser[9]/ Ser[21] *in vitro* is p70 ribosomal S6 kinase-1 (S6K1) [16], and recent evidence suggests that this may underlie the inhibition of GSK3 induced by amino acids in human myocytes. Thus the immunosuppressant drug rapamycin, which inactivates mammalian target of rapamycin (mTOR), a protein kinase required for the activation of S6K1, suppresses the inhibition of GSK3 induced by amino acids (Figure 2) [17]. The phosphorylation of GSK3 at Ser[9]/Ser[21] can also be induced by incubating cells with cAMP-elevating agents or cell-permeant cAMP analogues [18,19], although the physiological relevance is not yet clear.

GSK3 AS A KEY COMPONENT OF THE Wnt SIGNALLING PATHWAY

Many years after its discovery, GSK3 re-emerged as a kinase essential for the specification of cell fates in the early embryo, in studies conducted in *Drosophila* and *Xenopus*. Genetic analyses in *Drosophila* placed the GSK3 homologue, called shaggy or

zeste-white3, in a signalling pathway switched on by a secreted glycoprotein called wingless. This ligand sets up the pattern of segment polarity in *Drosophila*, which is characterized by alternating regions of naked bands and denticle belts. Thus loss-of-function mutations in shaggy led to a loss of denticle belts, while loss-of-function of wingless or armadillo (the *Drosophila* homologue of β-catenin) led to the opposite phenotype, with the loss of naked cuticle [20]. This implied that wingless suppressed GSK3 activity in this pathway. This was followed by studies in *Xenopus* that demonstrated that Li$^+$ induced the duplication of the dorsal axis [21] – the same phenotype observed when a dominant-negative version of GSK3 is expressed in the ventral side of the embryo [22–24]. The reason for this became clear when it was discovered that Li$^+$ ions inhibit GSK3 [25,26].

The canonical Wnt signalling pathway is highly conserved between *Drosophila*, *Xenopus* and vertebrates (Figure 3). In the absence of a Wnt signal, active GSK3 is present in a multiprotein complex that targets β-catenin for degradation via ubiquitin-mediated degradation [27]. The phosphorylation of β-catenin by GSK3 at a series of N-terminal serine residues is greatly enhanced by the presence of Axin, which acts as a scaffold by binding to several components of the complex, including GSK3, β-catenin and the product of the adenomatous polyposis coli (APC) gene [28–32]. Axin and APC are also substrates of GSK3. The phosphorylation of Axin by GSK3 stabilizes the protein [33], whereas the phosphorylation of APC by GSK3 appears to facilitate the interaction of β-catenin with APC [34]. In contrast, the phosphorylation of β-catenin by GSK3 creates a recognition site for binding of the F-box protein, βTrCP (or Slimb in *Drosophila*), which acts as the recognition subunit for the E3
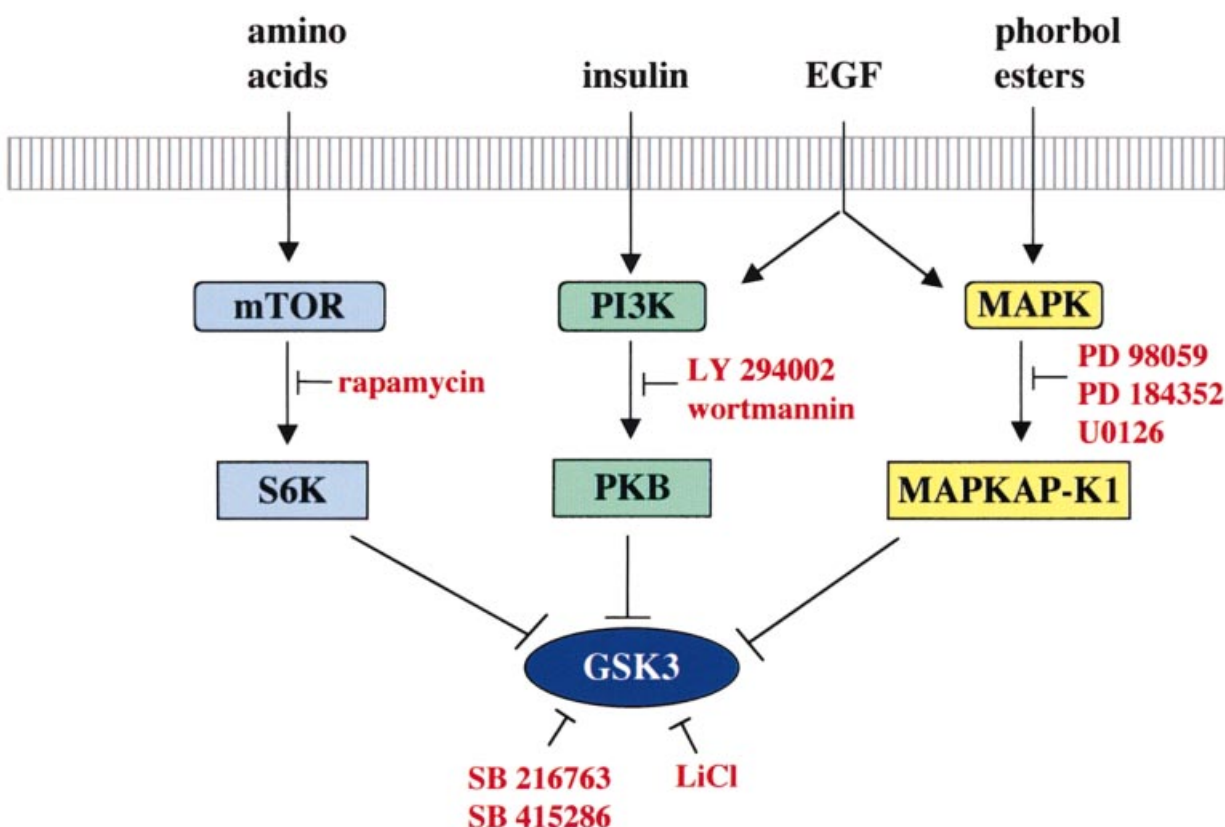
Figure 2   Several signalling pathways inhibit GSK3

GSK3 can be inhibited via several signal-transduction pathways, namely the PI 3-kinase–PKB pathway (green), the classical MAPK (yellow) cascade, where MAPKAP-K1 (also called RSK) inhibits GSK3, and the mTOR pathway, where p70 S6 kinase (S6K, lilac) inhibits GSK3 (purple). Insulin exerts its effects via the PI3K–PKB pathway, tumour-promoting phorbol esters via the classical MAPK cascade and amino acids via mTOR. However, some signals, such as EGF, can use more than one pathway to inhibit GSK3. The cell-permeant inhibitors indicated in the figure block one pathway selectively and have been critical in establishing the roles of these pathways in the regulation of GSK3 activity. Li^+ ions, which inhibit GSK3 relatively selectively *in vitro*, have been useful in identifying potential roles for GSK3 in cells. More potent cell-permeant inhibitors of GSK3, such as SB 216763 and SB 415286, have been developed recently.

ubiquitin ligase [35]. This causes β-catenin to be degraded by the proteasome and thus the transcriptional targets of Wnt remain off.

Surprisingly, the residues in Axin, β-catenin and APC that are phosphorylated by GSK3 have never been identified directly *in vitro* or *in vivo*, and dephosphorylation has mostly been assessed indirectly by inference from increases in the cytosolic levels of β-catenin. Indeed one group [36] reported that the sites phosphorylated by GSK3 *in vivo* differed from those found *in vitro* [28,33]. The sites in the N-terminus of β-catenin phosphorylated by GSK3 have never been mapped precisely and, although fragments of APC are phosphorylated by GSK3 *in vitro*, and this correlates with degradation of β-catenin, the sites have also not been mapped [34,37].

Following the binding of Wnts to their receptors, the activity of GSK3 towards Axin and β-catenin is prevented by a mechanism that is discussed in greater detail later. As a result, β-catenin becomes dephosphorylated and is no longer targeted for degradation. It accumulates in the cytoplasm and nucleus, where it activates the transcription of Wnt target genes by binding to transcription factors of the TCF/LEF (T-cell factor/lymphoid enhancer-binding factor-1) family (reviewed in [38] and [39]). In this complex, β-catenin provides the transactivation domain, while DNA binding specificity is provided by the individual TCF/LEF family members to which it is bound. Several excellent

reviews on Wnt signalling pathways have recently been published, to which readers are referred for more detailed accounts [40–42].

THE SUBSTRATE SPECIFICITY OF GSK3 AND ITS INHIBITION BY INSULIN AND GROWTH FACTORS ARE EXPLAINED BY THE EXISTENCE OF A COMMON PHOSPHATE BINDING SITE IN THE CATALYTIC DOMAIN OF GSK3

GSK3 is phylogenetically most closely related to the cyclin-dependent protein kinases (CDKs), such as CDK1 (also called cdc2) and CDK2. However, the specificity of GSK3 is unique in requiring a priming phosphate at $n+4$ (where n is the site of phosphorylation by GSK3) in order to phosphorylate many of its substrates, the optimal consensus site for phosphorylation being Ser/Thr-Xaa-Xaa-Xaa-pSer/pThr (where pSer and pThr are phosphoserine and phosphothreonine respectively and Xaa is any amino acid) [43]. This motif is found in several well-established substrates of GSK3, such as glycogen synthase, the ϵ-subunit of eIF2B, [7,8] and ATP citrate-lyase [44,45]. In the case of glycogen synthase, the C-terminal priming serine residue is phosphorylated by protein kinase CK2 (CK2; formerly 'casein kinase 2'), whereas in eIF2B it may be the dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK1A) [46]. For both of these substrates, the $n+4$ residue is phosphorylated nearly stoichiometrically *in vivo* under basal conditions, because
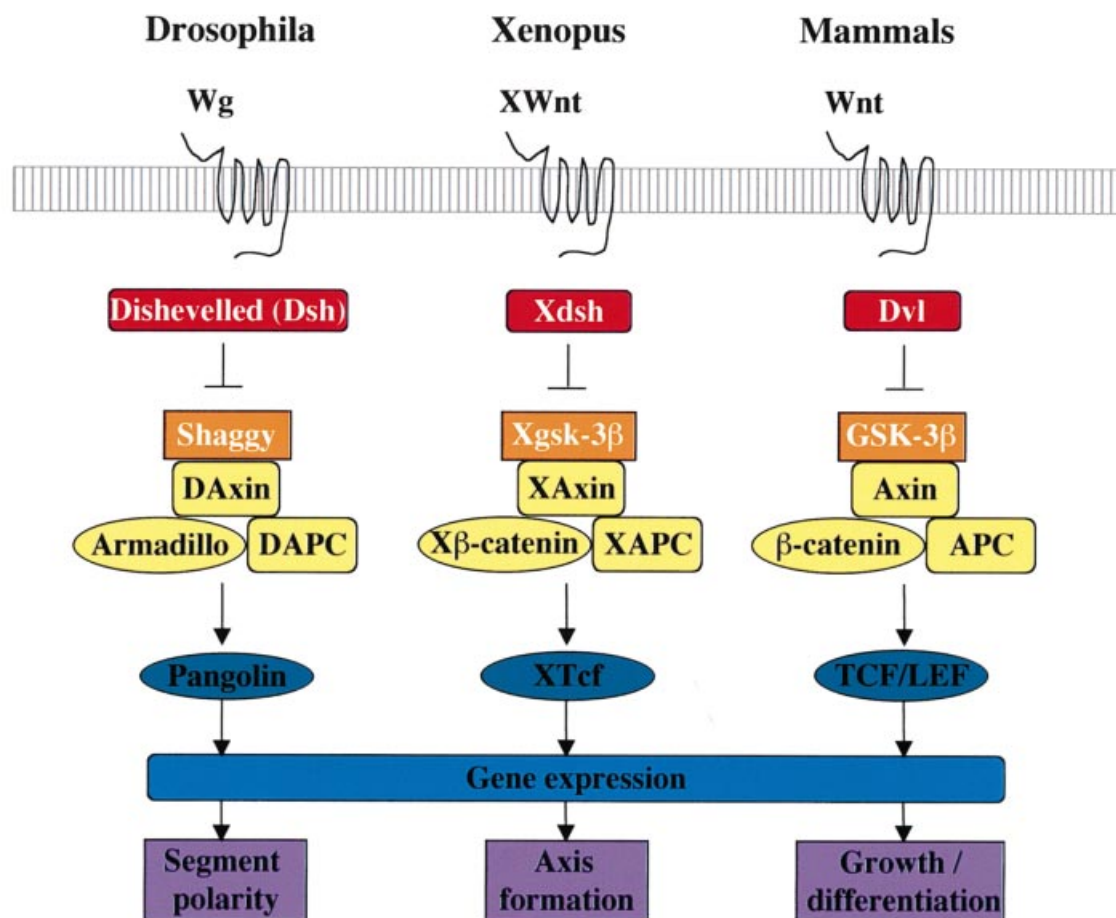
Figure 3   Wnt signalling components are highly conserved between diverse organisms

The Wnt signalling pathway specifies cell fate, proliferation and differentiation in *Drosophila*, *Xenopus* and mammals. It also plays essential roles in adult tissues, particularly in maintaining stem cells in their pluripotent state, for example in the skin and colon. Abbreviations: D, *Drosophila*; X, *Xenopus*; Wg, wingless; Dvl/Dsh, Dishevelled. Modified from [147] with permission © (1999) Elsevier Science.

the priming kinases, CK2 and DYRK1A, are constitutively active. However, there may be other substrates for GSK3 in which the activity of the priming kinase is regulated by extracellular signals; such signals could be identical with, or distinct from, those that regulate GSK3. This would provide a substrate-specific mechanism for signal integration. For example, the transcription factor CREB (cAMP-response-element-binding protein) is activated by the phosphorylation of Ser[133] by cAMP-dependent protein kinase, which has been found to prime the phosphorylation of Ser[129] by GSK3 *in vitro* [47]. If the phosphorylation of Ser[129] occurs *in vivo* (which has yet to be established), this would provide a potential mechanism by which the effects of insulin could antagonize the effects of signals that elevate cAMP.

In a recent study we identified a phosphorylation-dependent docking site for the priming phosphate of substrates that is required for substrate recognition and phosphorylation by GSK3 [48]. Through the use of molecular modelling and mutagenesis, we identified Arg[96] in the small lobe of the kinase catalytic domain as a residue that is critical for interaction with the primed phosphate of substrates. Independently, two other groups solved the three-dimensional structure of GSK3 [49,50] and found that sulphonate (a phosphate analogue present as a component of the

buffer used to purify GSK3) or a phosphate ion was bound to Arg[96], consistent with the importance of this residue in binding the primed phosphate. Both groups also found additional interactions between the sulphonate and phosphate anions and Arg[180] and Lys[205].

GSK3 is one of the few protein kinases that are inactivated by phosphorylation. We therefore wondered whether this unusual property was connected to its unique substrate specificity through the utilization of the same phosphate-binding site occupied by primed substrates. This prediction turned out to be correct, in that the mutation of Arg[96] to Ala not only drastically reduced the rate at which primed substrates were phosphorylated, but also prevented the inhibition of GSK3β resulting from the PKB-mediated phosphorylation of Ser[9]. This suggests that the phosphorylated N-terminus of GSK3 interacts with Arg[96] and acts as a pseudosubstrate to compete for the binding of primed substrates to the phosphate-binding site (shown schematically in Figure 4). That this was indeed the case was established by the finding that the extent of inhibition of GSK3 activity by PKB was diminished as the concentration of the primed substrate was increased [48]. Moreover, the activity of GSK3 could be inhibited specifically by synthetic phosphopeptides corresponding to the N-terminus of GSK3 [48,49].
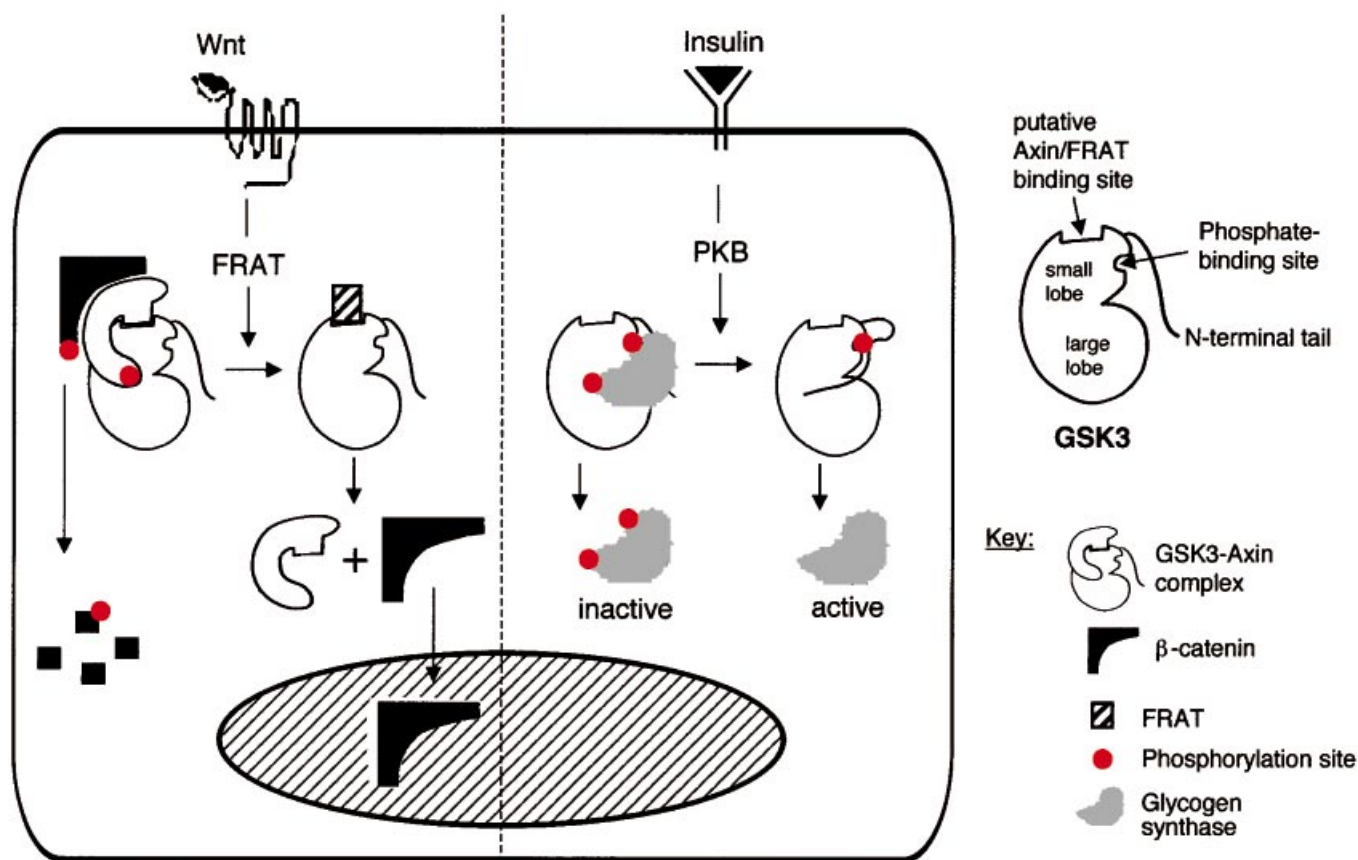
Figure 4   Wnts and insulin inhibit GSK3 by different mechanisms

There appear to be two distinct pools of GSK3 in cells under basal conditions. One is a free pool and the other is part of the Wnt signalling complex, containing Axin, β-catenin and other proteins. In the absence of Wnts, the latter pool of GSK3 phosphorylates Axin and β-catenin, triggering the stabilization of Axin and the degradation of β-catenin. Following the binding of Wnts to their receptors, Axin is displaced from GSK3 as a result of binding of FRAT to GSK3. This leads to the stabilization of β-catenin and its accumulation in the nucleus, where it stimulates the transcription of Wnt target genes. The residues on GSK3 forming the high-affinity interaction site for FRAT and Axin are depicted as being located in the small lobe, since mutation of Leu[128] in the small lobe of GSK3 impairs Axin phosphorylation [11]. However, the binding site for FRAT and Axin has not yet been identified. FRAT binding does not affect the phosphorylation of primed substrates of the insulin signalling pathway. In the absence of insulin, the free pool of GSK3 phosphorylates and inactivates glycogen synthase, inhibiting glycogen synthesis. Following stimulation by insulin, the free pool of GSK3 becomes phosphorylated at Ser[9] (GSK3β) and Ser[21] (GSK3α) by PKB. The phosphorylated N-terminus then inhibits GSK3 by acting as a pseudosubstrate, competing with primed substrates for the phosphate-binding and catalytic sites on GSK3. This leads to the dephosphorylation and activation of glycogen synthase, and hence to the stimulation of glycogen synthesis. GSK3 bound to Axin does not become phosphorylated on Ser[9]/Ser[21] in response to insulin, restricting the effects of insulin to a specific subset of GSK3 substrates. Compounds that target the ATP-binding site inhibit all the substrates of GSK3 and therefore mimic the effects of Wnts as well as insulin. In contrast, compounds that interact with the phosphate-binding site on GSK3 would be predicted to inhibit the phosphorylation of glycogen synthase selectively, without affecting the phosphorylation of Axin and β-catenin.

Our finding that the degree of inhibition depends on substrate concentration (and presumably on substrate affinity as well) has important methodological implications for measurement of the extent of GSK3 inactivation *in vitro*. It may also be of physiological significance, since it implies that the effect of Ser[9]/Ser[21] phosphorylation may vary depending on the substrate. For example, the widely reported 40–50 % reductions in GSK3 activity in response to insulin or growth-factor stimulation [16,51] may not reflect the true extent of inhibition *in vivo*, but simply be a function of the peptide substrate concentrations generally employed in the assay.

Arg[96], Arg[180] and Lys[205] are conserved in all GSK3 homologues identified to date, suggesting conservation of the priming phosphate-binding site and hence the substrate specificity of GSK3 in all organisms. In contrast, Ser[9]/Ser[21], and the PKB consensus sequence surrounding it, are only conserved in GSK3 homologues from mammals, the frog *Xenopus* and the fruitfly *Drosophila*, but not in yeast, higher plants, the cellular

slime mould *Dictyostelium* or the nematode worm *Caenorhabditis elegans*. Interestingly, both *Dictyostelium* and *C. elegans* employ different mechanisms to regulate GSK3, the enzyme becoming activated in response to particular agonists, rather than inhibited, as discussed below (and reviewed in [41]).

REGULATION OF GSK3 ACTIVITY DURING Wnt SIGNALLING

In contrast with glycogen synthase and eIF2B, there is not yet any evidence that the phosphorylation of Axin and β-catenin by GSK3 requires a priming phosphate *in vivo*. Instead the phosphorylation of these proteins may rely on high-affinity interactions in a multiprotein complex with GSK3. Consistent with this scenario, the Arg[96] mutants, which are defective towards primed substrates, phosphorylate Axin and β-catenin similarly to the wild-type enzyme [48]. Moreover, a phosphohexapeptide, derived from the N-terminus of GSK3β, which interacts with the phosphate-binding site and selectively inhibits the phosphoryl-

ation of primed substrates *in vitro*, does not impair the phosphorylation of Axin or β-catenin [48]. This implies that the phosphate-binding site, which is essential for interaction with primed substrates, is not necessary for phosphorylation of substrates of the Wnt signalling pathway.

GSK3 does not become phosphorylated at Ser[9] in response to Wnt signalling [52,53]. Thus the phosphorylation of substrates of GSK3 in the Wnt signalling pathway must be prevented by a different mechanism (Figure 4). One mechanism that has been proposed involves a GSK3-binding protein (GBP), whose mammalian homologues are the FRAT (frequently rearranged in advanced T-cell lymphomas) isoforms. These proteins appear to interact with an essential protein in the pathway called Dishevelled (Figure 3), and together disrupt the interaction of Axin with GSK3, thereby dissociating β-catenin from the complex and preventing its phosphorylation and degradation [54,55]. Another possibility is that the phosphorylation of GSK3 at as-yet-unidentified sites may be involved [52]. For example, one could envisage a phosphorylation event that weakened the interaction of GSK3 with Axin or strengthened the interaction of GSK3 with GBP/FRAT or Dishevelled. Dishevelled is itself reported to become phosphorylated in response to Wnts, and this may facilitate binding to GBP/FRAT and interaction of the complex with GSK3 (Figure 4). CK1 and CK2 are reported to be associated with the Wnt signalling complex and to phosphorylate Dishevelled [56–58]. However, CK1 and CK2 are thought to be constitutively active and evidence that their activity can be increased further by Wnts is lacking. It is therefore possible that Wnt-induced phosphorylation of Dishevelled is catalysed by (an) additional protein kinase(s).

Interestingly, a GSK3-binding peptide derived from FRAT1 (FRATtide) inhibits the phosphorylation of Axin and β-catenin, but does not affect the phosphorylation of primed substrates such as glycogen synthase or eIF2B [59], implying that the substrates of GSK3 in the Wnt signalling pathway may have a distinct binding site on this protein kinase. Consistent with this finding, the overexpression of FRATtide in HEK293 cells induces a robust accumulation of β-catenin, without affecting glycogen synthase activity ([59]; S. Frame, unpublished work). Others have also demonstrated in the same cell type that Wnt-1 increases the cytosolic pool of β-catenin without affecting glycogen synthase activity [53]. Thus the effects of Wnts are restricted to the specific subset of GSK3 substrates required to transduce this developmental signal, allowing other cellular functions of GSK3 to proceed normally.

Insulin and insulin-like growth factor-1 (IGF-1) do not elevate cytoplasmic β-catenin levels or activate β-catenin-dependent gene transcription in HEK293 cells [53]. This can be explained by the finding that the GSK3 bound to the Wnt signalling complex does not become phosphorylated in response to insulin [53]. This suggests that the dephosphorylated N-terminus of GSK3 may be inaccessible to PKB, perhaps because it interacts with Axin, APC, β-catenin or another component of the complex. This prevents insulin from triggering the Wnt signalling pathway, restricting its effects to the GSK3 substrates that control glycogen and protein synthesis. Other growth factors that inhibit GSK3 via the phosphorylation of Ser[9]/Ser[21] have been reported to stabilize β-catenin in some cells, but the involvement of GSK3 under these conditions has not been demonstrated [60–62].

The *Drosophila* homologue of GSK3 is more closely related to mammalian GSK3β than GSK3α and, in transgenic flies, mammalian GSK3β, but not GSK3α, can substitute for shaggy [63]. For this reason it has been assumed that only the β-isoform is involved in Wnt signalling in mammalian cells. However, early embryonic development occurred normally in a mouse 'knock-out' in which the gene encoding GSK3β had been disrupted, suggesting that GSK3α also plays a role in development, or at least that it can functionally substitute for the loss of GSK3β [64]. However, these animals die between E13.5 (embryonic day 13.5) and E14.5 due to liver apoptosis (as discussed below), suggesting at least one distinct biological role for the two isoforms.

PHOSPHORYLATION OF GSK3 ON TYROSINE RESIDUES

In unstimulated tissue-culture cells or rabbit skeletal muscle, GSK3 is phosphorylated on one tyrosine residue (Tyr[216] in GSK3β; Tyr[279] in GSK3α) [65], and the dephosphorylation of this residue, or its mutation to phenylalanine, reduces activity *in vitro* [65]. Tyrosine phosphorylation does not change in response to growth factors [66]. Nevertheless, two recent reports indicate that the phosphorylation of Tyr[216] may increase in neuronal cells after cerebral damage, nerve-growth-factor (NGF) withdrawal or treatment with staurosporine [67], or in response to transient increases in intracellular calcium [68]; this appears to be associated with increased GSK3 activity.

In *Dictyostelium* there is clear evidence that GSK3 is regulated by tyrosine phosphorylation. Binding of cAMP to one of its receptors (cAR3) activates zaphod kinase (ZAK1), which phosphorylates GSK3 on tyrosine, leading to its activation [69]. The precise tyrosine residue(s) on GSK3 have not yet been identified, but Tyr[216] in the activation loop is one candidate. Unfortunately, no mammalian homologue of ZAK1 has been identified, and a homologue does not appear to be present in the human genome.

Bacterially expressed mammalian GSK3β is capable of phosphorylating itself on tyrosine as well as serine and threonine residues, suggesting that GSK3 itself may be responsible for the phosphorylation of Tyr[216] in mammalian cells. In support of this conclusion, we have found that three different catalytically inactive mutants of GSK3 are not phosphorylated at Tyr[216] after their transfection into HEK293 cells, in contrast with the wild-type enzyme (S. Frame, unpublished work).

CRITERIA NEEDED TO ESTABLISH THAT A PROTEIN IS A PHYSIOLOGICAL SUBSTRATE FOR GSK3

The preceding sections have described the roles of GSK3 that are established most firmly. In recent years many more proteins have been proposed as potential substrates for GSK3 (Figure 5) and these will be discussed in the following sections. However, before reviewing these studies, we indicate the criteria that need to be met before a protein can truly be regarded as a physiological substrate for GSK3:

(1) The substrate should be shown, by phosphopeptide mapping and sequence analysis, to be phosphorylated by GSK3 *in vitro* at the same residue(s) that is (are) phosphorylated *in vivo*.

(2) Phosphorylation should be abolished by mutagenesis of this (these) site(s) to (a) non-phosphorylatable residue(s).

(3) The phosphorylation of the endogenous protein in cells should decrease in response to one or more signals known to inhibit GSK3; dephosphorylation should occur with similar kinetics to the inhibition of GSK3.

(4) The endogenous protein should become dephosphorylated at the relevant site(s) *in vivo* when cells are incubated with cell-permeant inhibitors of GSK3.

(5) Phosphorylation of the residues targeted by GSK3 should affect the function of the protein in a manner consistent with physiological effects of the agonist(s) that regulate(s) GSK3 activity.
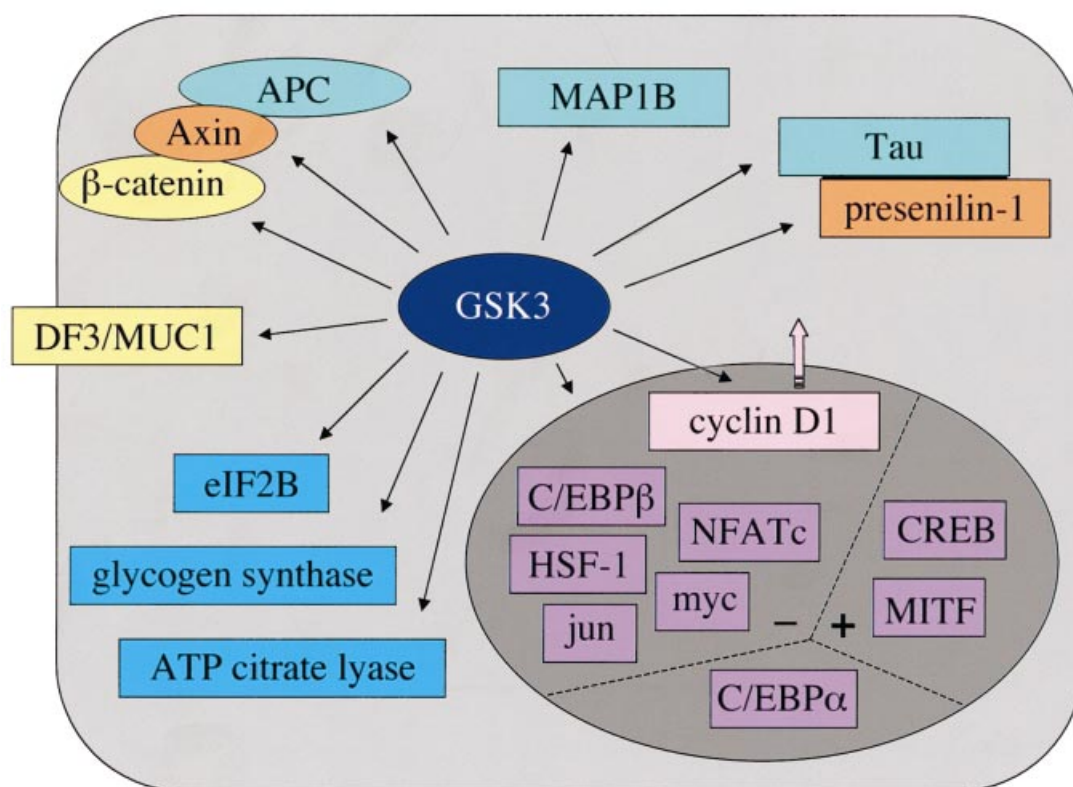
Figure 5   Proposed substrates of GSK3

Putative substrates are colour-coded according to their proposed function in the cell; transcription factors (mauve), enzymes that regulate metabolism (blue), proteins bound to microtubules (turquoise), scaffold proteins (orange), or components of the cell division cycle machinery (pink) or involved in cell adhesion (yellow). The evidence that these proteins are substrates of GSK3 is summarized in Table 1. Transcription factors are subdivided into those that are inhibited ($-$), activated ($+$) or unaffected by the phosphorylation by GSK3. A complex consisting of GSK3, Axin, APC and β-catenin (depicted by ellipses) is critical for regulating embryogenesis. The phosphorylation of cyclin D1 by GSK3, which is thought to occur in the nucleus of the cell, promotes its nuclear export and degradation. Presenilin-1 is reported to act as a scaffold, which facilitates the phosphorylation of tau by GSK3.

(6) Phosphorylation of the protein at the GSK3 site(s) should not occur in cells that carry targeted disruptions of the genes encoding GSK3, and be restored when GSK3 is replaced.

(7) Dephosphorylation of the protein at the GSK3 site(s) should not occur in response to the appropriate signal in cells that do not express one of the protein kinases that lies upstream of GSK3; for example, in PDK1$^{-/-}$ embryonic stem cells, PKB, MAPKAP-K1 and p70 S6 kinase are not activated, and GSK3 is not inhibited, in response to IGF-1 or tumour-promoting phorbol esters ([70]; see also Figure 2).

Table 1 summarizes how well these criteria have been met for proteins proposed to be GSK3 substrates. Surprisingly, few have met the first criterion, the evidence for phosphorylation being based largely on the effects of mutagenesis of the putative phosphorylation sites and on overexpression experiments. Much of the evidence that GSK3 may phosphorylate particular proteins in cells is based on the finding that Li$^+$ affect the function of the protein in a manner consistent with dephosphorylation having occurred. However, in almost every case, Li$^+$ have not yet been shown to promote dephosphorylation of the residues targeted by GSK3. In addition, although Li$^+$ is a relatively specific inhibitor of GSK3, it can also inhibit a few other protein kinases [71]. Small cell-permeant compounds that inhibit GSK3 relatively specifically have recently been developed by Glaxo-SmithKline but, as with other protein kinases, their *in vivo*

specificity will need to be validated. In particular, it will need to be shown that the effects of the drug disappear when a drug-resistant mutant is overexpressed [72,73] or replaces the endogenous wild-type enzyme. Finally, none of the substrates yet meet the sixth or seventh criteria. Thus, even for the substrates that are most well established, the genetic evidence required to prove that they are physiological substrates for GSK3 is lacking.

The presence of other contaminating protein kinases in preparations of GSK3 is a potential hazard when studying the phosphorylation of proteins *in vitro* (for example, a problem encountered in [77] and rectified in [78]). Phosphorylation of the transcription factor c-Jun *in vitro* was studied using tissue-purified GSK3, because GSK3 had not yet been cloned [79–81]. It would be worthwhile to re-examine phosphorylation using recombinant enzyme to check whether all three sites detected on c-Jun are phosphorylated by GSK3. However, even recombinant enzymes are not without their problems! Thus all preparations of His$_6$-tagged GSK3 (and indeed any His$_6$-tagged protein kinase) purified simply by affinity chromatography on Ni^{2+}-nitrilotri-acetate–agarose are contaminated with DYRK1A, a protein kinase that has a natural histidine tag in its sequence. This protein kinase phosphorylates serine and threonine residues that are followed by proline, a motif that frequently occurs at residues phosphorylated by GSK3. Moreover, DYRK1A acts as a 'priming' kinase for GSK3 in the case of eIF2B and tau [46]. It is therefore critical to remove DYRK1A and/or check that the

Table 1   Proteins reported to be substrates of GSK3

The table indicates which of the criteria needed to establish that a protein is a physiological substrate for GSK3 have been met. The genetic evidence (criteria 6 and 7) has not yet been obtained for any substrate. Therefore, these criteria have been omitted from the table. Three of the proteins included in the table have not been discussed in the text due to space limitations, and the evidence that ATP citrate lyase, MAP1B and presenilin-1 are substrates for GSK3 may be found in the following references: [43], [44], [74–76]. Abbreviations and references are given in the text.

Putative substrate	Biological process	Function proposed for phosphorylation	Criterion ...	1	2	3	4	5
Glycogen synthase	Glycogen synthesis	Inactivates		+	+	+	+	+
eIF2B	Protein synthesis	Inactivates		+	+	+	+	+
ATP citrate lyase	Fatty acid synthesis	None		+	−	+	−	−
Axin	Wnt signalling	Stabilizes protein and recruits β-catenin		−	+/−	+	+	+
β-Catenin	Wnt signalling/adhesion	Targets for degradation		−	+/−	+/−	+/−	+
APC	Wnt signalling	Facilitates binding of β-catenin to APC and decreases binding of APC to microtubules		−	−	−	−	−
MUC1/DF3	Glycoprotein	Decreases affinity of MUC1 for β-catenin		−	+	−	−	−
Cyclin D1	Cell division cycle	Nuclear export and targets for degradation		−	+	+/−	+	+
Jun	Transcription factor	Inhibits DNA binding and transactivation		+	+	+	−	+
Myc	Transcription factor	Targets for degradation		+	+	+	−	+
NFATc	Transcription factor	Promotes nuclear export, inhibits DNA binding		−	+	−	+/−	−
C/EBPα	Transcription factor	None		+	+	+	+	−
C/EBPβ	Transcription factor	Inhibits DNA binding and transactivation		−	−	+	+	+
CREB	Transcription factor	Enhances transcriptional activity		−	+	−	−	−
MITF	Transcription factor	Enhances binding to the tyrosinase promoter		−	+	−	−	−
HSF-1	Transcription factor	Inhibits DNA binding and transactivation		−	+	−	+/−	−
Tau	Microtubule-binding protein	Inhibits binding to microtubules		+	−	+	+	+
MAP1B	Microtubule-binding protein	Maintains microtubular instability		−	−	+	+	+
Presenilin-1	Transmembrane protein	Disposal of excess C-terminal fragments of presenilin, generated by endoproteolytic processing		−	+	−	+/−	−

substrate is not phosphorylated by purified DYRK1A. It is also important to establish that phosphorylation is blocked by inhibitors of GSK3 that do not affect DYRK1A, such as Li$^+$ (H. McLauchlan and P. Cohen, unpublished work).

POTENTIAL ROLE OF GSK3 IN REGULATING CELL ADHESION THROUGH THE PHOSPHORYLATION OF β-CATENIN AND MUC1

In addition to its key role in Wnt signalling, β-catenin is an essential component of cadherin-based adhesion junctions. Genetic analyses in *Drosophila* and *Xenopus* have indicated that the adhesive and Wnt signalling functions of β-catenin can be separated. Thus β-catenin mutants that are defective in adhesion can still induce axis duplication in *Xenopus* and rescue pattern formation in *Drosophila*, processes which are mediated by the Wnt signalling pathway [82,83]. However, the overexpression of cadherin can antagonize the signalling function of β-catenin, presumably by sequestering the signalling pool [82]. Moreover, expression of Wnts can, over the long term, increase cellular adhesion via an increase in E-cadherin transcription. This follows an initial transient decrease in adhesion caused by the Wnt-induced relocalization of β-catenin from the plasma membrane to the cytoplasm and nucleus [84].

Another proposed target of GSK3 involved in cell adhesion is MUC1/DF3. This glycoprotein spans the plasma membrane and its expression is restricted to the apical borders of secretory epithelial cells. The cytoplasmic domain of MUC1 is reported to interact with both GSK3 and β-catenin, the interaction with GSK3 occurring via the sequence STDRSPYEKV in MUC1. GSK3 can phosphorylate the cytoplasmic domain of MUC1, and mutagenesis of the second serine residue abolishes phosphorylation [85]. It was therefore suggested that GSK3 phosphorylates this second serine residue but, by analogy to glycogen synthase and eIF2B, there seems a strong possibility that the phosphorylation of this site is catalysed by another protein kinase, forming a priming site for phosphorylation by GSK3 at the first serine. This emphasizes the importance of identifying phosphorylation sites directly and the potential dangers of simply relying on mutagenesis for the identification of phosphorylation sites. Phosphorylation by GSK3 decreases the affinity of MUC1 for β-catenin, and it has been proposed that this might enhance binding between β-catenin and E-cadherin.

MUC1 is overexpressed in a variety of human carcinomas and is mislocalized in tumour cells, being found throughout the entire plasma membrane, as well as the cytoplasm. Such overexpression of MUC1 may sequester β-catenin, thereby inhibiting formation of the E-cadherin–β-catenin complex and contributing directly to the reduced cell–cell and cell–matrix interactions and increased motility/invasiveness of tumour cells.

These ideas are interesting, but at the present time almost none of the criteria needed to establish MUC1 as a *bona fide* substrate of GSK3 *in vivo* have been met.

POTENTIAL ROLE OF GSK3 IN REGULATING THE CELL-DIVISION CYCLE THROUGH THE PHOSPHORYLATION OF CYCLIN D1

Cyclin D1 binds to, and is required for, the activity of CDK4 and CDK6, which phosphorylate and inactivate the retinoblastoma gene product, facilitating entry into the S-phase of the cell-division cycle. Transcription of the cyclin D1 gene is dependent on mitogenic stimulation, via activation of the classical MAPK cascade, and the level is also controlled by ubiquitin-mediated proteolytic destruction. The latter is mediated by the phosphorylation of Thr^{286}, and phosphorylation at this residue is required for nuclear exit and subsequent proteolytic degradation [86].

The phosphorylation of cyclin D1 at Thr^{286} can be catalysed by GSK3 *in vitro*, but not by other protein kinases so far tested. However, Thr^{286} is only ten residues from the C-terminus of cyclin D1 and there are no Ser/Thr residues whose phosphorylation could prime the phosphorylation of Thr^{286}. It would therefore be interesting to determine the efficiency of phosphorylation relative to other physiological substrates, although it should be noted that the phosphorylation of cyclin D1 by GSK3 is enhanced if cyclin D1 is bound to CDK4 [87].

Consistent with a role for GSK3, the half-life of cyclin D1 is increased, and GSK3 activity decreased, in serum-stimulated cells, and this is prevented by wortmannin, an inhibitor of PI 3-kinase [87]. Moreover, in serum-starved cells, the half-life of cyclin D1 is increased by expression of oncogenic Ras (which activates PI 3-kinase), but not by an oncogenic mutant of Ras that does not activate PI 3-kinase [87]. However, it has not yet been demonstrated that Thr[286] becomes dephosphorylated in response to mitogens or oncogenic Ras, or that PI 3-kinase inhibitors prevent the effect of oncogenic Ras on the half-life of cyclin D1. Moreover, the ability of oncogenic Ras to extend the half-life of cyclin D1 could not be mimicked by the overexpression of a constitutively active form of MAPK kinase 1 (MKK1) (also called MEK) which activates the classical MAPK cascade [87] and should therefore inhibit GSK3 (Figure 2).

It has been reported that treatment of rat intestinal epithelial cells with 20 mM Li[+] increases the level of cyclin D1 protein over a period of several hours, similar to the time required to observe accumulation of β-catenin [88]. However, the effect of Li^{+} on the phosphorylation of Thr[286] was not examined in these studies. The effects of more specific inhibitors of GSK3 and studies in PDK1^{−/−} cells where GSK3 cannot be inactivated by phosphorylation at Ser[9]/Ser[21] are awaited with interest.

Wnt signalling also stimulates transcription of the cyclin D1 gene, which has a LEF-1 binding site in its promoter and therefore responds to the activated β-catenin–LEF-1 complex [89]. It would be interesting to know whether Thr[286] of cyclin D1 becomes dephosphorylated under these conditions.

POTENTIAL ROLE OF GSK3 IN THE PHOSPHORYLATION OF TRANSCRIPTION FACTORS

c-Jun

This was the first transcription factor suggested to be a substrate for GSK3. GSK3 was reported to phosphorylate c-Jun at Thr[239], Ser[243] and Ser[249] in a region proximal to the DNA-binding domain *in vitro* and in co-transfection experiments. This decreases the binding of c-Jun to DNA and its ability to stimulate transactivation [79–81]. These residues lie in the same tryptic peptide which is phosphorylated in cells, as judged by phosphopeptide mapping. Moreover, phosphorylation of the peptide is decreased by stimulation with phorbol esters, which is consistent with the inhibition of GSK3 via the classical MAPK cascade (Figure 2).

The mutation of Ser[243] to Phe prevents the phosphorylation of this peptide, consistent with a priming role for this site [79]. However, on the basis of the known specificity of GSK3, why this would prevent the phosphorylation of Ser[249] is unclear. The same sites are conserved in the JunB and JunD isoforms, as well as in the oncogenic form v-Jun, and are subject to similar regulation [81].

Interestingly, the expression of cyclin D1 can be induced by c-Jun through its ability to bind to the CRE element in the promoter [90]. Thus the mitogen-induced dephosphorylation of c-Jun may contribute to the mitogenic stimulation of cyclin D1 transcription.

These experiments were performed prior to the discovery of relatively specific inhibitors of GSK3 and the elucidation of the signalling pathways that trigger its inhibition. It therefore seems important to repeat these experiments using the more powerful reagents that are now available. In addition, it would be interesting to know whether insulin and growth factors also promote the dephosphorylation of Jun isoforms at the sites targeted by GSK3.

CCAAT/enhancer binding protein α (C/EBPα)

C/EBPα is a transcription factor that controls the expression of many genes and is required for preadipocyte differentiation. In 3T3-L1 preadipocytes, C/EBPα becomes dephosphorylated in response to insulin and this is mimicked by Li^{+} ions and prevented by wortmannin (a PI 3-kinase inhibitor) [91]. Moreover C/EBPα is phosphorylated by GSK3 at Thr[222] and perhaps Thr[226] in the sequence TPPPTPVPSP [91], suggesting that phosphorylation of Ser[230] (underlined) by an unknown kinase may prime the phosphorylation of Thr[226] by GSK3, which in turn primes the phosphorylation of Thr[222]. Li^{+} inhibit preadipocyte differentiation, but mutating Thr[222] and Thr[226] to Ala does not seem to affect the transcriptional activity of C/EBPα or its ability to induce spontaneous differentiation of preadipocytes [91]. Thus the role of Thr[222] and Thr[226] is still unclear.

The transcriptional activity of another member of this family, C/EBPβ, has recently been shown to be stimulated by the PI 3-kinase/PKB pathway in response to growth hormone. Li^{+} increases growth-hormone-dependent dephosphorylation and activation of C/EBPβ, suggesting an involvement of GSK3 in this process [92]. However, the phosphorylated residues have not yet been identified.

c-Myc

This transcription factor is an immediate early gene that is known to play important roles in the regulation of cell proliferation, differentiation and apoptosis. It can be phosphorylated at Ser[62] by the MAP kinase ERK2 (extracellular signal-related protein kinase 2), and at Thr[58] by GSK3, provided that Ser[62] is already phosphorylated [93]. Recent work suggests that the phosphorylation of Thr[58] may target c-Myc for ubiquitin-mediated proteolysis [93]. Thus mitogen-induced dephosphorylation of c-Myc at Thr[58] may increase its half-life, similar to the situation with cyclin D1 described above. The importance of these residues in the function of Myc are further underscored by the finding that they are absolutely conserved in all isoforms of Myc, with the exception of v-Myc [93]. Thr[58] is mutated in all v-Myc proteins, and replacement with the wild-type threonine severely inhibits its transforming potential [94,95]. Thr[58] is also frequently mutated in Burkitt's lymphomas and AIDS-associated lymphomas [96,97]. Moreover, mutation of Thr[58] to Ala in c-Myc potentiated focus formation, while mutation of Ser[62] to Ala had the opposite effect [98]. However, it is not clear whether GSK3 is really the kinase that phosphorylates Thr[58] *in vivo*, since inhibitors of GSK3 or genetic tools have not been employed for these studies. In addition, more work is needed to establish whether Ser[62] is phosphorylated by ERK1/ERK2 or another protein kinase. It has been reported that the drug PD 98059 (which inhibits the MAPK cascade and hence the activation of ERK1/ERK2; Figure 2) blocks Ras-mediated stabilization of Myc [93], but whether PD 98059 alters the phosphorylation of Thr[58] has not been reported.

The mutation of Ser[62] to Ala is reported to destabilize Myc, suggesting that the phosphorylation of this residue may stabilize Myc and hence play an opposing role to the phosphorylation of Thr[58]. Thus the regulation of Myc by serum presents an apparent paradox. On the one hand, this agonist activates PI 3-kinase and the classical MAPK cascade, which both inhibit GSK3 (Figure 1) and hence should decrease phosphorylation of Thr[58] or Ser[62]. This is consistent with the observation that the half-life of Myc increases in response to serum [99]. On the other hand, if ERK1 and ERK2 mediate the 'priming' phosphorylation at Ser[62], this should greatly enhance the phosphorylation of Thr[58] by GSK3 and destabilize Myc after serum stimulation. Since the opposite

is observed, it is possible that Ser^{62} is already phosphorylated, even in the absence of serum, by another protein kinase. If this is the case, then the phosphorylation of Ser^{62} should not be prevented by PD 98059 and other inhibitors of the classical MAPK cascade. The function of GSK3 is likely to be to ensure a low level of Myc in the absence of serum.

Nuclear factor of activated T-cells c (NFATc)

The dephosphorylation of NFATc by the calcium/calmodulin-dependent protein phosphatase, termed PP2B or calcineurin, stimulates its translocation into the nucleus and its binding to DNA, allowing it to regulate the production of interleukin-2, and hence T-cell proliferation. The phosphorylation of NFATc occurs at several sites clustered within the NFAT homology domain, and this inhibits DNA binding [100]. At least some of these sites can be phosphorylated by GSK3 in vitro. Mutation to Ala of all 11 serine residues whose phosphorylation is critical for mediating nuclear export of NFATc abolishes phosphorylation by GSK3 [101]. However, which of these are actually phosphorylated by GSK3 in vitro or in vivo has not been determined.

The overexpression of GSK3 promoted nuclear export, while incubation of T cells with LiCl had the opposite effect and antagonized nuclear translocation of NFATc [101]. It will clearly be important to examine whether the more specific cell-permeant inhibitors of GSK3 that have become available mimic the effect of Li$^+$ on the subcellular localization of NFATc, and whether these compounds actually promote dephosphorylation of NFATc.

Heat-shock factor-1 (HSF-1)

HSF-1 is the key transcriptional regulator of the heat-shock genes. Under basal conditions, its DNA binding and transcriptional activity is suppressed by the phosphorylation of Ser^{303}, which is dependent on prior phosphorylation of Ser^{307} [78]. The MAPK ERK1 phosphorylates Ser^{307} in vitro, allowing GSK3 to phosphorylate Ser^{303}. The overexpression of a constitutively active mutant of MKK1 (the immediate upstream activator of ERK1/ERK2), or the overexpression of GSK3, induces the phosphorylation of overexpressed HSF-1 at Ser^{303} and/or Ser^{307}, thereby repressing its transcriptional activity [78]. In Xenopus oocytes, relatively high concentrations of Li$^+$ (25 mM) were reported to elevate heat-shock-induced DNA binding of HSF-1 and to delay the attenuation of DNA binding during the recovery from heat shock [102]. However, further evidence is needed before the potential role of GSK3 in the regulation of HSF-1 can be assessed. Firstly, the effect of Li$^+$ on the phosphorylation of Ser^{303} was not reported, and it is therefore unclear whether the effects on HSF-1 DNA binding are mediated by the dephosphorylation of Ser^{303} or by another mechanism. Secondly, these investigators reported that the effects of Li$^+$ could be mimicked by the injection of mRNA encoding GBP into the oocytes [102]. However, this is surprising because, as described above, a GSK3-binding fragment of the mammalian homologue, FRAT, does not inhibit the activity of GSK3 towards 'primed substrates'. Furthermore, whether MAPK cascade inhibitors affect the transcriptional activity of the endogenous HSF-1 and whether they prevent the phosphorylation of Ser^{307} (and hence Ser^{303}) has not been reported.

Microphthalmia-associated transcription factor (MITF)

MITF is a member of the basic helix–loop–helix/leucine zipper ('bHLHLZip') family of transcription factors, which is mutated

in Waardenburg syndrome type 2 (WS2) and Tietz syndrome, which are characterized by deafness and albinism. Ser^{298} is mutated in a family with WS2 and such mutations impair its transcriptional activity [103]. Interestingly, it has been reported that MITF can be phosphorylated by GSK3, but not if Ser^{298} is changed to Ala. Phosphorylation by GSK3 enhances the DNA-binding activity of MITF in vitro, and overexpression of a catalytically inactive mutant of GSK3β (but not GSK3α) inhibits MITF-induced transactivation of the tyrosinase gene in cell-based assays. However, Ser^{298} has not been formally identified as the site of GSK3 phosphorylation, and it remains possible that this mutation induces conformational changes that impair phosphorylation at another site. Moreover, the mutation of Ser^{302} to Ala, which would be expected to impair phosphorylation by GSK3 at Ser^{298} by eliminating the putative priming phosphoserine residue, in fact had no effect on the ability of MITF to transactivate the tyrosinase promoter in cell-based assays. It therefore seems critical to examine whether the phosphorylation of MITF at Ser^{298} and its transcriptional activity are reduced in cells treated with GSK3 inhibitors, or altered by extracellular signals that are known to modulate GSK3 activity.

CREB

CREB becomes active when it is phosphorylated at Ser^{133}. This can occur as a result of phosphorylation by cAMP-dependent protein kinase in response to agonists that elevate cAMP or by isoforms of mitogen and stress-activated protein kinase ('MSK') in response to growth factors and cellular stresses [104,105]. The phosphorylation of Ser^{133} permits GSK3 to phosphorylate Ser^{129} in vitro and the mutation of Ser^{129} to Ala has been reported to impair the transcriptional activity of CREB in response to cAMP elevation [47]. These observations are interesting, but no evidence has yet been presented that Ser^{129} is phosphorylated in vivo or that GSK3 is a CREB kinase in vivo.

SOME GENERAL OBSERVATIONS ABOUT THE REGULATION OF TRANSCRIPTION FACTORS BY GSK3

One of the many functions of GSK3 may be to phosphorylate transcription factors such as Jun, Myc and HSF-1, maintaining them in an inactive state under basal conditions. Furthermore, like β-catenin, many of these proteins are subsequently degraded as a direct result of this phosphorylation. This suggests that the unique requirement of GSK3 for a priming phosphorylation may be linked to the recognition of these substrates by a particular ubiquitin ligase that recognizes proteins containing two phosphorylated residues that are four amino acids apart, causing them to be degraded by the ubiquitin-mediated proteasome [106].

GSK3 activity is suppressed by many different agonists, leading to the dephosphorylation and stabilization of these proteins. However, the inhibition of GSK3 activity must clearly be transient to ensure that the proteins are once again degraded, to switch the signal off when the appropriate response has been executed. Thus GSK3 may not only maintain the basal state, but also regulate the duration of a given response. Failure to do this may lead to transformation (β-catenin, cyclin D1, Jun and Myc) or cell death (HSF-1).

THE ROLE OF GSK3 IN APOPTOSIS

There is considerable evidence that many growth factors aid cell survival by activating PI 3-kinase and PKB [107]. Cooper and his colleagues were the first to provide evidence that GSK3 may contribute to the effects of PKB on apoptosis [108]. They found

that the overexpression of GSK3 induced apoptosis in pheo-chromocytoma (PC12) cells and Rat-1 fibroblasts, whereas the overexpression of a catalytically inactive mutant of GSK3 prevented apoptosis induced by LY 294002 (Figure 2) or by the overexpression of a catalytically inactive PI 3-kinase. Recent work using relatively specific GSK3 inhibitors has lent support to this hypothesis. Li$^+$ were reported to protect cells against apoptosis induced by staurosporine or heat shock in SH-SY5Y neuroblastoma cells, whereas the overexpression of GSK3 had the opposite effect and potentiated apoptosis [109]. Similarly, LiCl, or the GSK3 inhibitors SB 216763 or SB 415286, were found to protect cerebellar granule neurons from apoptosis induced by lowering the K$^+$ concentration in the medium [110]. The same compounds also protected chicken dorsal-root-ganglion sensory neurons from apoptosis caused by the with-drawal of NGF from the medium or by the inhibition of NGF-induced PI 3-kinase activity with LY 294002 [110].

The mechanism by which the inhibition of GSK3, via the PI 3-kinase cell-survival pathway, suppresses apoptosis remains elus-ive. Survival factors that inhibit GSK3 via PI 3-kinase and PKB do not stabilize β-catenin or stimulate β-catenin-dependent gene transcription. Therefore, not surprisingly, the overexpression of a mutant β-catenin that is not degraded by GSK3β did not protect against apoptosis in neurons, despite the fact that the overexpression of GSK3β in these neurons increased apoptosis [111].

However, the inhibition of GSK3 in Rat-1 fibroblasts via Wnt-1 signalling can inhibit apoptosis by activating β-catenin/TCF-mediated gene transcription [112], but the target genes whose increased transcription is responsible for enhancing resistance to apoptosis have yet to be identified. Such target genes may be important in the context of cancer, where β-catenin-dependent transcription is up-regulated. The identification of these genes is important, because Wnt-1-expressing cells are resistant to the chemotherapeutic drugs vincristine (VCR) and vinblastine (VBL), which normally induce apoptosis [112]. Thus the products of these genes, as well as components of the Wnt-1 pathway itself, may be attractive targets for the development of drugs to enhance the chemotherapeutic efficacy of agents like VCR and VBL. This has been elegantly demonstrated by the use of dominant negative versions of a TCF transcription factor, which greatly enhance VCR- and VBL-mediated killing of SW480 colon carcinoma cells [112].

In contrast with the pro-apoptotic effects of GSK3 in neuronal cells, mouse knockouts that do not express GSK3β die *in utero* due to apoptosis of the liver between E13.5 and E14.5. This appears to be due to an inability to elicit an anti-apoptotic response to tumour necrosis factor α (TNFα) [64], since cells lacking GSK3β fail to activate a nuclear factor κB (NF-κB)-dependent reporter gene efficiently, although they do exhibit normal degradation of IκB and normal nuclear translocation of the p65/RelA subunit of NF-κB in response to interleukin-1 or TNFα. Moreover, the reduced binding of NF-κB to DNA in response to TNFα in GSK3$\beta^{-/-}$ cells implies that GSK3 may positively regulate the DNA binding and transactivation potential of NF-κB. However, how GSK3 modulates NF-κB function in embryonic liver remains to be established. It could potentially phosphorylate an NF-κB subunit or an NF-κB co-activator.

In contrast with mammalian cells, studies in the *Drosophila* eye revealed that the overexpression of mutated forms of armadillo (the *Drosophila* homologue of β-catenin), which correspond to mutations found in human tumours, are pro-apoptotic [113]. Interestingly, this induction of apoptosis is delayed until EGF receptor/MAPK signalling is switched off during normal eye

development. Thus, in *Drosophila*, β-catenin-dependent gene transcription is antagonized by the EGF receptor/MAPK sig-nalling pathway.

Thus GSK3 seems to play an important, but tissue-specific, role in apoptosis. Future studies in this area will have to focus on identifying the substrates of GSK3 involved in regulating apop-tosis, which at the moment, remain a mystery.

THERAPEUTIC POTENTIAL OF GSK3 INHIBITORS FOR THE TREATMENT OF DIABETES

Diabetes is one of the leading causes of death in most developed countries, and the number of people affected is expected to double to around 300 million over the next 20 years, due to an increasingly sedentary lifestyle combined with excess energy intake. In addition, due to the many long-term complications of the disease, such as kidney damage, increased risk of heart disease and blindness, its impact on the healthcare expenditure of developed nations greatly exceeds its incidence.

Type II or non-insulin-dependent diabetes mellitus ('NIDDM') accounts for approx. 90 % of all cases of the disease. The initial stages are characterized by insulin resistance, i.e. an inability of the peripheral tissues (muscle, fat and liver) to respond correctly to the insulin that is secreted from the pancreas. However, as the disease progresses, the pancreas is no longer able to produce enough insulin to counteract the resistance and daily injections of exogenous insulin then become necessary. Thus drugs are urgently needed to combat resistance, allowing the tissues to respond much better to the insulin already present (reviewed in [114,115]).

Over the past few years there has been much interest within the pharmaceutical industry in identifying compounds that inhibit GSK3 as possible insulin mimetic/sensitizing drugs. This interest has been heightened by the report that the level and activity of GSK3 is moderately elevated in diabetic and obese strains of mice [116]. Investigators at GlaxoSmithKline have recently developed a class of maleimides that are potent and relatively selective inhibitors of GSK3. In liver cells, these compounds mimic insulin signalling, as expected by promoting the dephos-phorylation and activation of glycogen synthase, thereby facili-tating the conversion of extracellular glucose into glycogen [117]. However, interestingly, they also mimic a further action of insulin, namely its ability to repress the expression of the genes encoding glucose-6-phosphatase and phosphoenolpyruvate car-boxykinase [118], the enzymes that control hepatic gluconeo-genesis. These recent observations are exciting, since they imply that GSK3 inhibitors may suppress hepatic glucose output, as well as aiding glucose disposal by the tissues. Such drugs may therefore have greater therapeutic potential for the treatment of type II diabetes than recognized previously.

It has also been reported that Li$^+$ cause a modest stimulation of glucose uptake and slightly increase translocation of the glucose transporter GLUT4 to the plasma membrane in 3T3-L1 and rat adipocytes. Li$^+$ also enhanced glucose uptake stimulated by a submaximal dose of insulin [119–121]. However, the effects were much smaller and occurred more slowly than the activation of glycogen synthase or the effects of insulin on glucose transport. Moreover, the effects of Li$^+$ in 3T3-L1 adipocytes were blocked by inhibition of PI 3-kinase [121]. These observations are consistent with the report that GSK3 phosphorylates insulin receptor substrate-1 (IRS-1) *in vitro* (Figure 1) and inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 in over-expression experiments [122], suggesting that it may be a negative regulator of insulin signalling. However, the IGF-1-induced activation of PI 3-kinase is actually enhanced in embryonic stem

cells lacking PDK1, even though the inhibition of GSK3 is abolished [70], which is not consistent with this hypothesis. It is, however, consistent with another report that it is PKB, rather than GSK3, which phosphorylates IRS-1 and exerts the negative feedback control on PI 3-kinase activation [123]. Thus further work is needed to understand how Li$^+$ ions stimulate glucose uptake. It would also be interesting to examine the effects of other GSK3 inhibitors on glucose uptake.

However, there is a potential problem with the long-term use of GSK3 inhibitors to treat diabetes, namely that many of the components of the Wnt signalling pathway are overexpressed or mutated in many different tumour types [124,125]. For example, virtually all colon tumours arise from an initiating mutation in one of two Wnt signalling components (reviewed in [124,125]). Approx. 85 % of all colon tumours carry mutations in the APC gene product, while many of the remainder have mutations in β-catenin that render it resistant to degradation. Mutations in β-catenin have also been found in a wide variety of other tumour types, including ovarian, gastric and hepatocellular carcinomas, as well as pilomatricomas – a common hair-follicle tumour. Interestingly, Axin mutations, although not found in colon tumours, do occur in hepatocellular carcinomas, while viral activation of Wnt-3A causes mammary tumours in mice. As implied by its name, *FRAT1* was identified as a gene that co-operated with the transcription factor c-Myc in the progression of T-cell lymphomas in mice [126]. Alteration of any of these components would be predicted to lead to inappropriate accumulation of β-catenin.

It is widely believed that the oncogenic action of β-catenin is mediated by its ability to turn on the transcription of oncogenic target genes, such as c-Myc and cyclin D1. However, two recent reports suggest that β-catenin can now join the ranks of the most infamous oncogenes. Firstly, expression of a stabilized mutant of β-catenin transforms Madin–Darby canine kidney cells [127], which show enhanced proliferation and form colonies in soft agar. They are also resistant to detachment-induced apoptosis (anoikis), and show an attenuated cell-cycle block in response to radiation-induced DNA damage. This does not occur under normal circumstances because β-catenin is degraded [128,129]. Thus even moderate increases in the level of the wild-type protein are sufficient to promote oncogenesis [127]. Secondly, mice that express a mutant β-catenin that lacks the putative sites of GSK3 phosphorylation, and is therefore much more stable than wild-type β-catenin, developed as many as 3000 intestinal polyps per mouse only 3 weeks after birth [130].

It is also clear that other mechanisms exist to up-regulate the level of β-catenin in the nucleus and activate oncogenic gene transcription. Although only one out of 65 melanomas contained detectable mutations in β-catenin, nuclear localization was seen in one-third of the tumours [131], suggesting that components of the newly identified p53-inducible complex that degrades β-catenin [128,129] may well be mutated in the subset of tumours with elevated levels of β-catenin. This hypothesis is attractive in light of the well-established association between UV-induced DNA damage and the genesis of melanomas.

The GSK3 inhibitors developed by GlaxoSmithKline are ATP-competitive and would therefore be expected to inhibit the phosphorylation of all the substrates of GSK3, not just those involved in insulin signalling. Indeed, these compounds caused dramatic elevations in the level of β-catenin [110] and stimulated β-catenin-dependent gene transcription [117] in cell-based assays. There is therefore a danger that the prolonged use of such drugs would have the potential to be oncogenic. Information as to whether prolonged treatment with these and other GSK3 inhibitors stimulates the formation of tumours in animal models,

or enhances tumorigenesis induced by known carcinogens, is awaited with great interest.

However, the discovery of the unique way in which primed substrates dock on GSK3 may have opened up a new opportunity to develop compounds that target the phosphate-binding site on GSK3 [48]. These compounds should inhibit the phosphorylation of key proteins involved in mediating the metabolic actions of insulin without affecting the phosphorylation of Axin and β-catenin. The feasibility of this approach has been demonstrated by the identification of short (6–8-residue) phosphopeptides from the N-terminus of GSK3 that interact with the phosphate-binding site and differentially inhibit the activity of GSK3 towards primed, but not non-primed, substrates [48]. Such drugs may therefore be less likely to be oncogenic and hence more suitable for the long-term treatment of diabetes.

ADDITIONAL THERAPEUTIC USES FOR GSK3 INHIBITORS

Reducing neuronal apoptosis is an important therapeutic goal in the context of head trauma, stroke, epilepsy and motor neuron disease (reviewed in [132]). Therefore the emerging role of GSK3 as a pro-apoptotic factor in neuronal cells makes this protein kinase an attractive therapeutic target for the design of inhibitory drugs to treat these diseases. Moreover, in contrast with type II diabetes, the duration of treatment is relatively short, at least in the case of head trauma and stroke, so that potential oncogenic effects may be minimal.

GSK3 inhibitors may be able to prevent and/or reverse the abnormal hyperphosphorylation of the microtubule-associated protein tau that is an invariant feature of Alzheimer's disease and a number of other neurodegenerative diseases, such as progressive supranuclear palsy, corticobasal degeneration and Pick's disease. Mutations in the tau gene cause inherited forms of fronto-temporal dementia, further underscoring the relevance of tau protein dysfunction for the neurodegenerative process (reviewed in [133]).

Tau is thought to stabilize microtubules *in vivo* and promote their polymerization (reviewed in [134]). It is abundant in the central nervous system, being predominantly expressed in axons. The phosphorylation of tau is regulated developmentally, such that foetal tau is more highly phosphorylated than adult tau, and the degree of phosphorylation decreases with age. Many protein kinases have been shown to phosphorylate tau *in vitro* at multiple sites, including GSK3 [135,136], and several lines of evidence have implicated GSK3 in the phosphorylation of tau *in vivo*. In particular, Li$^+$ induced a partial dephosphorylation of tau in the brains of newborn animals and affected microtubule stability [137], while insulin and IGF-1 induce the dephosphorylation of tau in human neuronal NT2N cells, via a PI 3-kinase-dependent (and therefore presumably GSK3-dependent) pathway [138].

In normal brain, tau is a soluble protein. In the diseases mentioned above it is found in an abnormally hyper-phosphorylated, filamentous form. Hyperphosphorylation of tau is believed to be an early event and to precede its assembly into filaments. However, it is at present unclear whether hyper-phosphorylation of tau is either necessary or sufficient for filament assembly. What is clear, however, is that abnormal hyperphos-phorylation results in the inability of tau to bind to micro-tubules [139,140]. This will inappropriately free tau and render it available for aberrant filament assembly. The events leading from soluble tau to its ordered assembly into filaments are believed to underlie the degeneration of nerve cells that charac-terizes the tauopathies [133]. Many of the sites that are hyper-phosphorylated in filamentous tau are phosphorylated efficiently

by GSK3 *in vitro*. This raises the possibility that GSK3 inhibitors may reduce the abnormal hyperphosphorylatyion of tau, thereby leading to enhanced interactions with microtubules and reducing the pool of tau available for aberrant assembly.

Hair growth is controlled by the Wnt signalling pathway, in particular Wnt-3. In tissue-culture model systems of the skin, the expression of non-degradable mutants of β-catenin leads to a dramatic increase in the population of putative stem cells, which have greater proliferative potential [141]. *In vivo*, this population of stem cells expresses a higher level of non-cadherin-associated β-catenin- and TCF/LEF-driven reporter gene activity [142], which may contribute to their high proliferative potential. Moreover, transgenic mice overexpressing a truncated β-catenin in the skin undergo *de novo* hair-follicle morphogenesis, which normally is only established during embryogenesis. This raises the possibility that ectopic application of GSK3 inhibitors might be of use in the treatment of baldness and in restoring hair growth following chemotherapy-induced alopecia. In the latter case, it may be essential to begin treatment with a GSK3 inhibitor after the completion of chemotherapeutic treatment. Otherwise, the subsequent elevation in β-catenin levels in normal cells may confer resistance to the DNA-damage-induced cell-cycle block in response to chemotherapeutic agents, which would be dangerous. For this reason GSK3 inhibitors may be more useful as drugs to stimulate the regeneration or *de novo* synthesis of hair follicles, rather than as agents for preventing hair loss during cancer chemotherapy.

Inhibitors of CDKs have recently been reported to reduce chemotherapy-induced alopecia [143]. CDKs are the protein kinases most closely related in amino acid sequence to GSK3, and a number of compounds developed as CDK inhibitors have recently been shown to inhibit GSK3 with similar potency [140]. It is therefore possible that some of the effects of CDK inhibitors may result from the inhibition of GSK3. Conversely, some of the effects of GSK3 inhibitors may be explained by inhibition of CDKs. For example, CDK inhibitors, like GSK3 inhibitors, have been reported to inhibit neuronal apoptosis [144–146]. In order to ascribe a biological effect to GSK3, it is therefore crucial to use GSK3 inhibitors that do not inhibit CDKs and vice versa. Li$^+$ ions do not inhibit CDK2 (H. McLauchlan and P. Cohen, unpublished work).

CONCLUDING REMARKS

The study of GSK3 in the recent past has benefited from a forging of the fields of developmental biology, oncology, biochemistry, genetics, pharmacology and neurobiology, and a multidisciplinary approach will continue to be needed to make further progress in our understanding of this multi-faceted enzyme. Moreover, the role of GSK3 in the many different cellular processes in which it is currently implicated will continually have to be critically reviewed, as new pharmacological and genetic tools become available.

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