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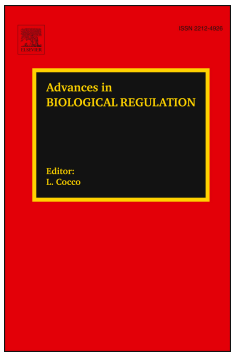

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GSK3 and its interactions with the PI3K/AKT/mTOR signalling network

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Abstract: Glycogen Synthase Kinase-3 (GSK3 or GSK-3) is a promiscuous protein kinase and its phosphorylation of its diverse substrates has major influences on many areas of physiology and pathology, including cellular metabolism, lineage commitment and neuroscience. GSK3 was one of the first identified substrates of the heavily studied oncogenic kinase AKT, phosphorylation by which inhibits GSK3 activity via the formation of an autoinhibitory pseudosubstrate sequence. This has led to investigation of the role of GSK3 inhibition as a key component of the cellular responses to growth factors and insulin, which stimulate the class I PI 3-Kinases and in turn AKT activity and GSK3 phosphorylation. GSK3 has been shown to phosphorylate several upstream and downstream components of the PI3K/AKT/mTOR signalling network, including AKT itself, RICTOR, TSC1 and 2, PTEN and IRS1 and 2, with the potential to apply feedback control within the network. However, it has been clear for some time that functionally distinct, insulated pools of GSK3 exist which are regulated independently, so that for some GSK3 substrates such as β-catenin, phosphorylation by GSK3 is not controlled by input from PI3K and AKT. Instead, as almost all GSK3 substrates require a priming phosphorylated residue to be 4 amino acids C-terminal to the Ser/Thr phosphorylated by GSK3, the predominant form of regulation of the activity of GSK3 often appears to be through control over these priming events, specific to individual substrates. Therefore, a major role of GSK3 can be viewed as an amplifier of the electrostatic effects on protein function which are caused by phosphorylation. Here we discuss these different aspects to GSK3 regulation and function, and the functions of GSK3 as it integrates with signalling through the PI3K-AKT-mTOR signalling axis.

Keywords: Protein Kinase, Phosphorylation, kinase inhibitor, GSK3, AKT, WNT

1. Introduction

Reversible phosphorylation is a mechanism of protein regulation which is ubiquitous amongst eukaryotes and almost all human proteins have recognised sites of phosphorylation (Gnad et al., 2011; Hornbeck et al., 2015; Hunter, 2012). Glycogen Synthase Kinase 3 (GSK3) is a serine/threonine directed protein kinase found widely throughout eukaryotes, with over a hundred specifically identified proteins proposed as GSK3 substrates and a much larger number predicted from bioinformatics (Kaidanovich-Beilin and Woodgett, 2011; Linding et al., 2007; Sutherland, 2011). In mammals GSK3 exists in two forms, GSK3α and GSK3β, paralogs which share a highly conserved catalytic domain but differ at both termini and are encoded by separate genes, in humans named GSK3A and GSK3B. Both GSK3α and GSK3β mRNA and protein are routinely detected in most human tissues and although firm quantitation of their concentration is rare, as an example, in human U2OS cells and the murine fibroblast cell line NIH3T3 quantitative proteomics indicated an abundance in the region of 50000 or 15000 molecules of GSK3β and around 5000 molecules of GSK3α per cell (Beck et al., 2011; Schwanhausser et al., 2011).

The 500 or so protein kinases encoded in the human genome, often termed the kinome, can be subdivided evolutionarily and functionally into families (Manning et al., 2002). Some kinases show relatively constitutive activity (e.g. CK2) whereas other groups display tightly regulated activity (e.g many members of the Receptor Tyrosine Kinase and Mitogen Activated Protein Kinase families). Within the kinome, GSK3 is most closely related to the Cyclin Directed Kinase (CDK) and Mitogen Activated Protein Kinase (MAPK) groups, with these latter groups sharing a substrate context preference for serine and threonine residues immediately followed (C-terminally) by a proline residue. In contrast, GSK3 shows a different substrate context. It will phosphorylate either serine or threonine residues, with a modestly higher activity against serine being reported (Stamos et al., 2014; Sutherland, 2011). Most importantly GSK3 has strong kinase activity only against serine and threonine residues which are followed 4 amino acids more C-terminally by another phosphorylated serine or threonine residue, in a S/T-X-X-X-pS/pT context (Figure 1). This activity is described as requiring 'priming' by phosphorylation at the C-terminal site. It also allows the sequential phosphorylation by GSK3 of multiple sites each 4 amino acids apart and is a key determinant of GSK3 function. Critically, it means that proteins are not GSK3 substrates until they have themselves been phosphorylated and in many cases the result of this is that the key factors influencing whether GSK3 will phosphorylate a target protein are not factors acting upon GSK3 but rather independent factors acting upon this substrate priming.

The biochemistry of GSK3 and its role in specific areas of physiology and pathology have been reviewed very well elsewhere (Beurel et al., 2015; Forde and Dale, 2007; Hur and Zhou, 2010; Kaidanovich-Beilin and Woodgett, 2011; McCubrey et al., 2014; Ricciardi et al., 2017; Ruvolo, 2017; Sutherland, 2011). Here we will focus on the regulation of GSK3 substrate phosphorylation and its connections with the PI3K-AKT signalling network. In some publications, we feel this latter connection has been over-emphasised and we will discuss the evidence that specific phosphorylation events catalysed by GSK3 are regulated upstream by PI3K and AKT and the interactions of GSK3 with components of the PI3K-AKT-mTOR signalling network.

2. The regulation of GSK3 activity: what controls whether GSK3 phosphorylates target proteins?

There are several factors which influence the measurable activity of the GSK3 kinase when it is assayed, most notably phosphorylation of the GSK3 N-terminus which will be discussed below. However, the existing data indicate that the dominant factors which control the phosphorylation by GSK3 of its best studied substrates are co-localisation of the kinase with these substrates ensured by protein-binding scaffolds, and the independently regulated priming of these substrates by other protein kinases.

2.1 Competing mechanisms of regulation: substrate priming and autoinhibition by N-terminal phosphorylation

GSK3 phosphorylates almost all of its recognised substrates at Ser/Thr residues which are 'primed' by phosphorylation at another Ser/Thr residue closely C-terminal to the GSK3 substrate residue. In most cases this priming site is four residues away (S/T-X-X-X-pS/pT) but priming 5 and 3 amino acids C-terminally has been demonstrated (Cole et al., 2004; Singh et al., 2012) and unprimed GSK3 mediated phosphorylation has been reported, apparently with acidic residues or other structural features seeming to fulfil this role (Boyle et al., 1991; Happel et al., 2009; Timm et al., 2008). This substrate specificity of GSK3 for Ser/Thr residues with adjacent priming phosphorylation appears to be enforced by a phosphate binding pocket made up of conserved basic amino acids and which lies adjacent to the catalytic site of the kinase domain (Bax et al., 2001; Dajani et al., 2001; Frame et al., 2001; ter Haar et al., 2001). This binding of a priming phosphorylated amino acid in the +4 position, assists the positioning of the substrate Ser/Thr residue within the GSK3 active site and also appears to stabilise a catalytically competent conformation (Frame et al., 2001; Stamos et al., 2014).

This basic pocket adjacent to the active site also seems to mediate one of the other key characteristics of GSK3, namely auto-inhibitory phosphorylation (Figure 1). Dynamic regulated phosphorylation which reduces the activity of GSK3 occurs on Ser 9 of GSK3β (Sutherland et al., 1993) and the analogous Ser 21 in GSK3α, a site which is conserved in vertebrates and *Drosophila* but not *C. elegans* and unicellular eukaryotes (Frame and Cohen, 2001). This N-terminal serine phosphorylation is induced by cellular stimulation with growth factors or insulin and is mediated by AKT and under some circumstances by other kinases including RPS6KA1 (p90RSK) and PKA (Cross et al., 1995; Kaidanovich-Beilin and Woodgett, 2011). This growth factor-stimulated reduction in GSK3 activity is in contrast to many other kinases which are growth factor-activated and leads to a modest (often around 50%) reduction in measurable GSK3 kinase activity and substrate phosphorylation. In many circumstances, growth factors and insulin therefore seem to be modifiers of GSK3 function rather than its key controllers. Perhaps the strongest evidence for this is from knockin mice expressing only mutant GSK3 alpha and beta proteins with an alanine residue replacing the N-terminal serine residue (GSK3α S21A / GSK3β S9A double homozygotes). These mice are viable and healthy and as expected do not show the normal inhibition of GSK3 activity by insulin and

accordingly, unlike wild-type mice, the inhibitory phosphorylation of muscle glycogen synthase (GS) is maintained after insulin injection. However, the mice develop and grow normally with no overt detrimental phenotype, show normal regulation of GS activity in response to muscle contraction and glucose and show no detectable effect on Wnt signalling, including the ability of GSK3 to phosphorylate β-catenin (McManus et al., 2005). Surprisingly, these mice are actually resistant to diet induced obesity, itself a phenotype usually associated with a failure in insulin signalling, probably related to the elevated adiponectin levels relative to wild-type mice and GSK3-mediated regulation of CEBPα (Chen et al., 2016). On the other hand, several further studies of these GSK3 $\alpha/\beta^{21A/21A/9A/9A}$ mice have shown additional GSK3 functions in which its activity appears to be independent of regulation through inhibitory N-terminal phosphorylation, including the regulation of hepatic gene expression, cellular polarisation and reorientation of the centrosome and Golgi, and cardiac stress remodelling and ischemic preconditioning (Lipina et al., 2005; Nishino et al., 2008; Schlessinger et al., 2007; Webb et al., 2010). Even the regulation of GS by insulin in muscle appears to be dominated by mechanisms other than GSK3 inhibition, most notably the allosteric activation of GS by glucose 6-phosphate (Bouskila et al., 2010).

Studies of these mice in which GSK3 cannot be inhibited by phosphorylation have however revealed important new aspects of physiology linked to GSK3. The GSK3 $\alpha/\beta^{21A/21A/9A/9A}$ mice have elevated blood pressure probably linked to changes in catecholamine release (Boini et al., 2009; Siraskar et al., 2011) and show reduced renal phosphate transport and elevated excretion (Foller et al., 2011). In keeping with studies of GSK3 function which have addressed its roles in the brain and a key finding was that N-terminal mutant GSK3 $\alpha/\beta^{21A/21A/9A/9A}$ adult mice show impaired neurogenesis. Neural precursor cell proliferation in the dentate gyrus of these mice was reduced by approximately 40% compared to wild-type mice, but interestingly this difference was not evident in vitro, suggesting that the effect was not cell autonomous, but caused by a lack of supportive environment/signals in this region of the hippocampus (Eom and Jope, 2009).

A deeper understanding of the structural basis for both autoinhibition and the preference for primed substrates was provided more recently by protein crystallographic studies which succeeded in obtaining structures for GSK3 with its phosphorylated N-terminus bound in the basic pocket adjacent to its active site. These researchers also obtained structures including an inhibitory peptide from LRP6 (low-density lipoprotein receptor-related protein 6), which is a Wnt co-receptor and proposed GSK3 inhibitor (Stamos et al., 2014). This work provides strong support for a competitive model for autoinhibition in which the active site and basic pocket can be bound either by the phosphorylated N-terminus or by other phosphorylated inhibitory sequences such as LRP6, competing with true substrates (Stamos et al., 2014).

Apparent GSK3 orthologs can be identified from across eukaryotes, in groups evolutionarily distant from Metazoa, including Plantae, Amoebazoa, Trypanosomatidae, Stramenopiles, Alveolatae and *Giardia*. Importantly, these encoded kinases show conservation of a loop unique to GSK3 which contributes to the primed substrate binding pocket, interacting with the phosphorylated priming residue in its substrates (Frame et al., 2001). This implies the emergence of GSK3 orthologs very early in eukaryotic evolution and the presence of the enzyme throughout the evolution of complex and multicellular organisms. It seems a key point to these authors that since the dominant factor influencing the phosphorylation of many individual substrates by GSK3 is a simple amino acid context which would be expected to evolve rapidly (Tan et al., 2009), this may help to explain the

large number and functional diversity of its substrates. One can speculate that whereas the substrate proteins of many protein kinases act in functionally related processes driven in part by shared regulation by the kinase in question, since the clearest shared characteristic of the substrates of GSK3 is that almost all display phosphorylation on primed amino acids, these proteins form a less functionally cohesive group. This hypothesis that the many GSK3 substrates share most notably aspects of protein chemistry rather than cellular function leads to the view that its key function may be to allow the amplification of individual phosphorylation events into clusters of sites with a much greater addition of negative charge to that region of a protein.

2.2 Not all GSK3 mediated phosphorylation events are influenced by AKT/PI3K: β-catenin phosphorylation, AXIN and the WNT pathway

β-catenin is the central signalling molecule in the canonical Wnt pathway, an important regulatory system which controls cell fate, and when disturbed is a major driver of cancer. β-catenin is a genuinely multifunctional protein, with two major cellular pools. It localises firstly at the plasma membrane as part of multiprotein cell-cell junction complexes (adherens junctions) and secondly free in the cytosol or nucleus. The free form is a vital transcriptional regulator of specific target genes, but in cells not stimulated with Wnt ligands, it is maintained at low level by its GSK3 phosphorylation, which targets β-catenin for ubiquitination and degradation (Nusse and Clevers, 2017).

The first demonstration of the relationship between endogenous GSK3 and Wnt/β-catenin was shown in *Xenopus*, where GSK3 acts as a negative regulator of dorsal axis formation (Dominguez et al., 1995) and subsequently, β-catenin was identified as a GSK3 substrate (Yost et al., 1996). The turnover of β-catenin is promoted by a scaffold protein, Axin, which mediates multiprotein complex formation consisting minimally of GSK3, β-catenin and Adenomatous Polyposis Coli (APC) (Hinoi et al., 2000; Nusse and Clevers, 2017). In this assembled destruction complex, GSK-3 phosphorylates β-catenin in its N-terminal region, thereby assigning it for polyubiquitination and subsequent proteasomal degradation (Hinoi et al., 2000). The N-terminal region of β-catenin consists of 4 serine (S)/threonine (T) residues (S33, S37, T41, and S45) the first three of which fit the GSK-3 phosphorylation consensus (Kimelman and Xu, 2006). S45 on the other hand, is not a target site for GKS3 phosphorylation (Hagen and Vidal-Puig, 2002; Wong et al., 2001) but instead β-catenin phosphorylation at S45 is mediated by an Axin-Casein Kinase I (CKI) complex independently of GSK3 and is required to prime for subsequent GSK3 phosphorylation of β-catenin at more N-terminal residues (Amit et al., 2002; Liu et al., 2002). However, the GSK-3 mediated phosphorylation of Axin and APC is required to increase their stability and increase their binding to β-catenin (Liu et al., 2002; Rubinfeld et al., 1996).The phosphorylation and hence destruction of β-catenin is blocked in cells stimulated by Wnt ligands acting on their cell surface receptors of the Frizzled family due to the ligand-induced phosphorylation of LRP5/6 co-receptors. This Wnt-ligand-induced phosphorylation of LRP6 in proline rich motifs allows

LRP6 to inhibit the GSK3 pool engaged in the Axin destruction complex and hence stabilise β-catenin (Niehrs and Shen, 2010; Nusse and Clevers, 2017; Stamos et al., 2014).

3. GSK3 action within the PI3K-AKT-mTOR signalling network: feedback and crosstalk

A key component of the cell regulatory mechanisms controlling cell growth and proliferation is represented by the PI3K (Class I Phosphoinositide 3-Kinase)-AKT-mTOR (Mechanistic Target Of Rapamycin) signalling network. Many diverse signals including a range of mitogens and growth factors activate cell surface receptors which drive cell growth and proliferation in part through the activation of PI3K, the synthesis of its primary lipid product, PIP_3, (phosphatidylinositol 3,4,5-trisphosphate) and downstream activation of the protein kinases AKT and mTOR (Figure 2 and (Kriplani et al., 2015; Manning and Toker, 2017; Vanhaesebroeck et al., 2012))

As discussed in Section 2.1, GSK3 is phosphorylated at its N-terminus, inhibiting its kinase activity, by several different protein kinases. Strong evidence indicates that multiple kinases are capable of mediating the phosphorylation of GSK3 under different circumstances, including for example, PKA probably scaffolded by AKAP220 (Li et al., 2000; Tanji et al., 2002). However, it is the AKT kinases, activated by many stimuli including growth factors and insulin via the class I PI 3-Kinases (PI3K) which have received the most attention and support as GSK3 kinases which phosphorylate its autoinihibitory N-terminal serine (Ser9/Ser21) (Buttrick and Wakefield, 2008; Freyberg et al., 2010; Laurent et al., 2014; Manning and Toker, 2017). GSK3 regulation by AKT (also known as PKB) was first demonstrated in L6 myotubes stimulated with insulin (Cross et al., 1995), but it has been shown in many cell types that diverse stimuli increase GSK3 phosphorylation by activation of PI3K and AKT and also that unstimulated 'basal' GSK3 phosphorylation is reduced by selective inhibition of PI3K and/or AKT (Green et al., 2008; Laurent et al., 2014; Leslie et al., 2001; Moore et al., 2013; Shaw et al., 1998; Zhang et al., 2013).

These data show that cellular stimulation with growth factors can lead to a robust reduction in measurable GSK3 activity (over 50% in some cases) and an even greater reduction in the phosphorylation of a few GSK3 substrates (e.g. Glycogen Synthase in muscle (McManus et al., 2005)) mediated via AKT. However, as mentioned in Section 2.1, this inhibition of GSK3 is partial and critically, the phosphorylation of some GSK3 substrates, such as β-catenin, is not affected by changes in AKT activity (Ding et al., 2000; McManus et al., 2005; Ng et al., 2009) and for most GSK3 substrates evidence addressing this point is lacking.

3.1 GSK3 substrates regulated via PI3K-AKT-GSK3

A diverse set of proteins have been shown to be phosphorylated by GSK3 and regulated at least in part through inhibitory N-terminal GSK3 phosphorylation by AKT (See Table 1). These proteins could be considered to be amongst the functional downstream effectors which are controlled in part by mitogenic/anabolic PI3K-AKT signalling and propagate the effects of its activity. Recent work has

identified the RNA binding protein and suppressor of p53 translation, RNPC1, as a GSK3 substrate (Zhang et al., 2013). This phosphorylation by GSK3 derepresses p53 translation and significantly, is influenced by input from PI3K-AKT. Selective pharmacological inhibition of AKT reduced GSK3 phosphorylation, increased RNPC1 phosphorylation and p53 expression but effects on p53 expression were absent in cells lacking RNPC1.

Equally, GSK3 has been shown to phosphorylate a number of proteins which are themselves recognised components of the PI3K-AKT-mTOR signalling network. The inhibition of the assayable GSK3 pool by growth factors and AKT therefore implies likely roles for GSK3 in feedback regulation of growth factor/PI3K/AKT signalling. Accordingly, as seen in other well-studied examples of signalling feedback within individual systems, these events seem likely to act in specific cell types under different conditions (Carracedo and Pandolfi, 2008; Copps and White, 2012).

One simple direct analysis of feedback between GSK3 and AKT was revealed by a human kinome siRNA knockdown screen looking for kinases which affected EGF-stimulated AKT activity in breast cancer cells (Lu et al., 2011). In two breast cancer cell lines stimulated with EGF and in IGF-treated mouse embryo fibroblasts (MEFs) genetically deleted for GSK3 isoforms, loss of GSK3 was associated with reduced activation of AKT (Lu et al., 2011). Similar effects have since been observed in prostate cancer cells with knockdown of GSK3β causing reduced AKT phosphorylation (Darrington et al., 2012) although the precise mechanisms are unclear.

GSK3 has been shown to phosphorylate the tumour suppressor phosphatase and functional PI3K antagonist, PTEN, on its regulatory C-terminal tail, at Thr366 and possibly Thr262, requiring priming at Ser370 probably mediated by CK2 (Al-Khouri et al., 2005; Maccario et al., 2007). Although the precise functional consequences of Thr366 phosphorylation on PTEN function are not clear and may be cell type specific, this broadly reduces PTEN function or abundance and also promotes interaction with the nucleolar protein MSP58 and potentially provides negative feedback suppression of PI3K-AKT signalling (Al-Khouri et al., 2005; Jang et al., 2013; Maccario et al., 2007; Masson et al., 2016; Okumura et al., 2005; Tibarewal et al., 2012).

In contrast, a potential positive feedback loop acting between GSK3 and AKT is the reported phosphorylation of the TORC2 component, and therefore AKT activator, RICTOR, by GSK3 in a recognised phosphodegron sequence. This GSK3-mediated phosphorylation of RICTOR seemed to promote an interaction with the E3 ubiquitin ligase FBXW7, targeting RICTOR for proteasomal degradation (Koo, J. et al., 2015).

Similarly, cellular sensitivity to insulin is tightly regulated, integrating many signals and environmental factors, several of which act at the level of the Insulin Receptor Substrate (IRS) proteins, which link the Insulin Receptor kinase to downstream signalling, including the activation of AKT and in many cells the N-terminal phosphorylation of GSK3 (Copps and White, 2012). GSK3 has been shown to phosphorylate both IRS1 and IRS2, reducing the insulin-stimulated activation of PI3K and AKT (Liberman and Eldar-Finkelman, 2005; Sharfi and Eldar-Finkelman, 2008). However, these GSK3 mediated phosphorylation events do not appear to be influenced by AKT and evidence for priming phosphorylation by PKCβ and JNK imply regulation by other signalling inputs (Liberman et al., 2008; Sharfi and Eldar-Finkelman, 2008).

A new twist, and possible reciprocal inhibition of AKT by GSK3 was reported in studies of Th17 primary T cells and HEK293 cells engineered to express the IL1 receptor (Gulen et al., 2012). This work showed that GSK3α could bind and phosphorylate AKT1 on threonine 312, which is adjacent to the key activating phosphorylation site T308 in the activation loop, leading to inhibition of AKT kinase activity (Gulen et al., 2012). This is in keeping with existing data from unbiased phosphoproteomic screens identifying AKT1 T312 and analogous residues on AKT2 and AKT3 as phosphorylation sites. Notably most of this phosphoproteomic data was obtained from T cells (www.phosphosite.org), although T312 has no priming residue in the +4 position. The authors propose that GSK3α inhibition, caused by its IL1 stimulated IKK mediated phosphorylation, thus leads to AKT activation and in turn canonical mTOR activation (Gulen et al., 2012).

As discussed in Section 2.1, an important mechanism by which peptide growth factors promote anabolic metabolism is by the PI3K dependent activation of AKT, which in turn phosphorylates and inhibits TSC2, allowing the downstream activation of the growth-promoting kinase mTOR. There is good evidence that GSK3 also mediates crosstalk between the canonical Wnt and AKT-mTOR signalling pathways, by phosphorylating distinct inhibitory sites on TSC2 in cells stimulated with Wnt ligands, and leading to Wnt-stimulated mTOR activation (Inoki et al., 2006; Mak et al., 2005). A key factor in this Wnt-mediated TSC phosphorylation is the apparent scaffolding of GSK3 and the TSC proteins by Axin, analogous to the role of Axin in GSK3 phosphorylation of β-catenin, with potential implications for other GSK3 mediated phosphorylation events (Beurel et al., 2015; Inoki et al., 2006; Mak et al., 2005).

4. Possible therapeutic use of GSK3 inhibitors: Bipolar disorder, Alzheimer's Disease, Cancer, Parasitic infections and more.

Lithium salts have been widely used as a mood stabilizer in patients suffering from bipolar disorder for more than 50 years. Although their mechanism of action is still not completely understood, lithium compounds have been found to inhibit GSK-3 in vitro and in vivo (Stambolic et al, Curr Biol, 1996; Caberlotto et al., 2013). Lithium, however, also inhibits several other protein kinases and there are many GSK3 independent mechanisms proposed to explain the clinical effects of lithium such as serotonin neurotransmission synergism (Scheuch et al., 2010), inhibition of inositol monophosphatases and decreases in IP_3 turnover (Berridge, 2016; Wallace, 2014) as well as phosphatases converting 3'-phosphoadenosine 5'-phosphate (PAP) (Yenush et al., 2000) or decrease in nitric oxide in serum (Ghasemi et al., 2009). Although it seems likely that the inhibition of GSK3 by lithium contributes to its efficacy in bipolar patients, more selective inhibitors of GSK3 have emerged, yet have not shown efficacy in these patients (Pandey and DeGrado, 2016).

The development of more selective small molecule inhibitors of GSK3 has allowed their consideration as drug candidates for pathologies in which GSK3 is known to play a role. Several such inhibitors have been developed of the last 15 years or so, including CT99021, NP031112, BRD1652, LY2090314 and BRD0209 and have been tested in pre-clinical disease models and in some cases clinical trials, particularly for a range of neurological conditions (Avrahami et al., 2013).

Of the above mentioned GSK3 selective drugs, two have gone into clinical trials, NP031112 and LY2090314. Tideglusib (also known as NP031112) is a non-ATP competitive GSK-3 inhibitor that has shown a potential effect for neurodegenerative disorders *in vivo* (Luna-Medina et al., 2007) and initially entered clinical trials for Alzheimer's disease and progressive supranuclear palsy (del Ser et al., 2013). Although initial studies have been discontinued after failing to reach its primary endpoint, further clinical trials including those for Myotonic Dystrophy and Autism Spectrum Disorder are ongoing (www.clinicaltrials.gov). LY2090314 has been studied in clinical trials for advanced cancers as a co-therapy. It was well tolerated and safe, but the lack of efficacy led to the termination of some of the trials and the authors did not report a therapeutic benefit (Zamek-Gliszczynski et al., 2013; Zamek-Gliszczynski et al., 2014). Although intense investment was required for the development of these existing, reasonably selective, GSK3 inhibitors, the lack of rapid success and realisation that GSK3 has a very large numbers of functionally diverse substrates appears to have reduced the investment in many of these programmes.

Interestingly, subsequent studies revealing the structure of substrate and inhibitor bound GSK3 conformations imply the possibility of developing inhibitors with some selectivity for specific substrates (Stamos et al., 2014), which may provide a route to translate the understanding of the importance of GSK3 in pathology into successful therapies.

Conflict of interest

The authors declare they have no conflict of interest with publication of this manuscript

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Table I. GSK3 substrates and evidence for the regulation of their phosphorylation by AKT mediated inhibition of GSK3. Recognised GSK3 substrates have been presented after assessment of the strength of evidence supporting the regulation of their phosphorylation by AKT inhibition of GSK3. Firstly, GSK3 substrates for which their phosphorylation has been analysed unambiguously (e.g validated phospho-specific antibodies) in cells expressing Ser9/21 mutated GSK3 and/or in which GSK3 mediated phosphorylation can be altered rapidly by selective activation or inhibition of AKT (Group I). Secondly, substrates which have been proposed to be regulated via AKT-GSK3 based upon evidence correlating their phosphorylation with GSK3 status (Group II). Thirdly proteins which are direct substrates of both AKT and of GSK3, which may confound analysis of independent direct and indirect influences of AKT (Group III). Group IV shows proteins for which extensive analysis by multiple methods in multiple cell types shows that GSK3 mediated phosphorylation is independent of AKT. Although this group contains only β-catenin, it seems likely that many other proteins are regulated similarly, but currently lack the necessary weight of evidence. We have not included within this table the many GSK3 substrates for which the role of AKT in their phosphorylation by GSK3 has not been tested experimentally.

Group I. GSK3 Substrates regulated by AKT inhibition of GSK3

Glycogen synthase	(McManus et al., 2005)
CEBPα	(Chen et al., 2016)
CRMP2	(Cole et al., 2006; Wakatsuki et al., 2011)
CRMP4	(Cole et al., 2006)
BCL3	(Viatour et al., 2004)
RNPC1	(Zhang et al., 2013)
TAU	(Jiang et al., 2015; Wang et al., 2015)
VDAC	(Pastorino et al., 2005)

Group II. Correlative evidence for AKT regulation of GSK3

CREB	(Tullai et al., 2007)
CTPS	(Higgins et al., 2007)
Dynamin I	(Smillie and Cousin, 2012)
C-JUN	(Lopez-Bergami et al., 2010)
MCL1	(Choudhary et al., 2015; Koo, Junghui et al., 2015)

C-MYC	(Vartanian et al., 2011)
Myocardin	(Abdalla et al., 2013; Liu et al., 2005)
Presenilin-1	(Maesako et al., 2011; McCubrey et al., 2017; Uemura et al., 2007)
PTEN	(Al-Khouri et al., 2005)
SNAIL	(Zhou et al., 2004)
SREBP1	(Kim et al., 2004)
VHL	(Thoma et al., 2007)

Group III. Direct Substrate of both GSK3 and AKT

ACLY	(Berwick et al., 2002; Covarrubias et al., 2016)
FAK	(Villa-Moruzzi, 2013)
Heat shock factor 1	(Carpenter et al., 2015; Dayalan Naidu and Dinkova-Kostova, 2017)
HIF1α	(Masoud and Li, 2015; Mottet et al., 2003)
MDM2	(Kulikov et al., 2005; Ogawara et al., 2002; Zhou et al., 2001)
MLK3	(Figueroa et al., 2003)
NDRG1	(Murray et al., 2004; Sommer et al., 2013; Zurashvili et al., 2013)
TSC2	(Inoki et al., 2006)

Evidence supports AKT-independence

β-catenin	(Ding et al., 2000; McManus et al., 2005; Ng et al., 2009)

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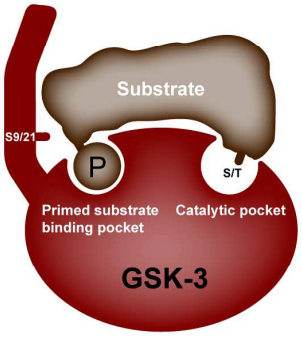
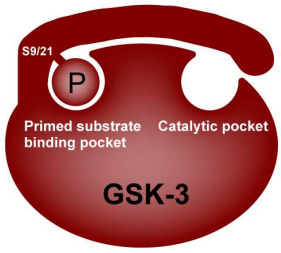
Figure legends

Figure 1. Mechanism of primed substrate selectivity and physiological inhibition of GSK3. The catalytic cleft and adjacent phosphate binding pocket are represented, showing the shared mechanisms of (A) primed substrate recognition, (B) autoinhibition in cis through occupation by the phosphorylated N-terminus of the phospho-binding pocket and (C) inhibition in trans by inhibitory binding partners such as LRP6 which have phosphorylated sequences capable of engaging the phospho-binding pocket.

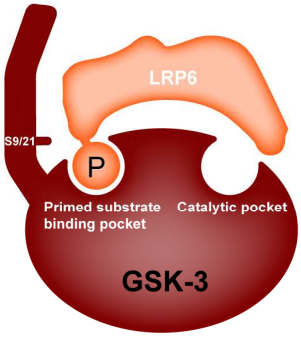
Figure 2. GSK3 and the PI3K-AKT-mTOR signalling network. A model for signalling network is shown, illustrating activation of PI3K by diverse cell surface receptors (including many mitogenic RTK, GPCR and Cytokine receptors), stimulated synthesis of PIP_3 and the downstream activation of signalling through PIP_3-binding proteins including AKT to promote cell growth, proliferation and anabolic metabolism. AKT phosphorylates and inhibits GSK3 in addition to many other substrates including TSC2, FOXO proteins, TBC1D4. GSK3 directly phosphorylates several components of the signalling network (including TSC2, RICTOR, PTEN and AKT itself, see text) with potential feedback effects on signalling.



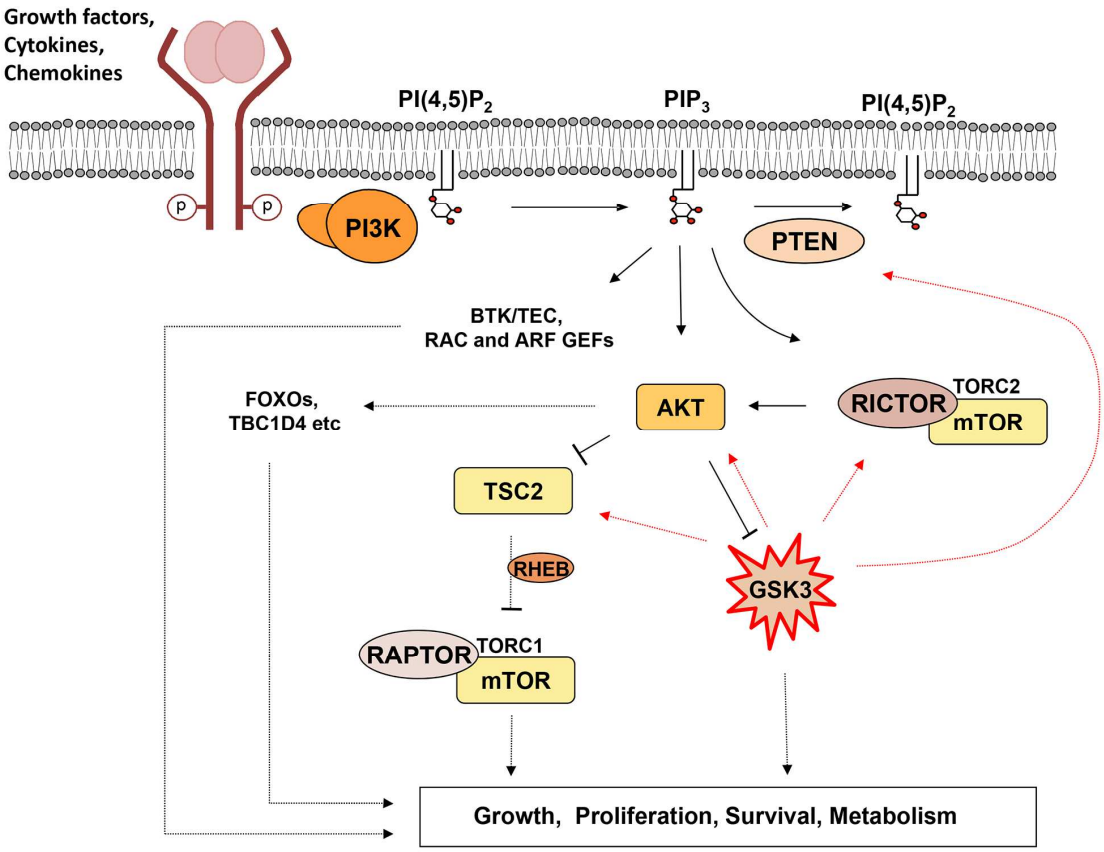
A. Substrate engaged



B. Autoinhibition - *in cis*



C. Inhibition - *in trans*



Conflict of interest

The authors declare they have no conflict of interest with publication of this manuscript

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