STRUCTURE-FUNCTION ANALYSIS OF
GLYCOGEN SYNTHASE KINASE (GSK-3) ISOFORMS

DISSERTATION

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Glycogen synthase kinase-3 (GSK-3) isoforms, GSK-3α and GSK-3β, are serine/threonine kinases involved in numerous cellular processes and diverse diseases, including Alzheimer’s disease, diabetes, and mood disorders. Accumulating evidence suggests that GSK-3 isoforms exhibit distinct activities and increasingly challenges the conventional belief that GSK-3 isoforms are functionally redundant. Despite abundant GSK-3-related research, the basis for differential functions of GSK-3 isoforms remains unresolved. Logically, the divergent regions of GSK-3, the N- and C-termini, might be predicted to mediate the differential activities of GSK-3 isoforms at the post-translational level. Herein we test the hypothesis that the divergent N- and C-terminal regions and conserved key residues of GSK-3 isoforms are functionally significant. To test our hypothesis we performed a structure-function analysis of GSK-3α and GSK-3β in mammalian cells. Deletion constructs of the non-catalytic N- and C-terminal domains in both GSK-3 isoforms were created as well as constructs containing point mutations of key regulatory residues. We examined the effect of these deletions and point mutations on GSK-3 activity, protein interactions, tyrosine autophosphorylation, and subcellular localization. We found that the N-termini of both GSK-3 isoforms are dispensable for activity, protein interaction with Axin GID, and tyrosine autophosphorylation, but are required for the GSK-3α-specific interaction with RACK1 WD4-7 and proper
localization. In turn, progressive C-terminal deletions resulted in a loss of activity, impaired ability to interact with protein binding partners, a gradual loss of autophosphorylation, and mislocalization. Taken together, these observations imply that deletion of the C-termini of GSK-3 isoforms compromises proper protein folding and suggest that the C-terminal regions make significant contributions to the structural integrity of GSK-3 enzymes. Furthermore, our data predict that the development of therapeutic modulators targeting the C-terminus may result in isoform-specific GSK-3 inhibition through destabilization of GSK-3 structure.
Dedicated to my parents, Pam Buescher and John Buescher, for providing inspiration and teaching me the value of hard work.
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LIST OF ABBREVIATIONS

Δ.................... deleted
AD.................... Alzheimer’s disease
APC............... adenomatous polyposis coli
Arg................. arginine
Asp............... asparagine
ATP............... adenosine triphosphate
BGS............... bovine growth serum
C-............... carboxyl
cAMP.............. cyclic AMP
Cdk................. cyclin-dependent kinase
CHO................ Chinese hamster ovary
CLK................. cyclin-dependent kinase like kinase
CK................. casein kinase
CMGC.............. cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinases, and Cdk-like kinases
CMV................ cytomegalovirus
Cpm................ counts per minute
CT.................. C-terminus
DMEM................ Dulbecco’s modified Eagle’s medium
Dsh.................. disheveled
DTT.................. dithiothreitol
ECL.................. enhanced chemiluminescence
EGF.................. epidermal growth factor
EGFP................. enhanced green fluorescent protein
EGR.................. early growth factor
ER.................. endoplasmic reticulum
ESC.................. embryonic stem cell
et al................ et alia (Latin)/and others
FL.................. full-length
FOP.................. Super 8X FOPFlash
FRAT............... frequently rearranged in T-cell lymphoma
GID.................. GSK-3 interaction domain
GSK-3.............. glycogen synthase kinase-3
GST.................. glutathione S-transferase
HEK............... human embryonic kidney

xiii
WCM........... Wnt-3a conditioned media
WT............... wild type
1.1 Eukaryotic Protein Kinases

Protein phosphorylation is a ubiquitous, reversible, post-translational modification that serves as a key biological regulatory mechanism. The protein kinase family is a large group of enzymes that mediate phosphorylation by catalyzing the covalent addition of a phosphate group to an amino acid side chain in a protein substrate. The introduction of a charged phosphate group often causes conformational modifications of protein structure due to changes in electrostatic interactions, ultimately resulting in alterations of protein function [1].

Most eukaryotic protein phosphorylation is accomplished by a single, large superfamily of kinases. Within this superfamily are two major subdivisions: serine/threonine kinases, which phosphorylate the hydroxyl group of serine or threonine residues, and tyrosine kinases, which phosphorylate the hydroxyl group of tyrosine residues. Despite differences in structure, regulation, and substrate specificity, all protein kinase family members share characteristic catalytic domains, flanked by distinct N- and C-termini. While the N- and C-termini confer discrete regulatory properties, the catalytic domain serves a ubiquitous function of binding and orientating ATP and protein
substrates in a manner conducive to the transfer of the ATP γ-phosphate to an amino acid side chain. The catalytic domain consists of twelve distinct subdomains and is not uniformly conserved, but rather, exhibits highly conserved features separated by regions of low conservation [2, 3]. The conservation of key catalytic features implies protein kinases share a common phosphotransfer mechanism and further suggests derivation of protein kinases from a single archetypal gene.

The initial discovery of a reversible protein phosphorylation regulatory mechanism is accredited to Edmond H. Fisher and Edwin G. Krebs who were awarded the 1992 Nobel Prize in Medicine and Physiology for their observations of the interconversion of the active and inactive forms of phosphorylase kinase [4-6]. The importance of protein phosphorylation in disease was later highlighted by the discovery that the transforming proteins of many oncogenic retroviruses possessed protein kinase activity [7-10]. While these initial discoveries propelled intensive investigation into the regulatory role of protein phosphorylation, the identification of remarkably conserved signature sequences in the catalytic domain of distinct protein kinases [11] led to molecular cloning strategies and sequencing techniques that dramatically accelerated the identification of protein kinases [2, 12].

It is now known that the human genome [13-15] encodes over 500 protein kinases, which represents 1.7% of all human genes. Nearly half of the putative protein kinases identified in the human genome map to disease loci [16, 17]. Additionally, aberrant protein phosphorylation has been linked to numerous human diseases, including cancer and neurodegenerative disorders. Thus, the physiological importance of protein kinase activity cannot be underestimated. Further, since protein kinases are major drug targets
for disease intervention [18], a greater knowledge of protein kinase structure and function may lead to improved therapeutics.

1.2 Discovery of Glycogen Synthase Kinase-3 (GSK-3) Isoforms

Within the large and diverse protein kinase superfamily, is the CMGC group of protein kinases [19], which includes cyclin-dependent kinases (CDKs), mitogen-activated protein kinase (MAPKs), glycogen synthase kinases (GSKs), and CDK-like kinases (CLKs). Of special interest to our lab are the GSKs, particularly GSK-3s. GSK-3 was originally identified as the third kinase that possessed activity towards glycogen synthase, the rate-limiting enzyme in glycogen metabolism [20, 21]. GSK-3 activity inhibits glycogen synthase through ordered phosphorylation of multiple serine residues within a proline-rich stretch of amino acids, defining GSK-3 as a proline-directed member of the serine/threonine kinase family [21-24]. Ten years after its discovery, GSK-3 was cloned and two mammalian isoforms were identified in rat brain as products of distinct genes, GSK-3α and GSK-3β [25]. In human, GSK-3α and GSK-3β have been mapped to chromosomes 19q13.2 and 3q13.3, respectively [26, 27]. At the protein level, GSK-3α and GSK-3β are highly homologous, sharing a 98% amino acid identity within their internal kinase domains but become divergent in sequence outside this region (Figure 1.1). At the amino terminus, GSK-3α contains a glycine-rich extension of unknown significance accounting for its larger size of 51 kD (Figure 1.1). This glycine-rich region is absent from GSK-3β resulting in its smaller size of 47 kD (Figure 1.1). In the carboxyl terminus, GSK-3 isoforms share a mere 36% identity in amino acid sequence (Figure 1.1).
**Figure 1.1** Amino acid sequence alignment of human GSK-3α and human GSK-3β. Identical residues are shaded in dark gray and similar residues are shaded in light gray. Conserved amino acid residues with functional significance are circled. The catalytic domain is boxed. Numbering refers to amino acid sequence of GSK-3α.
While splice variants of GSK-3α have not been identified, alternative splicing of GSK-3β has been reported. A GSK-3β splice variant, termed GSK-3β2, contains the splice insert of exon 8b, which encodes an extra 13 amino acid sequence in the kinase domain [28, 29]. GSK-3β2 has been detected in human and rodent species and represents a minor fraction of total GSK-3β. The insert in GSK-3β2 may alter kinase activity, protein interactions and subcellular localization and has also been implicated in mediating neuronal specific functions [28-31]. Alternative splicing of exon 10 has also been reported for GSK-3β; exclusion of both exon 8b and exon 10 has been associated with increased GSK-3 activity in neurons [29, 32].

GSK-3 has been widely conserved throughout evolution and homologues have been identified many eukaryotic model organisms, including Danio rerio [33], Xenopus laevis [34-36], Drosophila melanogaster [36-38], Caenorhabditis elegans [39, 40], Dictyostelium discoideum [41], Schizosaccharomyces pombe [42] and Schizosaccharomyces cerevisiae [43-46]. In general, vertebrate genomes exhibit two gene products, one of which resembles human GSK-3α while the other resembles human GSK-3β [47]. Conversely, invertebrate species often express a single homologue that more closely resembles human GSK-3β, suggesting a more recent evolution of GSK-3α [47]. All GSK-3 homologues share a significant degree of homology, particularly within the kinase domain and key regulatory residues are also highly conserved, indicating an evolutionary conservation of protein function and a fundamental role for GSK-3 activity.

Although originally isolated from rabbit skeletal muscle, GSK-3α and GSK-3β mRNA and protein have been detected in many other tissues. Both isoforms are believed to exhibit ubiquitous expression [25, 48, 49]. At the cellular level, GSK-3β localizes
predominantly to the cytosol [50] but has also been detected in the mitochondria and nucleus [51, 52]. Nuclear and mitochondrial pools of GSK-3β exhibit particularly high activities relative to cytoplasmic GSK-3β [52]. Dynamic nuclear localization of GSK-3β has been demonstrated in response to cell cycle progression [53], apoptotic stimuli [54, 55], and through interaction with the GSK-3 binding partner FRAT (frequently rearranged in T-cell lymphoma) [56]. The subcellular localization of GSK-3α has been studied less extensively. In rat cerebellum, GSK-3α localizes to the cytoplasm and nucleus but is excluded from mitochondria [57]. GSK-3α also exhibits dynamic nuclear localization through interaction with FRAT [56], but responses to other stimuli have not been reported. GSK-3α and GSK-3β both display a homologous nuclear localization signal within the catalytic domain that influences nuclear localization of GSK-3β [58]. The first nine amino acids of GSK-3β are also important for nuclear localization [58] and may serve a similar role in GSK-3α since these residues are largely conserved.

1.3 GSK-3 Structure

Although the crystal structure of GSK-3α has not been reported, several groups have determined the crystal structure of GSK-3β at 1.8 Å - 2.9 Å by molecular replacement using X-ray diffraction data [59-63]. Structural analysis demonstrated that GSK-3β adopts a common kinase fold structure that consists of two domains: an N-terminal β-strand domain that forms a β-barrel and a C-terminal α-helical domain (Figure 1.2). Proper alignment of these two domains is essential for optimal positioning of the catalytic groups involved in the γ-phosphate transfer [59, 60]. Two conserved lysine residues,
Lys$^{85/86}$ in GSK-3β and Lys$^{148/149}$ in GSK-3α, are found at the interface of the two domains and are important for binding ATP and stabilizing the γ-phosphate during transfer to the substrate [2, 59, 62-64]. Single or double mutation of Lys$^{85/86}$ in GSK-3β or Lys$^{148/149}$ in GSK-3α is commonly employed to generate catalytic inactive variants of GSK-3 isoforms [34, 35, 65, 66]. Like many kinases, GSK-3 isoforms have a particular requirement for divalent metal ions that are important for coordinating ATP for γ-phosphate transfer. GSK-3 has a unique requirement for two divalent magnesium ions [67] and structural characterization indicates these divalent magnesium ions bind at the interface of the two domains [62, 63] (Figure 1.2). Outside the catalytic core structure, a distinct region in the carboxyl terminus (approximate GSK-3β amino acids 342-388) is described as having a unique structure consisting of short helices and loops that pack against the large core helical domain [59, 60, 62] (Figure 1.2). The most distal N- and C-terminal regions of GSK-3β are structurally disordered and believed to be very flexible [59, 60, 63].
Figure 1.2 Overall structure of GSK-3β (amino acids 23-386) in complex with an ATP analogue as determined by Aoki et al. (protein data bank (PDB) reference 1j1c) [62]. The N-terminus of GSK-3β is comprised of β-sheets while the C-terminus consists largely of α-helices. At the interface of the N- and C-terminal domains is the binding site for ATP, magnesium, and substrate. The glycine-rich loop defines the beginning of the catalytic domain. The activation loop contains a conserved tyrosine residue believed to enhance GSK-3 activity when phosphorylated.
Proper alignment of the N-terminal and C-terminal domains is important for optimal configuration of the substrate-binding site and catalytic site of a kinase. In many kinases, favorable conformations of both sites are influenced by the conformation of an activation loop structure, which is modulated by phosphorylation. In GSK-3 isoforms, the activation loop contains a conserved tyrosine residue, Tyr$^{279}$ and Tyr$^{216}$ in human GSK-3α and GSK-3β respectively, which, when phosphorylated, is believed to facilitate GSK-3 activity [68] (Figure 1.2). Indeed, crystal structure analysis of GSK-3β demonstrates that phosphorylation of Tyr$^{216}$ induces rotation of the tyrosine side chain from the substrate-binding site, thereby promoting substrate accessibility [59, 60, 69]. However, structural analysis also indicates that in the absence of Tyr$^{216}$ phosphorylation, the activation loop is still able to achieve a conformation receptive to substrate binding and catalytic activity [59, 60, 62], strongly resembling the phosphorylated activation loop structure [61, 69]. Furthermore, numerous functional studies suggest Tyr$^{279/216}$ phosphorylation is important for GSK-3 activity [68, 70-78], while other reports indicate the effect is modest [69], and even insignificant [79].

The mechanism of Tyr$^{279/216}$ phosphorylation has also been debated. Several kinases have been implicated in mediating GSK-3α/β Tyr$^{279/216}$ phosphorylation [74, 80, 81], but mounting evidence suggests that GSK-3α/β autophosphorylate Tyr$^{279/216}$ using an intramolecular tyrosine kinase activity, which converts GSK-3 isoforms to intermolecular serine/threonine kinases [66, 68, 72, 77]. Recently, GSK-3β autophosphorylation was shown to occur post-translationally during protein folding in a heat-shock protein 90 (Hsp90)-dependent manner [72]. Post-translational autophosphorylation was also shown to be resistant to phosphatase activity [72] though other data demonstrate susceptibility of
phosphorylated Tyr\(^{279/216}\) to phosphatase treatment [55, 68, 75, 77]. Thus, the significance and regulation of GSK-3\(\alpha\) Tyr\(^{279}\) and GSK-3\(\beta\) Tyr\(^{216}\) phosphorylation is unclear. Yet, conventional wisdom holds that high levels of Tyr\(^{279}\) and Tyr\(^{216}\) phosphorylation in resting cells confer constitutive activity to GSK-3 isoforms [68].

### 1.4 GSK-3 Substrate Recognition

Although GSK-3 was originally named based on its discovery as a glycogen synthase kinase, GSK-3 isoforms are now recognized as broadly influential, multifunctional serine/threonine kinases that regulate many fundamental cellular processes. GSK-3\(\alpha\) and GSK-3\(\beta\) activity towards substrates is generally inhibitory and a panoply of substrates have been reported including numerous transcription factors [50, 82-87], metabolic proteins [20, 21, 51, 88, 89], and structural proteins [90-92].

GSK-3 activity towards many substrates is regulated through a synergistic priming phosphorylation mechanism [23, 24]. Priming involves hierarchical pre-phosphorylation of the substrate by another kinase at a site four amino acids C-terminal to the GSK-3 phosphorylation site, generating the GSK-3 recognition motif \(\text{Ser/Thr-X-X-X-Ser/Thr}-P\). The N-terminal \(\text{Ser/Thr}\) is the GSK-3 target site, while the C-terminal \(\text{Ser/Thr}\) is the priming phosphorylation site [23, 24]. The GSK-3 recognition motif must be accessible for efficient phosphorylation and is commonly located within a proline-rich region in the substrate as originally noted in the glycogen synthase substrate [22].

Structural analysis clarified the mechanism of GSK-3 primed substrate recognition by demonstrating that GSK-3\(\beta\) utilizes a triad of conserved basic residues, Arg\(^{96}\), Arg\(^{180}\) and Lys\(^{205}\) corresponding to Arg\(^{159}\), Arg\(^{243}\), and Lys\(^{268}\) in GSK-3\(\alpha\), for recognition [59-61,
The side chains of these residues provide a positively charged binding pocket specific for the negatively charged phosphate group on the primed residue which, upon recognition, serves to position the substrate into the substrate binding groove while also stabilizing alignment of the N- and C-terminal domains of GSK-3 into a catalytically active conformation [59, 60]. Priming is common among GSK-3 substrates and considerably enhances the efficiency of GSK-3 activity by increasing the affinity of GSK-3 for the substrate [94]. Thus, GSK-3 activity can be regulated indirectly by the activity of other kinases and the availability of the primed substrate. Though some substrates require priming, it is not obligatory for the GSK-3-dependent phosphorylation of every substrate [50, 82, 95, 96] and substrates that lack a priming phosphorylation site often mimic phosphorylation through display of a negatively charged residue at the site of priming.

1.5 Inhibition of GSK-3 Activity

Many kinases are activated through effector molecules or second messengers. By contrast, GSK-3 isoforms exhibit high basal levels of activity [68] and require stimuli for inhibition. Numerous signaling pathways inhibit GSK-3 isoforms, including the well-characterized insulin and Wnt signaling pathways. However, the mechanisms of insulin and Wnt signaling-mediated inhibition are distinct [73, 97].

The insulin signaling cascade results in GSK-3 inhibition through protein kinase B (PKB)/Akt-mediated phosphorylation of an N-terminal serine residue, Ser\(^{21}\) in GSK-3\(\alpha\) and Ser\(^{9}\) in GSK-3\(\beta\) [98]. GSK-3\(\alpha/\beta\) Ser\(^{21/9}\) is situated within a conserved seven amino acid motif in each isoform; this sequence context is also important for GSK-3 inhibition
The structural basis for GSK-3α/β Ser<sup>21/9</sup> phosphorylation-mediated inhibition involves an autoinhibitory mechanism in which the phosphorylated Ser<sup>21/9</sup> residue undergoes a conformational transition to occupy the same phosphate-binding pocket that the phosphorylated residue of the primed substrate utilizes [59, 60, 93]. Thus, inhibition of GSK-3α/β through Ser<sup>21/9</sup> phosphorylation results from intramolecular interactions, involving the acquisition of a pseudosubstrate structure that competes with and may preclude substrate binding.

In addition to insulin, a variety of other stimuli can result in GSK-3α/β inhibition through Ser<sup>21/9</sup> phosphorylation. For example, phorbol esters signal through protein kinase C (PKC) to inhibit GSK-3β [100-102]; growth factors, such as epidermal growth factor (EGF), can stimulate p<sup>90<sup>R</sup>RSK</sup>/MAPK-activated protein (MAPKAP) kinase-1β-mediated inhibition of GSK-3α and GSK-3β [103-107]; amino acids result in GSK-3α and GSK-3β inhibition through p<sup>70<sup>S6K</sup></sup> phosphorylation of Ser<sup>21/9</sup> [104, 105, 108]; cyclic AMP (cAMP) activation of protein kinase A (PKA) also inhibits GSK-3 isoforms [109, 110]. Further, dephosphorylation by protein phosphatase 1 [107] and protein phosphatase 2A [104, 105] reverse inhibition although little is known about the regulation of such dephosphorylation events. Taken together, phosphorylation of GSK-3α/β Ser<sup>21/9</sup> represents a common mechanism employed by diverse signaling pathways to inhibit constitutive GSK-3α/β activity, alleviate negative regulation, and promote various downstream events.

The canonical Wnt signaling pathway regulates GSK-3α/β activity through a distinct mechanism that does not involve Ser<sup>21/9</sup> phosphorylation [73, 97], but instead relies on protein interactions. Canonical Wnt signaling is important during development and is
aberrantly activated in numerous human cancers. Investigation in *Drosophila* [111-115] and *Xenopus* [34, 35, 65, 79, 116] established a key regulatory role for GSK-3 isoforms in the highly conserved canonical Wnt signaling pathway. Wnt signaling regulates the phosphorylation and destruction of the GSK-3α/β substrate β-catenin, a transcriptional co-activator. In the absence of Wnt stimulation, β-catenin exists in a cytoplasmic destruction complex formed by the scaffold protein Axin, and includes the tumor suppressor adenomatous polyposis coli (APC), casein kinase 1α (CK1α), and GSK-3α/β [96, 117-122]. In the destruction complex, β-catenin is first primed at Ser45 by CK1 [120, 121] enabling subsequent and sequential GSK-3α/β phosphorylation at Ser41, Ser37, and Ser33 [120, 121], which targets β-catenin for ubiquitination and proteasomal degradation [116, 123, 124]. Signaling is initiated when a secreted Wnt ligand binds the co-receptor Frizzled and low-density lipoprotein receptor-related proteins 5 or 6 (LRP5/6) [125-129]. Wnt ligand binding to co-receptors results in downstream dissociation of the destruction complex [130] and GSK-3 inhibition which leads to β-catenin stabilization [111, 112, 115]. Stabilized β-catenin accumulates and translocates to the nucleus where it binds members of the lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) family of transcription factors to activate transcription of target genes [131-133], such as the proto-oncogene c-MYC [134].

While GSK-3 inhibition through Ser21/9 phosphorylation is not involved in Wnt signaling, phosphorylation of Thr390 in GSK-3β by p38 MAPK inhibits GSK-3β activity, resulting in β-catenin accumulation and increased expression of Wnt target genes [135]. This phosphorylation event is not a direct consequence of activated Wnt signaling per se but due to persistent activation of p38 MAPK. Inhibition by Thr390 phosphorylation may
involve the acquisition of a pseudosubstrate conformation, similar to the inhibitory mechanism of phosphorylated Ser\textsuperscript{21/9}.

Numerous signaling pathways converge on inhibition of GSK-3 through select mechanisms, yet pathway crosstalk is occluded. In Wnt signaling, GSK-3 is sequestered into an Axin-mediated scaffold complex that insulates GSK-3 from other pathway agonists. How other signaling pathways specifically couple pathway agonist to downstream events is unclear but may involve analogous yet undefined compartmentalization strategies.
CHAPTER 2

INTRODUCTION AND THESIS RATIONALE

2.1 GSK-3 as a Therapeutic Target

GSK-3 isoforms are multifunctional kinases involved in fundamental cellular processes, including cytoskeletal integrity, cell survival, proliferation, and cell fate determination. Dysregulation of GSK-3 isoforms is linked to the pathogenesis of multiple diseases including diabetes and neurological diseases such as Alzheimer’s disease and mood disorders. Thus, modulation of GSK-3 activity has great therapeutic potential, which has spurred the identification and development of many small molecule GSK-3 inhibitors [136-148]. The therapeutic potential of some of these compounds has been tested *in vitro* and *in vivo* with promising results. For example, GSK-3 inhibitors confer protection, particularly in neurons, from apoptosis in response to various toxic insults including prion peptide-treatment [149], poly-glutamine-induced toxicity [150], glutamate-induced toxicity [151], and toxicity from amyloid-β [144]. In rodent models of non-insulin-dependent diabetes mellitus (NIDDM), several GSK-3 inhibitors improve glucose tolerance and insulin resistance [145, 146, 152-154]. Additionally, lithium, the highly effective and primary therapeutic agent for bipolar disorder for over 50 years [155] is also a GSK-3 inhibitor [136, 137]. Lithium treatment enhances stroke outcome
in ischemic rats [156, 157]. In Alzheimer’s disease and tauopathy mouse models respectively, lithium reduces the formation of amyloid-β [158] and aggregated tau [159, 160], components of characteristic pathological lesions. Taken together, therapeutic targeting of GSK-3 activity in human disease has great potential.

Though some small molecule GSK-3 inhibitors compete with substrate [143] or magnesium ions [161], the majority of the small molecule inhibitors identified thus far function by targeting the ATP-binding region within the catalytic domain [138-142, 144, 145, 162]. ATP-competitive inhibitors often lack specificity because they target the phospho-transfer features common among the protein kinase superfamily [163]. Not surprisingly, currently available inhibitors also fail to discriminate between GSK-3 isoforms due to the high homology of GSK-3α and GSK-3β within the catalytic domain [163-165]. Thus, a more selective approach to GSK-3 inhibition is highly desirable.

### 2.2 Differential Roles of GSK-3 Isoforms

Long-standing dogma holds that GSK-3 isoforms function redundantly. However, accumulating evidence suggests that GSK-3 isoforms are subject to distinct regulatory mechanisms and exhibit discrete activities in certain settings. This suggests that therapeutic utilization of isoform-specific GSK-3 inhibitors may be beneficial. For example, despite detectable mRNA levels in nearly all tissue, high levels of GSK-3α protein is observed in human testis while GSK-3β protein is especially abundant in human brain, suggesting unique translational regulation [48]. Post-translationally, certain PKC isotypes mediate inhibition of GSK-3β by phosphorylating Ser⁹, but do not modify Ser²¹ of GSK-3α [101]. Further, p38 MAPK phosphorylates and inhibits GSK-3β
specifically, but not GSK-3α despite the presence of an analogous phosphorylation motif [135].

Compelling evidence of differential GSK-3 isoform activity was demonstrated *in vivo* by disruption of the *Gsk-3β* gene in mice, which resulted in extensive hepatocyte apoptosis due to defective NF-κB signaling [166]. *Gsk-3α* is unable to compensate for *Gsk-3β*, as *Gsk-3β* null embryos die around E13.5. Knock-out of *Gsk-3β* in mice is also associated with congenital heart defects [167]. Further, chemical regulation of *Gsk-3β* gene expression in mice has revealed a temporal requirement for *Gsk-3β* in midline development [168] [unpublished data], suggesting additional *Gsk-3β*-specific roles for which *Gsk-3α* is unable to compensate. Conversely, disruption of *Gsk-3α* in mice is not lethal, but results in insulin sensitivity [169]. Male *Gsk-3α* null mice are infertile due to sperm motility defects [unpublished data]. Thus, *GSK-3α* serves biological roles for which *GSK-3β* cannot compensate. Additionally, isoform-specific *gsk-3* morpholino-mediated knock-down in zebrafish reveals divergent functions for *gsk-3* isoforms during cardiogenesis [170].

Differential activity of GSK-3 isoforms is also apparent *in vitro*: 1) in Chinese hamster ovary (CHO) cells *Gsk-3* isoforms exhibit diverse effects on the production of amyloid-β peptides associated with Alzheimer’s disease [158]; 2) in cultured rat cortical neurons *Gsk-3* isoforms differentially regulate transcriptional activity of Smad3/4 and early growth response (EGR)-1 [171]; and 3) in cultured human epidermal keratinocyte cells, *GSK-3* isoforms demonstrated distinct affects on cell migration [172]. Such demonstrations of GSK-3 isoform-specificities indicate that GSK-3 isoforms are not functionally identical, despite their high homology. Reasonably, the divergent regions of
GSK-3 proteins, the N- and C-termini, might be predicted to mediate the differential activities of GSK-3 isoforms at the post-translational level. Thus, a better of understanding of the functional significance of the divergent N- and C-termini might provide insight into mechanisms of isoform-specific GSK-3 activities and may also facilitate the identification of specific target regions for future development of isoform-specific GSK-3 inhibitors.

Herein we test the hypothesis that the divergent N- and C-terminal regions and conserved key residues of GSK-3 isoforms are functionally significant. To test our hypothesis, we performed a comprehensive structure-function analysis of \( \text{GSK-3}\alpha \) and \( \text{GSK-3}\beta \) in mammalian cells. Based on the definition of the protein kinase catalytic domain, previously defined by characteristic conserved sequences [2, 3], we dissected GSK-3 isoforms into N-terminal, catalytic, and C-terminal regions (Figure 2.1). We created deletion constructs of the non-catalytic N- and C-terminal domains in both GSK-3 isoforms to examine the functional significance of these regions. Constructs were also generated containing point mutations of key regulatory residues in the context of the full-length protein to examine the contribution of these residues to GSK-3 function. Specifically, mutation of GSK-3\( \alpha \) Lys\(^{148} \) and GSK-3\( \beta \) Lys\(^{85} \) to arginine is expected to impair catalytic activity of GSK-3 isoforms; mutation of GSK-3\( \alpha \) Ser\(^{21} \) and GSK-3\( \beta \) Ser\(^{9} \) to alanine is predicted to prevent Ser\(^{21/9} \) phosphorylation-mediated inhibition of GSK-3 isoforms; mutation of GSK-3\( \alpha \) Arg\(^{159} \) and GSK-3\( \beta \) Arg\(^{96} \) to alanine is anticipated to impair GSK-3 recognition of primed substrates; mutation of GSK-3\( \alpha \) Tyr\(^{279} \) and GSK-3\( \beta \) Tyr\(^{216} \) to phenylalanine is expected to impair GSK-3 activity; mutation of GSK-3\( \beta \) Thr\(^{390} \) to alanine is predicted to prevent Thr\(^{390} \) phosphorylation-mediated inhibition of GSK-3\( \beta \);
lastly, mutation GSK-3α Pro$^{442/443}$ and GSK-3β Pro$^{379/380}$ to alanine was generated to examine the contribution of these residues to GSK-3 structure.
Figure 2.1 Schematic of GSK-3 mutants – Deletion and point mutants of GSK-3α (A.) and GSK-3β (B.) were created using PCR based cloning and site-directed mutagenesis, respectively. GSK-3 enzymes were deleted from either the N-terminus or consecutively from the C-terminus. Point mutants were generated in the full-length enzyme, namely GSK-3α S21A (Ser²¹ → Ala), K148R (Lys¹⁴⁸ → Arg), R159A (Arg¹⁵⁹ → Ala), Y279F (Tyr²⁷⁹ → Phe), and P442A/P443A (Pro⁴⁴²/⁴⁴³ → Ala), and GSK-3β S9A (Ser⁹ → Ala), K85R (Lys⁸⁵ → Arg), R96A (Arg⁹⁶ → Ala), Y216F (Tyr²¹⁶ → Phe), and P379/380A (Pro³⁷⁹/³⁸⁰ → Ala). The N-terminus is designated by white. The catalytic domain is colored light gray and the C-terminus is indicated in dark gray. Numbering refers to amino acid residues of respective human GSK-3 isoforms. (WT, wild type; Δ, deleted; NT, N-terminus; CT, C-terminus)
We studied the effect of deletion and point mutations on 1) GSK-3 activity a) towards tau and b) in the canonical Wnt signaling pathway, 2) protein interactions, 3) tyrosine phosphorylation, and 4) subcellular localization. Examination of deletion mutants revealed that the N-termini of both GSK-3 isoforms are dispensable for activity, protein interaction with Axin GID, and tyrosine autophosphorylation, but are required for the GSK-3α-specific interaction with RACK1 WD4-7 and proper localization. In turn, progressive C-terminal deletion results in a loss of activity, impaired ability to interact with protein binding partners, a gradual loss of autophosphorylation, and mislocalization within the cell. Taken together, these observations imply that deletion of the C-termini of GSK-3 isoforms compromises proper protein folding and suggest that the C-terminal regions make significant contributions to the structural integrity of GSK-3 enzymes. These data predict that the development of therapeutic modulators targeting the C-terminus may result in isoform-specific GSK-3 inhibition through destabilization of GSK-3 structure.

Examination of point mutants demonstrated that mutation of GSK-3α/β Ser^{21/9} and GSK-3β Thr^{390} did not influence activity and therefore phosphorylation of these sites is not involved in regulation of GSK-3 activity in canonical Wnt signaling; GSK-3α R159A and GSK-3β R96A exhibit different activities towards the substrate tau which reflects differential priming requirements of GSK-3 isoforms; mutation of GSK-3α/β Tyr^{279/216} did not affect activity and thus, phosphorylation of GSK-3α/β Tyr^{279/216} is not essential for activity; catalytic inactive mutants GSK-3α K148R and GSK-3β K85R ectopically activate canonical Wnt signaling despite an impaired interaction with axin GID. Thus, GSK-3α K148R and GSK-3β K85R function as dominant-negatives in canonical Wnt
signaling through an axin-independent mechanism; mutation of GSK-3α Pro\textsuperscript{442/443} and GSK-3β Pro\textsuperscript{379/380} only slightly affected the ability of GSK-3 isoforms to interact with axin GID and are therefore not essential for structural integrity.
Chapter 3

3. CATALYTIC ACTIVITY OF GSK-3 MUTANTS

3.1 Catalytic Activity of Mutant GSK-3α and GSK-3β towards the Microtubule-Associated Protein Tau

3.1.1 Introduction

The GSK-3 substrate tau is a microtubule-associated protein widely distributed in human brain [173, 174]. Tau normally functions to promote tubulin polymerization and stabilize microtubules [175-178] and is negatively regulated by phosphorylation [179, 180]. Abnormally hyperphosphorylated tau has been implicated in the pathogenesis of a class of neurodegenerative diseases termed tauopathies [181, 182]. Alzheimer’s disease (AD) is one such tauopathy characterized, in part, by the accumulation of insoluble intracellular neurofibrillary tangles (NFTs) in post-mortem brain tissue [181, 183]. NFTs are formed from aggregates of paired helical filaments (PHFs) whose major component is the tau protein [184-187]. In AD, PHF tau is abnormally hyperphosphorylated [188-191]. Such hyperphosphorylation is believed to be a major determinant in the formation of the PHF tau and the pathogenesis of AD.
The identification of protein kinases responsible for the conversion of normal tau to PHF tau has been extensively investigated and GSK-3 isoforms have been implicated as prime candidates. Collectively, GSK-3 isoforms phosphorylate more than half of the nearly 40 phosphorylated sites identified in PHF tau [192-201]. Select phosphorylation of some of these sites generates particular epitopes that distinguish PHF tau from normal tau [202]. One such site, Ser\(^{396}\), is implicated in the formation of PHF tau [191, 203, 204] and may even serve as a diagnostic marker of AD [205]. Both GSK-3 isoforms phosphorylate Ser\(^{396}\) [194-197, 200, 201, 206, 207]. Thus, a thorough understanding of the structural requirements for GSK-3 phosphorylation of tau at Ser\(^{396}\) is of considerable interest.

To gain insight into the mechanism of GSK-3-mediated tau phosphorylation, we assessed the ability of GSK-3\(\alpha\) and GSK-3\(\beta\) deletion and point mutants to phosphorylate the substrate tau at Ser\(^{396/404}\) in a mammalian cell culture system by immunoblot analysis. We discovered that the N-termini are not required, while the C-termini are essential for the activity of both GSK-3\(\alpha\) and GSK-3\(\beta\) towards tau Ser\(^{396/404}\). Further examination of point mutants demonstrated that GSK-3\(\alpha\) phosphorylation of Ser\(^{396/404}\) requires recognition of a primed phosphorylation site whereas GSK-3\(\beta\) does not exhibit this restraint.

3.1.2 Materials and Methods

*Cloning and Site-Directed Mutagenesis* – Wild-type (WT) and deletion mutant GSK-3\(\alpha\) and GSK-3\(\beta\) were PCR-amplified from human GSK-3\(\alpha\) (Origene, accession number NM_019884) and human GSK-3\(\beta\) respectively (obtained from Peter Klein, University of
Pennsylvania) and TA-cloned into Gateway entry vector, *pCR8GW TOPO* (Invitrogen). Point mutant *GSK-3* constructs were generated in WT human *GSK-3α pCR8GW TOPO* and WT human *GSK-3β pCR8GW TOPO* using site-directed mutagenesis (Stratagene). Oligonucleotides used for PCR amplification and site-directed mutagenesis are listed in Table 3.1A and B, respectively. DNA integrity and mutations were confirmed by sequence analysis of *pCR8GW TOPO* clones. Subsequent directional cloning from *pCR8GW TOPO* into Gateway destination vector *pDEST27* (Invitrogen) generated N-terminal GST-tagged GSK-3α (Figure 2.1A) and GSK-3β proteins (Figure 2.1B).
### Table 3.1
Oligonucleotides used for cloning (A.) and site-directed mutagenesis (B.) of GSK-3α and GSK-3β constructs. Underlined nucleotides denote mutated codons in B.
**Plasmids** - Flag-tagged human tau, the longest tau isoform, was obtained from Hemant Paudel, McGill University. *pMAX EGFP* was obtained from Amaxa. Empty vector *pcDNA3.1* was obtained from Invitrogen.

**Cell Culture and Transient Transfections** – Human embryonic kidney (HEK) 293T cells (ATCC) were maintained in Dulbecco’s Modified Eagle’s Medium (Cellgro) supplemented with 10% bovine growth serum (BGS) (Hyclone) and 1% penicillin-streptomycin solution (Cellgro) at 37°C and 5% CO₂. Cells were plated under reduced (2.5%) serum conditions at 1.0 x 10⁶ in 6-well plates (Corning) 24 hours prior to transfection. Subconfluent HEK 293T cells were maintained in reduced (2.5%) serum conditions during transient transfection with polyethylenimine (PEI) (Polysciences, Inc.) dissolved in 50 mM HEPES buffer, pH 7.05. Each transfection included 1 µg Flag-tau, 0.2 µg *pMAX EGFP*, and either 1 µg *pcDNA3.1* or GSK-3 as indicated. All transfections were normalized with empty vector *pcDNA3.1*. Co-transfection with vector encoding enhanced green fluorescent protein (EGFP) allowed for visual assessment of transfection efficiency.

**Cell lysis** - Approximately 24 hours post-transfection, cells were collected with trypsin and pelleted by centrifugation at 1,500 rpm for two minutes. After one wash with 1× phosphate buffered saline (PBS), cells were lysed in IP/lysis buffer (137 mM NaCl, 10 mM Tris pH 7.4, 1% NP40) supplemented with Protease Inhibitor Cocktial (PIC) (Sigma). Lysate was incubated on ice for approximately 20 minutes with intermittent vortexing. Lysate was then cleared by high-speed centrifugation at 4°C for 15 minutes. The supernatant fraction was collected and boiled in sample buffer (50 mM Tris pH 6.8, 12% glycerol, 4% SDS, 0.1 M DTT, 0.01% Coomassie Blue R-250) for five minutes.
Immunoblotting and Antibodies - Approximately equal amounts of denatured protein lysate was separated by Tricine-SDS-PAGE, transferred to nitrocellulose (Whatman), and immunoblotted. The following primary antibodies were used: rabbit polyclonal anti-GST (Cell Signaling) at a 1:1000 dilution, mouse monoclonal anti-Flag M2 (Sigma) at a 1:1000 dilution, and mouse monoclonal PHF1 (obtained from Peter Davies, Albert Einstein College of Medicine) at a 1:250 dilution. Corresponding secondary antibodies, either horseradish peroxidase (HRP)-linked anti-mouse or HRP-linked anti-rabbit (GE Healthcare), were used at a 1:5000 dilution. Detection was facilitated using enhanced chemiluminescence (ECL) Western Blotting Substrate (GE Healthcare).

3.1.3 Results

To gain insight into the mechanism of GSK-3-mediated tau phosphorylation, we examined the ability of GSK-3α and GSK-3β deletion and point mutations to phosphorylate the well-characterized GSK-3 substrate tau. HEK 293T cells were transfected with a Flag-tagged tau construct and either a GST-tagged GSK-3α construct or a GST-tagged GSK-3β construct. Cell lysates were collected and analyzed by immunoblot. Antibodies against GST revealed comparable expression of GSK-3α (Figure 3.1A) and GSK-3β (Figure 3.1B) deletion and point mutants. Likewise, antibodies against Flag demonstrated similar expression levels of tau (Figure 3.1A-B). As expected, co-expression of WT GSK-3α (Figure 3.1A) and WT GSK-3β (Figure 3.1B) enhanced tau phosphorylation at Ser\textsuperscript{396} and Ser\textsuperscript{404} as recognized by the phosphorylation-specific antibody PHF1, which correlated with a band shift in the Flag immunoblot. N-terminal deletion mutants, GSK-3α ΔNT (Figure 3.1A) and GSK-3β
ΔNT (Figure 3.1B), phosphorylated tau similar to WT GSK-3α and WT GSK-3β, respectively. Likewise, C-terminal deletion mutants GSK-3α ΔCT-1, GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3β ΔCT-1, GSK-3β ΔCT-2 and GSK-3β ΔCT-3 also enhanced tau phosphorylation (Figure 3.1A-B). However, activity of the most extensive C-terminal deletion mutants, GSK-3α ΔCT-4 and GSK-3β ΔCT-4, was nearly abolished (Figure 3.1A-B).
Figure 3.1 C-terminal deletion of GSK-3 isoforms impairs GSK-3 activity towards tau – Deletion and point mutants of GST-tagged GSK-3α (A.) and GSK-3β (B.) were co-expressed with Flag-tagged human tau in HEK 293T cells. Protein was separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize GSK-3 (GST, top panel), Ser\textsuperscript{396} and Ser\textsuperscript{404} phosphorylated tau (PHF1, middle panel), and total tau (Flag, bottom panel). Results shown are representative of at least three independent experiments. Untransfected and empty vector transfections serve as negative controls as indicated. WT GSK-3α and WT GSK-3β serve as positive controls as indicated. Arrows denote GSK-3 mutants that exhibit impaired activity towards tau phosphorylation at Ser\textsuperscript{396/404}. (Un, untransfected; Vec, vector; WT, wild type; Δ, deleted; NT, N-terminus; CT, C-terminus).
As anticipated, the point mutants expected to inactivate GSK-3, GSK-3α K148R (Figure 3.1A) and GSK-3β K85R (Figure 3.1B), demonstrated little activity towards tau, unlike positive control WT GSK-3α and WT GSK-3β, but analogous to negative control vector (Vec) alone transfection. Furthermore, under basal conditions, no external stimuli was present to induce inhibitory phosphorylation of GSK-3α at Ser21, GSK-3β at Ser9, or GSK-3β at Thr390. Thus, point mutants GSK-3α S21A (Figure 3.1A), GSK-3β S9A (Figure 3.1B), and GSK-3β T390A (Figure 3.1B) did not exhibit any obvious differences in activity when compared to positive control WT counterparts. Additionally, strong impairment of GSK-3α R159A (Figure 3.1A) activity was detected in contrast to GSK-3β R96A (Figure 3.1B), which exhibited activity similar to WT GSK-3β. Mutation of the tyrosine residue implicated in potentiating GSK-3 activity in GSK-3α Y279F (Figure 3.1A) and GSK-3β Y216F (Figure 3.1B), unexpectedly, had no effect.

3.1.4 Discussion

The N-termini were not required for activity by either GSK-3 isoform. However, GSK-3α and GSK-3β demonstrate similar requirements for the C-termini. The strong impairment in activity of C-termini deletion mutants, GSK-3α ΔCT-4 and GSK-3β ΔCT-4, indicate that amino acids 417-483 of GSK-3α and 345-420 of GSK-3β are essential for activity towards tau.

We observed a significant reduction in GSK-3α R159A activity, whereas GSK-3β R96A activity was unaffected, indicating GSK-3α and GSK-3β exhibit distinct prerequisites for priming at the PHF-1 epitope. Mutation of this conserved arginine
residue impairs the ability of GSK-3 isoforms to recognize primed substrates with the consensus motif S-X-X-X-S-p [93]. Thus, GSK-3α appears to have a specific prerequisite for priming phosphorylation that must be met in order for GSK-3α-mediated phosphorylation of tau at Ser^{396/404}. GSK-3β, however, is not subject to this additional mode of regulation and may represent a more influential tau kinase.

The PHF-1 epitope of tau encompasses Ser^{396-404}. Phospho-specific PHF-1 antibody can reportedly recognize individually phosphorylated Ser^{396} or Ser^{404} and exhibits a significant increase in immunoreactivity when both residues are phosphorylated [208]. The PHF-1 immunoreactivity of tau was not completely abolished, but rather, greatly reduced, with GSK-3α R159A co-transfection. Thus, it is possible that one site, either Ser^{396} or Ser^{404}, was phosphorylated by GSK-3α R159A while phosphorylation of the other site requires priming. According to the published sequence of human tau [173], a lysine residue exists at position 408, the predicted priming site for subsequent GSK-3α phosphorylation at Ser^{404}. Since lysine is not a phosphorylatable residue, Ser^{404} cannot be subject to a priming mechanism. Ser^{396} does have a potential priming site at Ser^{400}, suggesting the reduction in PHF-1 immunoreactivity with GSK-3α R159A co-transfection may be due to a reduction in Ser^{396} phosphorylation. Thus, GSK-3α R159A may elicit the observed PHF-1 immunoreactivity by readily phosphorylating Ser^{404}, while phosphorylation of Ser^{396} is prevented due to the inability of GSK-3α R159A to recognize the primed Ser^{400} site.

This mechanism, however, does not conform to the C- to N-terminal processivity of GSK-3 phosphorylation of primed substrates. Such processivity was first noted in the sequential multi-site GSK-3-mediated phosphorylation of glycogen synthase. Priming of
glycogen synthase by casein kinase II (CKII) at site 5 in the sequence PRPAS(3a)VPPS(3b)PSLS(3c)RHSS(4)PHQS(5)EDEEEP, enables processive and ordered phosphorylation by GSK-3 at site 4, followed by site 3c, then site 3b, and lastly site 3a [22, 23, 209, 210]. According to such an ordered preference for GSK-3 activity, it is more likely that phosphorylation of Ser\(^{404}\), Ser\(^{400}\), and Ser\(^{396}\) occurs sequentially, with Ser\(^{404}\) serving as the priming site and enabling subsequent phosphorylation at Ser\(^{400}\) and Ser\(^{396}\). Such a mechanism for tau phosphorylation by GSK-3\(\beta\) has been described previously in which cyclin dependent kinase 5 (Cdk5) primes Ser\(^{404}\) for GSK-3\(\beta\) phosphorylation at Ser\(^{400}\) and Ser\(^{396}\) [211]. Though we did not observe this priming phosphorylation mechanism for GSK-3\(\beta\) in our experimental setting, such a mechanism may exist for GSK-3\(\alpha\). The reduction in PHF-1 immunoreactivity observed with GSK-3\(\alpha\) R159A may, therefore, merely represent an overall impairment in phosphorylation.

The priming site required by GSK-3\(\alpha\) is likely phosphorylated in our cell culture conditions, as indicated by the ability of WT GSK-3\(\alpha\) to greatly enhance PHF-1 immunoreactivity. If phosphorylation of the priming site were absent, WT GSK-3\(\alpha\) activity would be impaired analogously to GSK-3\(\alpha\) R159A due to the apparent priming prerequisite GSK-3\(\alpha\). We did not investigate which priming kinase(s) is/are responsible for regulating GSK-3\(\alpha\) phosphorylation at the PHF-1 epitope. Unlike GSK-3, most kinases require a stimulus for activation and, although we did not add exogenous factors to stimulate kinase activity, it is possible that the reduced-serum conditions of our transfections activated a priming kinase. Regardless of the mechanism or priming kinase, our data demonstrate a novel isoform-specific difference in GSK-3 activity at an important tau epitope.
3.2 Catalytic Activity of Mutant GSK-3α and GSK-3β in the Canonical Wnt Signaling Pathway

3.2.1 Introduction

The canonical Wnt signaling pathway is important during development and is aberrantly activated in some cancers. GSK-3 isoforms negatively regulate the canonical Wnt signaling pathway through association with the cytoplasmic scaffold protein Axin (Figure 3.2A) [96, 118, 119, 122]. The axin scaffold complex mediates GSK-3 phosphorylation of the transcriptional co-factor β-catenin, thereby targeting it for degradation [116, 123, 124] (Figure 3.2A). Induction of canonical Wnt signaling inhibits GSK-3 activity, stabilizes β-catenin and consequently induces β-catenin-dependent activation of Wnt target genes [111, 112, 115, 131-133]. The mechanism of GSK-3 inhibition by Wnt stimuli is poorly established, but recent observations have provided important insights. The current model suggests that Wnt stimulation initiates signaling through Disheveled (Dsh)-mediated recruitment of an axin-GSK-3 complex to the membrane co-receptor Frizzled-LRP-5/6 [130, 212-214], which disrupts the cytoplasmic destruction complex thereby inhibiting GSK-3-mediated phosphorylation of β-catenin [213] (Figure 3.2B). Membrane recruited GSK-3 further amplifies the Wnt signal in conjunction with CK-1γ through phosphorylation of LRP-5/6 [212, 213, 215] (Figure 3.2B). The dual roles of GSK-3 in Wnt signaling are confounding, but it appears these opposing activities are tightly controlled by protein interactions and subcellular distribution.
Figure 3.2  Schematic of canonical Wnt signaling depicting the dual roles of GSK-3 – In the absence of a Wnt ligand (A.) GSK-3 exists in a cytoplasmic destruction complex formed by the scaffold protein axin, which mediates GSK-3 phosphorylation of β-catenin resulting in β-catenin degradation. In the presence of Wnt ligand (B.), the destruction complex is disrupted by Disheveled (Dsh)-mediated re-localization of the axin-GSK-3 complex to the membrane where GSK-3 phosphorylates the co-receptor LRP-5/6 leading to propagation of the Wnt signal. β-catenin stabilization leads to transcription of Wnt target genes.
In order to obtain insight into the specific requirements for GSK-3 activity in the canonical Wnt signaling pathway, we assessed the effect of GSK-3α and GSK-3β deletion and point mutants on downstream transcriptional activation of a β-catenin-inducible LEF/TCF luciferase reporter in a mammalian cell culture system. Consistent with the tau phosphorylation data, we observed that deletion of the N-terminus did not affect GSK-3 activity, while deletion of the C-terminus greatly impaired the activity of both GSK-3α and GSK-3β. Further, we noted that GSK-3α exhibited a greater sensitivity to C-terminal deletion and lost activity more readily than GSK-3β. To verify these results, we also measured mRNA levels of the Wnt target gene Axin2 by quantitative real-time PCR (qRT-PCR) in Gsk-3α-/ Gsk-3β-/ mouse embryonic stem cells (ESCs), which exhibit constitutively active Wnt signaling [216]. Though the qRT-PCR data for GSK-3β was inconclusive, GSK-3α did exhibit an analogous, though not identical, impairment of activity when the C-terminus was deleted.

3.2.2 Materials and Methods

*Plasmids* – Human GSK-3α and GSK-3β WT and mutant constructs were generated as described in section 3.1.2. *Super 8X TOPFlash* and *Super 8X FOPFlash* were obtained from Randall Moon, University of Washington. *Renilla* luciferase plasmid pRL SV40 was obtained from Promega. *pMAX EGFP* was obtained from Amaxa. Empty vector *pcDNA3.1* was obtained from Invitrogen.

*Cell Culture and Transient Transfections* – HEK 293T cells were maintained in DMEM supplemented with 10% BGS and 1% penicillin-streptomycin solution at 37°C and 5% CO₂. Cells were plated under reduced (2.5%) serum conditions at 1.0 x 10⁶ in 6-
well plates 24 hours prior to transfection. Subconfluent HEK 293T cells were maintained in reduced (2/5%) serum conditions during transient transfection with PEI dissolved in 50 mM HEPES buffer, pH 7.05. Each transfection included 1 µg Super 8X TOPFlash or Super 8X FOPFlash, 0.01 µg pRL SV40, 0.2 µg pMAX EGFP and either 1 µg pcDNA3.1 or GSK-3 construct as indicated. All transfections were normalized with empty vector pcDNA3.1. Co-transfection with vector encoding EGFP allowed for visual assessment of transfection efficiency.

Gsk-3α−/− Gsk-3β−/− mouse ESCs (obtained from Dr. Brad Doble, McMaster University, and Dr. James Woodgett, University of Toronto) were grown directly on 0.1% gelatin (Sigma)-coated tissue culture-treated dishes (Corning) and maintained in DMEM with high (4.5 g/L) glucose (Cellgro) supplemented with 1 mM non-essential amino acids (Cellgro), 1 mM sodium pyruvate (Cellgro), 2 mM L-glutamine (Cellgro), 15% fetal bovine serum (Fisher), 1% penicillin-streptomycin solution, 55 µM 2-mercaptoethanol (Sigma), and 1000 U/ml ESGRO (Millipore) at 37°C and 5% CO2. Cells were transfected in suspension with 4 µl Lipofectamine2000 (Invitrogen) diluted in 96 µl OptiMEM (Cellgro). Each transfection included 0.2 µg pMAX EGFP and either 1 µg or pcDNA3.1 or GSK-3 construct as indicated. Co-transfection with vector encoding EGFP allowed for visual assessment of transfection efficiency.

**LEF/TCF Luciferase Reporter Assay** - LEF/TCF reporter constructs containing eight optimal (Super 8X TOPFlash) or eight mutant (Super 8X FOPFlash) LEF/TCF binding sites driving firefly luciferase [217], were co-transfected into HEK 293T cells in conjunction with pRL SV40, and either empty vector, WT GSK-3α or WT GSK-3β construct, or mutant GSK-3α or mutant GSK-3β construct. Twenty-four hours later,
transfected cells in a 6-well plate were collected and equally transferred to a 24-well plate. Twenty-four hours after transfer, cells were treated in triplicate for six hours with either 50% Wnt-3a conditioned media (WCM), to stimulate canonical Wnt signaling, or 50% L-cell conditioned media (LCM) for control. Cells were then lysed with 1× passive lysis buffer supplemented with PIC. Firefly and Renilla luciferase activities were measured using Firefly and Renilla Luciferase Assay kit (Biotium) in a Veritas microplate luminometer (Turner Biosystems). Firefly luciferase was normalized to Renilla luciferase and samples were assayed in triplicate.

qRT-PCR – Forty-eight hours after transfection, total RNA was isolated from Gsk-3α−/−Gsk-3β−/− mouse ESCs with TRIzol Reagent (Invitrogen) and 250 ng total RNA was used to synthesize cDNA using the High-Capacity Reverse Transcription Kit (Applied Biosystems). Resulting cDNA was then analyzed by qRT-PCR on a 7500 Real Time PCR System (Applied Biosystems) using a pre-developed Axin2 Taqman assay (Applied Biosystems). A pre-developed GAPDH Taqman assay (Applied Biosystems) was also employed and used for normalizing. Samples were assayed in triplicate.

3.2.3 Results

To gain insight into the structural requirements for GSK-3 activity in the canonical Wnt signaling pathway, we examined the ability of GSK-3α and GSK-3β deletion and point mutations to modulate the transcriptional activity of a β-catenin-inducible LEF/TCF firefly luciferase reporter, Super 8X TOPFlash [217]. HEK 293T cells were co-transfected with Super 8X TOPFlash, a Renilla luciferase reporter for normalizing, and either empty vector, WT or mutant GSK-3α construct, or WT or mutant GSK-3β
construct. Transfected cells were treated in triplicate with either Wnt-3a conditioned media (WCM) to activate canonical Wnt signaling or negative control L-cell media (LCM) [218, 219]. Subsequent examination of luciferase activity in cell lysates demonstrated that addition of WCM specifically induced reporter expression in cells transfected with Super 8X TOPFlash alone (TOP) (Figure 3.3A-B), but not in cells transfected with a reporter containing mutated LEF/TCF binding sites, Super 8X FOPFlash (FOP) (Figure 3.3A-B). Thus, WCM successfully activated canonical Wnt signaling resulting in the accumulation of β-catenin protein and reporter expression whereas negative control LCM had no effect. Over-expression of WT GSK-3α (Figure 3.3A) and WT GSK-3β (Figure 3.3B) suppressed reporter expression, indicating GSK-3 isoforms negatively regulate canonical Wnt signaling in this setting. N-terminal deletion mutants, GSK-3α ΔNT (Figure 3.3A) and GSK-3β ΔNT (Figure 3.3B), and the least extensive C-terminal deletion mutants, GSK-3α ΔCT-1 (Figure 3.3A) and GSK-3β ΔCT-1 (Figure 3.3B), also suppressed reporter expression. Contrary to GSK-3β ΔCT-2 and GSK-3β ΔCT-3 (Figure 3.3B), GSK-3α ΔCT-2 and GSK-3α ΔCT-3 (Figure 3.3A) were slightly impaired in their ability to suppress reporter, suggesting a distinct sensitivity of GSK-3α to C-terminal deletion. Finally, the most extensive C-terminal deletion mutants, GSK-3α ΔCT-4 (Figure 3.3) and GSK-3β ΔCT-4 (Figure 3.3B), were unable to suppress reporter expression. Interestingly, GSK-3β ΔCT-4 synergizes with WCM indicated by excess reporter expression as compared to reporter alone (TOP).
Figure 3.3 C-terminal deletion of GSK-3 isoforms impairs GSK-3 ability to suppress the canonical Wnt signaling pathway – Deletion and point mutants of GST-tagged GSK-3α (A.) and GSK-3β (B.) were co-expressed with β-catenin-dependent luciferase reporter, Super8X TOPFlash, and Renilla reporter and in HEK 293T cells. Cells were treated for 6 hours with either negative control media (LCM) or conditioned medium containing the Wnt-3a ligand (WCM) to activate canonical Wnt signaling. Firefly and Renilla luciferase levels were measured from cell lysates. The mean and standard error of the mean (SEM) was determined from three independent experiments performed in triplicate (n=9). Negative control reporter, FOPFlash, is non-responsive to both LCM and WCM. TOPFlash alone transfection is responsive only to WCM. Positive and negative controls are indicated. Arrows denote GSK-3 mutants that exhibit impaired activity in the canonical Wnt signaling pathway. (FOP, Super 8X FOPFlash; TOP, Super 8X TOPFlash; WT, wild type; Δ, deleted; NT, N-terminus; CT, C-terminus; LCM, L-cell conditioned media; WCM, Wnt3a conditioned media)
Catalytically inactive point mutants GSK-3α K148R (Figure 3.3A) and GSK-3β K85R (Figure 3.3B) function as dominant-negatives by activating reporter expression in the absence of Wnt stimuli (LCM). GSK-3β K85R was a much more potent dominant-negative than GSK-3α K148R. Further, Wnt stimuli do not induce GSK-3 phosphorylation at GSK-3α Ser21, GSK-3β Ser9, or GSK-3β Thr390 [73, 97, 135] and thus, GSK-3α S21A (Figure 3.3A), GSK-3β S9A (Figure 3.3B), and GSK-3β T390A (Figure 3.3B) functioned similar to their WT counterparts. Mutation of the primed recognition site in GSK-3α R159A (Figure 3.3A) and GSK-3β R96A (Figure 3.3B) impaired the ability of GSK-3 to suppress reporter, indicating defective recognition of the primed substrate β-catenin, and also conferred a slight dominant-negative activity. Lastly, contrary to a previous report [78], mutation of the tyrosine residues implicated in enhancing GSK-3 activity, GSK-3α Y279F (Figure 3.3A) and GSK-3β Y216F (Figure 3.3B), once again had no apparent effect on GSK-3 activity.

We further attempted to validate the effect of C-terminal deletion on GSK-3 activity in the canonical Wnt signaling by analyzing gene expression of the Wnt target Axin2 by qRT-PCR. Gsk-3α−/− Gsk-3β−/− ESCs, which exhibit constitutively active Wnt signaling [216], were transfected with select GSK-3α or GSK-3β WT or mutant constructs and total RNA was extracted for cDNA synthesis and real-time quantification. Though the data obtained with GSK-3β constructs (Figure 3.4B) was unconvincing, analysis of GSK-3α constructs (Figure 3.4A) did replicate the luciferase reporter data to an extent. Reconstitution of WT GSK-3α activity repressed Axin2 mRNA levels, as did GSK-3α ΔCT-1 (Figure 3.4A). Contrary to the reporter assay, GSK-3α ΔCT-2 and GSK-3α ΔCT-
3 exhibited activity similar to WT GSK-3α (Figure 3.4A). GSK-3α ΔCT-4, however, was unable to repress Axin2 transcript levels (Figure 3.4A). Catalytically inactive mutant GSK-3α K148R also did not alter the amount of Axin2 transcript (Figure 3.4A). Finally, mutation of GSK-3α Tyr279 did not impair GSK-3α activity and GSK-3α Y279F retained the ability to repress Axin2 transcript levels (Figure 3.4A).
A.

![Bar chart showing RNA expression levels for different constructs.](image)

B.

![Bar chart showing RNA expression levels for different constructs.](image)

**Figure 3.4** C-terminal deletion of GSK-3α impairs the ability of GSK-3α to repress Wnt target Axin2 gene expression – Gsk-3α−/− Gsk-3β−/− mouse ESCs, which display constitutive active Wnt signaling, were transfected with 1 µg GSK-3α construct (A.) or GSK-3β construct (B.). After 48 hours, RNA was isolated and subjected to reverse transcription. Resulting cDNA was then analyzed by qRT-PCR for Axin2. Axin2 values were normalized to GAPDH. Empty vector (Vec) serves as negative control and WT GSK-3 serves as positive control as indicated. Arrows denote GSK-3α mutants that exhibit impaired ability to repress axin2 expression. Statistical significance was not calculated. (RQ, relative quantity; WT, wild type; CT, C-terminus)
3.2.4 Discussion

Though the N-terminus was not required by either GSK-3α or GSK-3β for activity in canonical Wnt signaling, GSK-3α and GSK-3β both demonstrated a requirement for the C-terminus. The reporter assay suggests GSK-3α displays a greater requirement for the C-terminus than GSK-3β, indicated by the impaired ability of GSK-3α ΔCT-2 and GSK-3α ΔCT-3 to suppress reporter expression, which was not obvious with GSK-3β ΔCT-2 and GSK-3β ΔCT-3. This enhanced sensitivity of GSK-3α was not observed when Axin2 gene expression was assayed, in which GSK-3α ΔCT-2 and GSK-3α ΔCT-3 functioned similar to WT GSK-3α. Nevertheless, GSK-3α ΔCT-4 and GSK-3β ΔCT-4 were greatly impaired in their ability to suppress reporter and GSK-3α ΔCT-4 lacked the ability to repress Axin2 levels. Together, these data further argue that amino acids 417-483 in GSK-3α and 345-420 in GSK-3β are essential for GSK-3 activity.

Interestingly, unlike GSK-3α ΔCT-4, GSK-3β ΔCT4 synergizes with Wnt-3a by enhancing reporter expression to levels above those of reporter alone (TOP). This is not due to a gain of dominant-negative activity, as reporter expression in LCM control is not affected. The recently proposed model for initiation of Wnt signaling contends that GSK-3 positively regulates signaling by phosphorylating and activating co-receptor LRP-5/6, which promotes downstream signaling. It is tempting to speculate, therefore, that the observed synergy of GSK-3β ΔCT4 with WCM is due to enhanced positive regulation of Wnt signaling through modifications in LRP-5/6 activity. It is unlikely that these alterations are directly due to GSK-3β ΔCT4 catalytic activity per se, since activity of GSK-3β ΔCT4 is apparently impaired towards GSK-3 substrates β-catenin and tau. A
more likely mechanism may involve alterations in endogenous GSK-3 activity, protein interactions, or subcellular distribution that ultimately result in a synergistic effect.

Disruption of the primed recognition site in GSK-3α R159A and GSK-3β R96A conferred a slight dominant-negative activity to GSK-3 but the dominant-negative activity of catalytically inactive mutants GSK-3α K148R and GSK-3β K85R was significantly greater. Previous reports have also described the dominant-negative activity of GSK-3α Arg^{159} and GSK-3β Arg^{96} point mutants [78] and particularly of GSK-3α Lys^{148} and GSK-3β Lys^{85} point mutants in the canonical Wnt signaling pathway [34, 35, 65, 79]. Dominant-negative GSK-3 activity re-localizes β-catenin to the nucleus [116] and it has been suggested that such activity is dependent upon the interaction of GSK-3 with axin [220]. Thus, dominant-negative activity in our setting likely resulted from the displacement of endogenous GSK-3 from the Axin-mediated destruction complex. Since GSK-3α R159A and GSK-3β R96A are impaired in β-catenin recognition and GSK-3α K148R and GSK-3β K85R lack catalytic activity, displacement would effectively reduce or inhibit scaffold-mediated β-catenin degradation, resulting in constitutively activated Wnt signaling.

GSK-3α R159A, GSK-3β R96A, GSK-3α K148R and GSK-3β K85R might be expected to have similar dominant-negative effects since both point mutations result in an impaired ability to phosphorylate the primed β-catenin substrate. However, their dominant-negative abilities were quite different. This discrepancy may be explained by yet another role of GSK-3 in Wnt signaling. Axin is a GSK-3 substrate and GSK-3 phosphorylation of axin promotes axin stability and the interaction of axin with β-catenin [221-223]. Phosphorylation of axin by GSK-3 may not require priming [93] and thus,
GSK-3\(\alpha\) R159A and GSK-3\(\beta\) R96A, would be expected to retain activity towards axin. Despite the inability of GSK-3\(\alpha\) R159A and GSK-3\(\beta\) R96A to phosphorylate \(\beta\)-catenin, their ability to phosphorylate axin effectively retains \(\beta\)-catenin in an intact destruction complex where it is unable to activate transcription. Conversely, GSK-3\(\alpha\) K148R and GSK-3\(\beta\) K85R, which lack catalytic activity, would be unable to phosphorylate axin. Unphosphorylated axin exhibits reduced affinity for \(\beta\)-catenin [222, 223], which would result in release of \(\beta\)-catenin from the destruction complex and axin destabilization. According to this theory, GSK-3\(\alpha\) R159A and GSK-3\(\beta\) R96A maintain an intact destruction complex while GSK-3\(\alpha\) K148R and GSK-3\(\beta\) K85R promote destabilization of the destruction complex, which results in maximal \(\beta\)-catenin-dependent transcription.

It is not clear why GSK-3\(\beta\) K85R exhibited such a greater dominant-negative effect than GSK-3\(\alpha\) K148R. It may simply be due to greater expression of GSK-3\(\beta\) K85R although expression levels of GSK-3 proteins were not quantified. Alternatively, it could also be due to greater levels of endogenous GSK-3\(\beta\) expression and a select domination of GSK-3\(\beta\) K85R for endogenous GSK-3\(\beta\) and GSK-3\(\alpha\) K148R for endogenous GSK-3\(\alpha\).

3.3 Catalytic Activity of GSK-3\(\beta\) in vitro

3.3.1 Introduction

We demonstrated that GSK-3\(\alpha\) and GSK-3\(\beta\) require their C-termini for activity towards tau and for negative regulation of the canonical Wnt signaling pathway using
end-point assays in a cell culture system. Such end-point assays, however, do not examine enzyme kinetics. Further, GSK-3 activity in a cell culture system may also be subject to unidentified restraints. Thus, in order to gain insight into potential differences in enzyme kinetics of GSK-3 mutants, and to verify our data in a cell-free system, we attempted to determine GSK-3 activity in vitro. Using bacterially expressed and purified GSK-3 enzyme we tried to examine activity towards a primed peptide substrate by measuring incorporation of $[^{\gamma-32}\text{P}]$ ATP. Despite repeated attempts, our efforts proved futile due to apparent inactivity of the isolated enzyme.

3.3.2 Materials and Methods

**Plasmids** – Human WT GSK-3β pCR8GW TOPO was directionally cloned into Gateway destination vector pBAD-DEST49 for bacterial expression (Invitrogen). pBAD-DEST49 contains an araBAD promoter, which facilitates L-arabinose (Sigma) protein induction, and a C-terminal 6xHis tag for purification.

**Bacterial protein induction and protein purification** – One Shot TOP10 chemically competent E. coli (Invitrogen) transformed with WT GSK-3β pBAD DEST49 were grown to OD$_{600}$ at 37°C. L-arabinose was then added to induce protein expression and cultures were grown at 16°C overnight. Bacterial protein was purified under native conditions by metal affinity using a cobalt resin according to the HisTALON (Clontech) protein purification procedure.

**Purified Protein Analysis** – A fraction of each HisTALON eluate was denatured by boiling in sample buffer for five minutes, followed by separation on tricine-SDS-PAGE and transfer to nitrocellulose. Protein was analyzed by fluorescent Sypro Ruby protein
blot stain (Molecular Probes). Protein was also analyzed by immunoblot using a primary mouse monoclonal antibody against GSK-3α/β (Calbiochem) at a 1:1000 dilution and corresponding secondary antibody, HRP-linked anti-mouse, at a 1:5000 dilution. Detection was facilitated using ECL Western Blotting Substrate.

In vitro kinase assay – WT GSK-3β in HisTALON elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, pH 7.4) was mixed with 25 μM primed glycogen synthase peptide 2 substrate, 100 μM ATP, 2.3 μCi [γ-32P] ATP, 10 mM MgCl₂, 5 mM DTT, and 20 mM Tris-HCl in a 20 μl reaction. Reactions were incubated 15 minutes at 30°C and spotted onto P81 phosphocellulose paper (Whatman). The P81 paper was washed in 75 mM phosphoric acid, immersed in acetone, and allowed to air dry. Radioactivity was counted in the presence of scintillant. Recombinant GSK-3β, purchased from New England Biolabs, was used for positive control.

3.3.3 Results

6xHis-tagged WT GSK-3β protein expression was induced in transformed bacterial cultures by addition of L-arabinose. Induced protein was purified by metal affinity using a cobalt resin. Five eluate fractions (E1-E5) were collected and a fraction of each eluate was denatured, separated by tricine-SDS-PAGE and transferred to nitrocellulose. Blots were examined by total protein stain with Sypro Ruby (Figure 3.5A) and by immunoblot with an anti-GSK-3α/β antibody (Figure 3.5B). Total protein staining revealed an intense band in fraction E2 around 65 kDa, the predicted size of WT GSK-3β (Figure 3.5A). However, the detection of other staining patterns indicated the presence of contaminating and/or denatured protein products. Specific detection of GSK-3 by
immunoblotting also revealed an intense band around 65 kD in input and all five eluate fractions (Figure 3.5B). Numerous other bands of higher mobility indicated denaturation of GSK-3β protein despite the presence of bacterial protease inhibitor throughout the purification procedure.
Figure 3.5 Analysis of bacterially expressed and cobalt-purified 6xHis-GSK-3β protein – WT GSK-3β tagged with 6xHis was expressed in bacteria and purified by metal affinity using a cobalt resin. Input and eluate (E) fractions one through five were separated by tricine-SDS-PAGE, transferred to nitrocellulose, and either examined by total protein stain (A.) or immunoblotted with antibody against GSK-3α/β (B.). Arrows indicate expected size of tagged GSK-3β protein at 65 kD. Input serves as positive control as indicated.
We proceeded to measure the incorporation of $[\gamma^{32}\text{P}]$ ATP into a primed glycogen synthase (GS) peptide substrate using a gradient of eluate from E2 (Figure 3.5). As a negative control we included a reaction without substrate. As a positive control, we included recombinant GSK-3β, purchased from New England Biolabs (NEB). Though NEB GSK-3β successfully incorporated $\gamma^{32}\text{P}$ into the GS peptide substrate, bacterially expressed and affinity purified GSK-3β failed incorporate $\gamma^{32}\text{P}$, and resembled negative control (Figure 3.5).
Figure 3.6 *Purified GSK-3β fails to phosphorylate glycogen synthase peptide substrate in vitro* – Bacterially expressed and affinity purified WT GSK-3β was examined for its ability to incorporate \([\gamma-^{32}\text{P}]\) ATP into a primed glycogen synthase peptide substrate. Eluate fraction two (E2) was used in increasing amounts. A reaction lacking substrate (no substrate) was used for negative control as indicated. Recombinant GSK-3β purchased from New England Biolabs (NEB) was used for positive control as indicated. (cpm, counts per minute)
3.3.4 Discussion

Several decades of literature describing in vitro kinase reactions with GSK-3 enzyme exist. Thus, it is a well-characterized reaction that can proceed with different sources of GSK-3 enzyme, including bacterially purified enzyme [77] and even crude cell extract [224]. In addition to the cobalt-purified 6xHis-tagged GSK-3β described here, we also utilized crude bacterial extract containing 6xHis-tagged GSK-3β enzyme, glutathione sepharose-purified GST-tagged GSK-3β enzyme from mammalian cell extract, and untagged GSK-3β enzyme from crude mammalian cell extract. Store purchased NEB GSK-3β consistently yielded positive results, but numerous attempts with different GSK-3β enzyme preparations from distinct sources repeatedly failed. Thus, the problem appears to lie, not within the reaction itself, but rather within our isolated GSK-3 enzyme.
CHAPTER 4

THE ABILITY OF GSK-3 MUTANTS TO INTERACT WITH PROTEIN BINDING PARTNERS

4.1 GSK-3 Interaction with Axin

4.1.1 Introduction

Negative regulation of Wnt signaling through GSK-3-mediated β-catenin phosphorylation is dependent on the protein interaction of GSK-3 with the destruction complex scaffold protein axin [96, 118, 119, 122]. Axin can be dissected into various domains including binding regions for GSK-3 [GSK-3 Interaction Domain (GID)], and regions that mediate β-catenin interaction [96, 118, 122, 225, 226]. The axin GID peptide fragment interacts strongly with GSK-3 [226]; its removal disrupts negative regulation of Wnt signaling by GSK-3 [122, 225, 226]. Likewise, elimination of β-catenin binding regions from axin also prevents GSK-3-mediated negative regulation of Wnt signaling [122, 225, 227]. Thus, axin brings β-catenin and GSK-3 into close proximity to promote GSK-3-dependent phosphorylation of β-catenin and stimulate β-catenin degradation.
C-terminal deletion of GSK-3 isoforms destroys their ability to negatively regulate Wnt signaling in the TOPFlash reporter assay, indicating that the activity of GSK-3α and GSK-3β to modify β-catenin was compromised. In addition, we observed dominant-negative activities of GSK-3α/β point mutants Arg^{159/96} and Lys^{148/85} in the TOPFlash reporter assay, albeit to different extents. We suggest that the alterations of activity for these mutants are due to alterations in the protein interaction of these mutants with axin. Here, we examined the ability of GSK-3 mutants to interact with the axin GID. Indeed, we observed that the GSK-3α and GSK-3β C-terminal deletion mutants that were unable to suppress the TOPFlash reporter were also unable to interact with the axin GID. However, a direct correlation between negative regulation of Wnt signaling by GSK-3 and the ability of GSK-3 to interact with axin is lacking. Furthermore, we unexpectedly observed discrete abilities of GSK-3α/β Lys^{148/85} and GSK-3α/β Arg^{159/96} mutants to interact with the axin GID. Such binding differences of GSK-3α/β Lys^{148/85} and GSK-3α/β Arg^{159/96} mutants to the axin GID may account for the dramatically different dominant-negative potencies of these mutants in the luciferase reporter assay.

4.1.2 Materials and Methods

*Plasmids* – Human GSK-3α and GSK-3β WT and mutant constructs were generated as described in section 3.1.2. The GSK-3 interaction domain (GID, amino acids 321-429) of *Xenopus axin* (obtained from Peter Klein, University of Pennsylvania) was PCR-amplified and TA-cloned into Gateway entry vector *pCR8GW TOPO*. Subsequent directional cloning into Gateway destination vector, *pDEST-myc* (obtained from BCCM/LMBP plasmid collection), generated N-terminal 6× myc-tagged protein.

DNA
integrity was confirmed by sequence analysis. *pMAX EGFP* was obtained from Amaxa. Empty vector *pcDNA3.1* was obtained from Invitrogen.

*Cell Culture and Transient Transfections* – HEK 293T cells were maintained in DMEM supplemented with 10% BGS and 1% penicillin-streptomycin solution at 37°C and 5% CO₂. Cells were plated under reduced (2.5%) serum conditions at 1.0 x 10⁶ in 6-well plates 24 hours prior to transfection. Subconfluent HEK 293T cells were maintained in reduced (2.5%) serum conditions during transient transfection with PEI dissolved in 50 mM HEPES buffer, pH 7.05. Each transfection included 1 µg *Axin GID* construct, 0.2 µg *pMAX EGFP* and either 1 µg *pcDNA3.1* or *GSK-3* construct as indicated. All transfections were normalized with empty vector *pcDNA3.1*. Co-transfection with vector encoding EGFP allowed for visual assessment of transfection efficiency.

*Cell lysis* - Approximately 24 hours post-transfection, cells were collected with trypsin and pelleted by centrifugation at 1,500 rpm for two minutes. After one wash with 1x PBS, cells were lysed in IP/lysis buffer supplemented with PIC. Lysate was incubated on ice for approximately 20 minutes with intermittent vortexing. Lysate was then cleared by high-speed centrifugation at 4°C for 15 minutes. The supernatant fraction was either collected and boiled in sample buffer for five minutes or utilized in subsequent glutathione S-transferase (GST) pull-down assays.

*GST Pull-down Assay* - Cleared cell lysate was incubated with Glutathione Sepharose 4B beads (GE Healthcare) for 2 hours at 4°C. Subsequently, reactions were centrifuged to pellet beads, which were then washed 4 times with two volumes IP/lysis buffer supplemented with PIC. Following the final wash, protein was eluted by boiling in sample buffer.
**Immunoblotting and Antibodies** - Approximately equal amounts of denatured protein lysate was separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted. Primary antibodies included rabbit polyclonal anti-GST (Cell Signaling) at a 1:1000 dilution and mouse monoclonal 9E10 (anti-Myc, developed by J. Michael Bishop and obtained from the Developmental Studies Hybridoma Bank, The University of Iowa) at a 1:1000 dilution. Corresponding secondary antibodies, either HRP-linked anti-mouse or HRP-linked anti-rabbit (GE Healthcare), were used at a 1:5000 dilution. Detection was facilitated using ECL Western Blotting Substrate (GE Healthcare).

**Densitometry** - Chemiluminescence was quantified by measuring area density using an UltraLum documentation system and software (UltraLum Inc., Claremont, CA). Values represent immunoreactivity values of ((9E10 PD:9E10 TL):GST PD).

4.1.3 Results

To determine if the impaired ability of GSK-3 C-terminal deletion mutants to negatively regulate Wnt signaling was due to a compromised interaction with axin, we characterized the interaction of GSK-3 mutants with the axin GID under basal conditions. HEK 293T cells were transfected with Myc-tagged axin GID and either a GST-tagged GSK-3α construct or a GST-tagged GSK-3β construct. Cell lysates were collected and either processed as total lysate for immunoblot or purified on glutathione sepharose and then processed for immunoblot. Antibodies against Myc (9E10) demonstrated similar expression levels of the axin GID in total lysate (Figure 4.1A-B). Further, antibodies against GST revealed comparable levels of purified GSK-3α (Figure 4.1A) and GSK-3β (Figure 4.1B) deletion and point mutants. As expected, the axin GID strongly interacted
with WT GSK-3α (Figure 4.1A) and WT GSK-3β (Figure 4.1B). The axin GID also interacted strongly with GSK-3α ΔNT, GSK-3α ΔCT-1, GSK-3β ΔNT, and GSK-3β ΔCT-1 (Figure 4.1A-B). However, other C-terminal deletion mutants, GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3β ΔCT-2, and GSK-3β ΔCT-3, exhibited a dramatic reduction in their ability to interact with the axin GID (Figure 4.1A-B); two mutants, GSK-3α ΔCT-4 and GSK-3β ΔCT-4, displayed no interaction with the axin GID (Figure 4.1A-B).
Figure 4.1 C-terminal deletion of GSK-3 isoforms abolishes GSK-3 interaction with axin GID – Deletion and point mutants of GST-tagged GSK-3α (A.) and GSK-3β (B.) were co-expressed with 6× Myc-tagged axin GID in HEK 293T cells. Total lysate (TL) and glutathione sepharose affinity-purified protein (PD) were separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize GSK-3 (GST, top panel) and the axin GID (9E10, middle and bottom panels). Results are representative of three independent experiments. Numerical values were derived from densitometry experiments and error represents standard deviation. Untransfected (Un) and empty vector (Vec) transfections represent negative controls and WT GSK-3α and WT GSK-3β represent positive controls as indicated. Arrows denote GSK-3 mutations that impair the GSK-3 interaction with the axin GID. (WT, wild type; Δ, deleted; NT, N-terminus; CT, C-terminus; PD, pull-down; TL, total lysate)
Our assay was conducted under basal conditions in which GSK-3 exhibits constitutive activity. Thus, no stimulus was present to promote phosphorylation of GSK-3α Ser^{21}, GSK-3β Ser^{9}, or GSK-3β Thr^{390}. Effects of the point mutations in GSK-3α S21A, GSK-3β S9A, and GSK-3β T390A were therefore not evident and these mutants interacted with the axin GID similarly to their WT counterparts (Figure 4.1A-B). In contrast to previous reports [96, 220], GSK-3α Y279F and GSK-3β Y216F interacted strongly with the axin GID (Figure 4.1A-B). Point mutants that exhibited dominant-negative activity in the reporter assay, presumably by displacing endogenous GSK-3 from the axin-mediated destruction complex, exhibited distinct binding abilities: GSK-3α R159A and GSK-3β R96A retained the ability to interact with the axin GID whereas GSK-3α K148R and GSK-3β K85R unexpectedly demonstrated a strong impairment (Figure 4.1A-B). Our qualitative observations were verified by quantitative densitometry (Figure 4.1A-B).

4.1.4 Discussion

We anticipated the inability of the axin GID to interact with GSK-3α ΔCT-4 and GSK-3β ΔCT-4, which accounts for their inability to suppress expression of the β-catenin-inducible LEF/TCF luciferase reporter. The impaired ability of deletion mutants GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3β ΔCT-2, and GSK-3β ΔCT-3 to interact with axin GID was unexpected as each of these mutants suppressed expression of the β-catenin LEF/TCF luciferase reporter. Interestingly, a similar observation was reported by Fraser and colleagues who described a GSK-3β point mutant, V267G/E268R that failed to interact with the axin GID, but retained the ability to repress β-catenin-inducible TCF luciferase reporter activity [220]. These data suggest that the interaction of GSK-3
isoforms with axin is not required for the negative regulation of Wnt signaling by GSK-3. Details of an alternate axin-independent mechanism for the GSK-3 negative regulation of Wnt signaling have not been explored. Examination of the activity of GSK-3α K148R, GSK-3β K85R, and GSK-3β V267G/E268R point mutants in canonical Wnt signaling in Axin deficient cells may provide further support for an axin-independent mechanism of GSK-3 regulation of Wnt signaling.

Our data also revealed distinct mechanisms for dominant-negative GSK-3 activity by GSK-3α K148R and GSK-3β K85R in comparison to GSK-3α R159A and GSK-3β R96A. The less effective dominant-negatives, GSK-3α R159A and GSK-3β R96A, strongly interact with the axin GID suggesting that they are capable of disrupting endogenous GSK-3 activity by displacing axin-associated GSK-3 from the scaffold. GSK-3α K148R and GSK-3β K85R, however, exhibit a very weak interaction with axin GID indicating their dominant-negative activity is largely independent of an interaction with axin.

Our data is supported by other studies in which the conversion of GSK-3β Lys⁸⁵ to methionine (K85M) generates dominant-negative activity of GSK-3β in Wnt signaling [65] despite an inability of GSK-3β K85M to associate with the axin GID [96, 220, 228]. The deficiency of axin GID binding by GSK-3β K85M has been attributed to disruptions in proper folding of GSK-3β [220]. Indeed GSK-3α Lys¹⁴⁸ and GSK-3β Lys⁸⁵ are not predicted surface residues [220] and are thus unlikely to be directly involved in binding to regulatory proteins. However, GSK-3β K85R is a more conservative mutation and in contrast to our data, Fraser and colleagues previously demonstrated GSK-3β K85R does
interact with the axin GID [220]. This interaction was proposed to mediate the dominant-negative activity of the GSK-3β K85R [220].

Nonetheless, the axin-independent mechanism of the potent dominant-negative effects of GSK-3α K148R and GSK-3β K85R remains elusive. It is also not clear how C-terminal deletion mutants suppress the β-catenin reporter by an axin-independent mechanism. It is appealing to speculate that these mutants are functioning through a similar mechanism but in opposing manners. We recognize, however, that a minimal interaction of GSK-3 with axin, undetectable by our methods, may account for GSK-3 activity in the reporter assay. We also acknowledge that full-length axin may interact with GSK-3 differently than axin GID and that taken together, our data may not accurately reflect an endogenous mechanism.

4.2 GSK-3α Interaction with Receptor for Activated Protein Kinase C 1 (RACK1)

4.2.1 Introduction

A yeast two-hybrid screen used to identify and examine novel GSK-3α interacting proteins identified the receptor for activated C-kinase 1 (RACK1) as a GSK-3α interacting partner (unpublished data). RACK1 was not identified in an analogous screen for novel GSK-3β interacting proteins and represents a potential GSK-3α-specific interacting protein (unpublished data). RACK1 was originally identified as an anchoring protein for protein kinase C (PKC) [229] but is also a scaffold protein that interacts with a number of distinct proteins [230]. Protein interactions of RACK1 are mediated by seven Trp-Asp 40 (WD40) repeats. Structurally, each repeat comprises a blade of a larger
propeller structure [230]. GSK-3α interacts strongly with WD repeats 4-7 (unpublished data). The functional significance of this interaction is not known but is currently under investigation.

To determine the GSK-3α structural requirements for the GSK-3α interaction with RACK1, we examined the ability of GSK-3α mutants to interact with RACK1 WD4-7. GSK-3α required both the N- and C-termini for interaction with RACK1, although its catalytic activity of GSK-3α was dispensable.

4.2.2 Materials and Methods

Cloning and Site-Directed Mutagenesis – Wild-type (WT) GSK-3α and GSK-3β and deletion mutants of GSK-3α were PCR-amplified from respective human GSK-3α and human GSK-3β without stop codons to generate C-terminal fusion proteins. PCR products were TA-cloned into Gateway entry vector, pCR8GW TOPO. Point mutant GSK-3α constructs compatible with C-terminal fusion were generated in WT human GSK-3α pCR8GW TOPO using site-directed mutagenesis. Oligonucleotides used for PCR amplification and site-directed mutagenesis are listed in Table 4.1A and Table 3.1B respectively. DNA integrity and mutations were confirmed by sequence analysis of pCR8GW TOPO clones. Subsequent directional cloning from pCR8GW TOPO into Gateway destination vector pDEST40 (Invitrogen) generated C-terminal V5-tagged GSK-3α and GSK-3β proteins.

Full-length RACK1 and deletion mutant RACK1 constructs were PCR-amplified from human RACK1 (ATCC, accession number BC021993) using the oligonucleotides listed in Table 4.1B. PCR products were TA-cloned into pCR8GW TOPO. DNA integrity was
confirmed by sequence analysis of *pCR8GW TOPO* clones. Subsequent directional cloning into *pDEST27* generated N-terminal GST-tagged RACK1 proteins.
Table 4.1  Oligonucleotides used for cloning of GSK-3α C-terminal fusions (A.) and RACK1 (B.).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK 3α FL</td>
<td>ATGACGCGCGCAGCGGCTCCAGGA</td>
<td>GGAGGAAGTTAGTGAGGTTAGGTG</td>
</tr>
<tr>
<td>GSK 3α ΔN1</td>
<td>ATGAGGACACACAGTGAGCCCA</td>
<td>GGAAGAGTTAGTGAGGTTAGGTG</td>
</tr>
<tr>
<td>GSK 3α ΔCT1</td>
<td>ATGACGCGCGCAGCGGCTCCAGGA</td>
<td>TGAGACCTTCAAGTGAGGAGGAT</td>
</tr>
<tr>
<td>GSK 3α ΔCT2</td>
<td>ATGACGCGCGCAGCGGCTCCAGGA</td>
<td>GTTGTAAGGCAGCTGAGGTTCAG</td>
</tr>
<tr>
<td>GSK-3α ΔCT3</td>
<td>ATGACGCGCGCAGCGGCTCCAGGA</td>
<td>AAAGAACGTGCGCACAGGCTC</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RACK1 FL</td>
<td>ATGAGTGCAGCAATGACCTCCGT</td>
<td>CTAGCGGTGCTGCAATGTCACCAG</td>
</tr>
<tr>
<td>RACK1 WD4-7</td>
<td>ATGACGCAATACACTGTCCAGGAT</td>
<td>CTAGCGGTGCTGCAATGTCACCAG</td>
</tr>
</tbody>
</table>
**Plasmids**: *pMAX EGFP* was obtained from Amaxa. Empty vector *pcDNA3.1* was obtained from Invitrogen.

**Cell Culture and Transient Transfections** – Neuro-2A cells (N2A) (ATCC) were maintained in 50:50 DMEM:Opti-MEM supplemented with 5% BGS and 0.5% penicillin-streptomycin solution at 37°C and 5% CO₂. N2A cells were seeded in a 6-well plate at 8.0 x 10⁵ cells/well 24 hours prior to transfection. Cell transfections were performed under reduced (2.5%) serum conditions with PEI dissolved in 50 mM HEPES, pH 7.05. Transfections included 0.2 µg *pMAX EGFP*, 2 µg *GSK-3* construct as indicated, and either 2 µg *pDEST27* or 2 µg *RACK1* construct as indicated. Cotransfection with vector encoding EGFP allowed for visual assessment of transfection efficiency.

**Cell lysis** - Approximately 24 hours post-transfection, cells were lysed in IP/lysis buffer supplemented with PIC. Lysates were incubated on ice for approximately 30 minutes with intermittent vortexing. Lysates were then cleared by high-speed centrifugation at 4°C for 15 minutes. The supernatant fraction was collected and either boiled in sample buffer for five minutes or used for subsequent GST pull-down.

**GST Pull-down Assay** - Cleared cell lysates were incubated with glutathione sepharose 4B beads for 2.5 hours at 4°C. Subsequently, reactions were centrifuged to pellet beads, which were then washed four times with IP/lysis buffer supplemented with PIC. Following the final wash, protein was eluted by boiling in sample buffer.

**Immunoblotting and Antibodies** - Approximately equal amounts of denatured protein lysate were separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted. The following primary antibodies were used: rabbit polyclonal anti-GST
at a 1:1000 dilution and mouse monoclonal V5 (Invitrogen) at a 1:5000 dilution. Corresponding secondary antibodies, either HRP-linked anti-mouse or HRP-linked anti-rabbit, were used at a 1:5000 dilution. Detection was facilitated using ECL Western Blotting Substrate.

4.2.3 Results

We examined the ability of GST-tagged full length (FL) RACK1 and deletion mutant RACK1 WD4-7 to interact with V5-tagged WT GSK-3α or GSK-3β to verify that the interaction of RACK1 with GSK-3α was physiologically significant and specific for GSK-3α. Cell lysates from transfected N2A cell were collected and either processed as total lysate for immunoblot or purified on glutathione sepharose and then processed for immunoblot. Antibodies against V5 demonstrated an obvious greater expression of WT GSK-3α as compared to WT GSK-3β in total lysate (TL), though GSK-3β still expressed at substantial and detectable levels (Figure 4.2A). Antibodies against GST revealed successful purification of FL and WD4-7 RACK1 (Figure 4.2A). Consistent with the yeast two-hybrid data, GSK-3α, but not GSK-3β, interacted with RACK1 as evidenced by V5 immunoreactivity in the glutathione sepharose purified (PD) fraction (Figure 4.2A). The interaction of GSK-3α with FL RACK1 was barely detectable but was significantly enhanced with RACK1 WD4-7 (Figure 4.2A). Thus, RACK WD4-7 was used in subsequent experiments.
**Figure 4.2** The interaction of GSK-3α with RACK1 WD4-7 requires the N- and C-termini of GSK-3α but not GSK-3α catalytic activity – GST-tagged full-length (FL) RACK1 or WD4-7 RACK1 was co-expressed with V5-tagged WT GSK-3α or GSK-3β (A.), WT or deletion mutant GSK-3α (B.) or WT or point mutant GSK-3α (C.) in N2A cells. Total lysate (TL) and glutathione sepharose-purified protein (PD) were separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize RACK1 (GST, top panel) and GSK-3 (V5, middle and bottom panels). Negative controls include empty vector transfection (GST) and vector co-transfection with WT GSK-3α as indicated in (A.) and (B.) and untransfected and WT GSK-3α transfection as indicated in (C.). Positive controls include WT GSK-3α co-transfection with RACK1 WD4-7 in (B.) and (C.). Arrows denote interaction and impairment of interaction in (A.) and (B.), respectively. (WT, wild type; FL, full-length; Δ, deleted; NT, N-terminus; CT, C-terminus; PD, pull-down; TL, total lysate)
We then examined the ability of N- and C-terminal deletion mutants to interact with RACK1 WD4-7 to determine the significance of these regions in maintaining the GSK-3α-RACK1 WD4-7 interaction. Cell lysates from transfected N2A cell were collected and either processed as total lysate for immunoblot or purified on glutathione sepharose and then processed for immunoblot. V5 immunoreactivity of TL demonstrated comparable expression of deletion mutants though WT GSK-3α expressed at somewhat higher levels (Figure 4.2B). Antibodies against GST revealed successful purification of RACK1 WD4-7 and V5 antibodies demonstrated that WT GSK-3α interacted strongly with RACK1 WD4-7 (Figure 4.2B). Only deletion mutant GSK-3α ΔCT-1 interacted strongly with RACK1 WD4-7 (Figure 4.2B). We did not detect an interaction of RACK1 WD4-7 with either GSK-α ΔCT-2 or GSK-3α ΔCT-3 (Figure 4.2B). The interaction of RACK1 WD4-7 with GSK-3α ΔNT was also impaired (Figure 4.2B).

Finally, we examined the ability of GSK-3α point mutants to interact with RACK1 WD4-7 to determine if key regulatory residues were important for the GSK-3α interaction with RACK1 WD4-7. Again, cell lysates from transfected N2A cell were collected and either processed as total lysate for immunoblot or purified on glutathione sepharose and then processed for immunoblot. V5 immunoreactivity of TL demonstrated comparable expression of WT and point mutant GSK-3α (Figure 4.2C). Antibodies against GST revealed successful purification of RACK1 WD4-7 and V5 antibodies demonstrated that all of the GSK-3α point mutants, GSK-3α K148R, GSK-3α R159A, GSK-3α S21A, and GSK-3α Y279F, interacted strongly with RACK1 WD4-7 (Figure 4.2C).
4.2.4 Discussion

The interaction of RACK1 WD4-7 with GSK-3 is specific for the GSK-3α isoform, as RACK1 WD4-7 does not interact with GSK-3β. We expected this isoform-specific interaction to be mediated by the non-homologous regions of GSK-3 isoforms, namely the N- and C-termini. Indeed, the interaction of GSK-3α with RACK1 WD4-7 required both the N- and C-termini of GSK-3α for optimal interaction. These requirements are distinct from the GSK-3α requirements for interaction with the axin GID, which involves only the C-terminus. These results indicate RACK1 WD4-7 may interact with GSK-3α by a mechanism distinct from that of axin GID.

Our analysis was conducted under basal conditions and thus stimulation of GSK-3α Ser21 phosphorylation was lacking and the effect of the GSK-3α S21A mutant wasn’t obvious. GSK-3α Y279F exhibited an interaction with RACK1 WD4-7 similar to WT GSK-3α and may even interact with a slightly greater affinity than WT GSK-3. These results, however, are not quantitative and as such, these conclusions are tentative. In addition, the GSK-3α interaction with RACK1 WD4-7 does not require GSK-3α catalytic activity, as noted by the ability of catalytically inactive GSK-3α K148R to interact with RACK1 WD4-7. The interaction also did not require the primed recognition ability of GSK-3α demonstrated by the interaction of GSK-3α R159A with RACK1 WD4-7.

The RACK1 WD4-7 interacted strongly with GSK-3α although this interaction does not reflect the endogenous interaction with FL RACK1. Indeed, the interaction of FL RACK1 with GSK-3α was very weak and nearly undetectable. It is possible that
endogenous RACK1 may require a particular conformation for interaction with GSK-3α that is not induced by the basal culture conditions. Alternatively, rather than serving as a scaffold for GSK-3α, RACK1 may serve as a GSK-3α substrate, which would likely result in a very transient interaction. RACK1 WD4-7 would then stabilize the interaction due to deletion of the GSK-3α phosphorylation site(s).

To our knowledge, this is the first report of a GSK-3α-specific protein interaction. This observation adds further support to the proposal that GSK-3 isoforms exhibit discrete biological activities. Indeed, such discrete activities may be mediated by distinct protein interactions, such as the RACK1 interaction with GSK-3α demonstrated here. Determining whether RACK1 is a GSK-3α substrate and/or a scaffold for GSK-3α will be important in determining the biological significance of this interaction.
CHAPTER 5

EXAMINATION OF THE STRUCTURAL INTEGRITY OF GSK-3 MUTANTS

5.1 Mutation of Conserved Proline Residues GSK-3α Pro^{442/443} GSK-3β Pro^{379/380}

5.1.1 Introduction

GSK-3β was crystallized in association with a minimal 18 amino acid peptide derived from the axin GID, which binds GSK-3β as a single alpha helix [69]. This crystal structure suggests that the minimal axin GID peptide occupies a hydrophobic surface groove in GSK-3β formed by amino acid residues 262-273, which form an alpha helix, and residues 285-299, which form an extended loop of conformational plasticity [69]. The crystal structure of GSK-3α has not been solved, although corresponding GSK-3α residues, 325-336 and 348-362, are identical in sequence to GSK-3β with a completely conserved surrounding region as well. Thus, axin GID would be expected to localize to the same region in GSK-3α that it does in GSK-3β. This region was not deleted from our GSK-3 C-terminal deletion mutants. In fact, the most extensive carboxyl termini deletion we created, GSK-3α ΔCT-4 and GSK-3β ΔCT-4, extended to amino acid 416 of GSK-3α and amino acid 344 of GSK-3β (Figure 2.1). Thus, we speculated that GSK-3α and
GSK-3β C-terminal deletion mutants were unable to bind the axin GID peptide due to compromised structurally integrity.

We noted an axin GID binding impairment with GSK-3α ΔCT-2 and GSK-3β ΔCT-2, but not with GSK-3α ΔCT-1 and GSK-3β ΔCT-1, indicating the seven amino acids distinguishing GSK-3α ΔCT-1 and GSK-3β ΔCT-1 from GSK-3α ΔCT-2 and GSK-3β ΔCT-2 are important for maintaining an Axin GID interaction (Figure 4.1A-B). *In silico* sequence analysis revealed two conserved proline residues, Pro\(^{442/443}\) of GSK-3α and Pro\(^{379/380}\) of GSK-3β, exist within this seven amino acid stretch (Figure 1.1). The distinctive side chains of proline residues confer conformational rigidity to protein structure. Therefore, we sought to determine the structural importance of GSK-3α Pro\(^{442/443}\) and GSK-3β Pro\(^{379/380}\) by converting them to alanine in the context of full-length GSK-3. We examined GSK-3α P442/443A and GSK-3β P379/380A activity towards tau, ability to suppress β-catenin-dependent LEF/TCF luciferase reporter, and ability to interact with the axin GID peptide. Together, our data indicate GSK-3α Pro\(^{442/443}\) and GSK-3β Pro\(^{379/380}\) are not important for GSK-3 activity, nor are they essential for the GSK-3 interaction with axin GID.

5.1.2 Materials and Methods

Materials and Methods have been described previously. For information on Materials and Methods used to measure GSK-3 activity towards tau see Section 3.1.2. Information regarding Materials and Methods used for analysis of the ability of GSK-3 to suppress β-catenin-dependent LEF/TCF luciferase reporter can be found in Section 3.2.2. Lastly, for
Materials and Methods information related to the examination of GSK-3 interaction with the axin GID refer to Section 4.1.2.

5.1.3 Results

To examine the activity of GSK-3α P442/443A and GSK-3β P379/380A towards tau Ser\(^{396/404}\), HEK 293T cells were transfected with Flag-tagged human tau and either a GST-tagged GSK-3α construct or a GST-tagged GSK-3β construct. Cell lysates were collected and analyzed by immunoblot. Antibodies against GST revealed comparable expression of GSK-3α WT and P442/443A (Figure 3.1A) and GSK-3β WT and P442/443A (Figure 3.1B). Likewise, antibodies against Flag demonstrated similar expression levels of tau (Figure 3.1A-B). Co-expression of WT GSK-3α and WT GSK-3β enhanced tau phosphorylation at Ser\(^{396}\) and Ser\(^{404}\) as recognized by the phosphorylation-specific antibody PHF1, which correlated with a band shift in the Flag immunoblot (Figure 3.1A-B). Co-expression of GSK-3α P442/443A and GSK-3β P442/443A also enhanced tau phosphorylation at Ser\(^{396}\) and Ser\(^{404}\), as indicated by PHF-1 immunoreactivity (Figure 3.1A-B).

Next, we examined the ability of GSK-3α P442/443A and GSK-3β P379/380A to suppress the β-catenin-inducible LEF/TCF firefly luciferase reporter, Super 8X TOPFlash [217]. HEK 293T cells were co-transfected with Super 8X TOPFlash, a Renilla luciferase reporter for normalizing, and either a GST-tagged GSK-3α construct or a GST-tagged GSK-3β construct. Transfected cells were treated in triplicate with either negative control L-cell media (LCM) or Wnt-3a conditioned media (WCM) to activate canonical Wnt signaling [218, 219]. Subsequent examination of luciferase activity in cell
lysates demonstrated that over-expression of WT GSK-3α (Figure 3.3A) and WT GSK-3β (Figure 3.3B) suppressed reporter expression. Similarly, GSK-3α P442/443A and GSK-3β P379/380A also suppressed reporter expression (Figure 3.3A-B).

Lastly, we examined the ability of GSK-3α P442/443A and GSK-3β P379/380A to interact with axin GID. HEK 293T cells were transfected with Myc-tagged axin GID and either a GST-tagged GSK-3α construct or a GST-tagged GSK-3β construct. Cell lysates were collected and either processed as total lysate or purified on glutathione sepharose. Immunoblot analysis with antibodies against GST revealed comparable levels of purified GSK-3α WT and P442/443A (Figure 4.1A) and GSK-3β WT and P379/380A (Figure 4.1B). Further, antibodies against Myc (9E10) demonstrated similar expression levels of axin GID expression in total lysate (Figure 4.1A-B). Myc antibodies also revealed a strong interaction of the axin GID with WT GSK-3α (Figure 4.1A) and WT GSK-3β (Figure 4.1B), while GSK-3α P442/443A (Figure 4.1A) and GSK-3β P379/380A (Figure 4.1B) appeared to exhibit a slight impairment in their ability to interact with the axin GID.

5.1.4 Discussion

Mutation of GSK-3α Pro^{442/443} and GSK-3β Pro^{442/443} did not negatively affect GSK-3 activity towards tau nor did mutation affect the ability of GSK-3 to negatively regulate canonical Wnt signaling. Thus, GSK-3α P442/443A and GSK-3β P379/380A are not required for GSK-3 activity. They may be important for the optimal interaction of GSK-3 with the axin GID as suggested by the slight reduction in affinity of GSK-3α P442/443A and GSK-3β P379/380A for Axin GID. It is unlikely that GSK-3α Pro^{442/443}
and GSK-3β Pro$^{379/380}$ directly mediate the interaction of axin GID with the hydrophobic groove of GSK-3 considering the spatial separation of the hydrophobic groove and GSK-3α Pro$^{442/443}$ and GSK-3β Pro$^{379/380}$ in the three-dimensional GSK-3β structure (PDB ID: 1o9u). Rather, an indirect mechanism may involve a role for GSK-3α Pro$^{442/443}$ and GSK-3β Pro$^{379/380}$ in structuring the hydrophobic groove that the axin GID occupies.

It should be noted that the 109 amino acid axin GID (Axin GID$^{109}$) peptide we used to assay the interaction of GSK-3 with the axin GID was significantly larger than the minimal 18 amino acid axin GID (Axin GID$^{18}$) peptide crystallized in association with GSK-3β. Though the axin GID$^{109}$ peptide does encompass the axin GID$^{18}$ sequence, the extraneous sequence of the axin GID$^{109}$ peptide may interact with regions of GSK-3 outside of the hydrophobic groove that are not evident from crystal structure analysis. Such interactions would represent a more physiological scenario, but whether they involve the C-termini of GSK-3 isoforms is not known.

5.2 Analysis of GSK-3α Tyr$^{279}$ and GSK-3β Tyr$^{216}$ Phosphorylation

5.2.1 Introduction

The subtle reduction of the interaction of GSK-3α P442/443A and GSK-3β P379/380A with the axin GID hinted at the potential importance of the C-terminus in structuring the GSK-3 molecule. However, the interaction of GSK-3α P442/443A and GSK-3β P379/380A with the axin GID was much greater than GSK-3α ΔCT-2 and GSK-3β ΔCT-2. Thus, removal of Pro$^{442/443}$ from GSK-3α ΔCT-1 and Pro$^{379/380}$ from GSK-3β ΔCT-1 when generating GSK-3α ΔCT-2 and GSK-3β ΔCT-2 cannot, in itself, account
for the dramatic reduction in the ability of GSK-3α ΔCT-2 and GSK-3β ΔCT-2 to interact with the axin GID. Therefore, we reasoned that the loss of an interaction with the axin GID peptide observed with GSK-3α ΔCT-2 and GSK-3β ΔCT-2 and subsequent C-terminal deletions may result from a general effect on protein structure, such as misfolding, and not from deletion of any particular residue(s).

Next, we sought to examine protein structure of GSK-3α and GSK-3β mutants by measuring the phosphorylation status of Tyr279 and Tyr216 respectively. Phosphorylation of GSK-3β Tyr216 has been shown to occur through an intramolecular autophosphorylation event during protein folding [68, 72, 231]. We predicted a structurally compromised GSK-3α/β mutant may exhibit reduced Tyr279/216 phosphorylation due to defective protein folding. Indeed, analysis of transfected cell lysates demonstrated a progressive reduction of GSK-3α/β Tyr279/216 phosphorylation in C-terminal deletion mutants that culminated in complete loss in GSK-3α ΔCT-4 and GSK-3β ΔCT-4.

5.2.2 Materials and Methods

Plasmids – Human GSK-3α and GSK-3β WT and mutant constructs were generated as described in section 3.1.2. pMAX EGFP was obtained from Amaxa. Empty vector pcDNA3.1 was obtained from Invitrogen.

Cell Culture and Transient Transfections – HEK 293T cells were maintained in DMEM supplemented with 10% BGS and 1% penicillin-streptomycin solution at 37°C and 5% CO2. Cells were plated under reduced (2.5%) serum conditions at 1.0 x 10^6 in 6-well plates 24 hours prior to transfection. Subconfluent HEK 293T cells were maintained
in reduced (2.5%) serum conditions during transient transfection with PEI dissolved in 50 mM HEPES buffer, pH 7.05. Each transfection included 0.2 µg pMAX EGFP and 1 µg pcDNA3.1 or GSK-3 construct as indicated. Co-transfection with vector encoding EGFP allowed for visual assessment of transfection efficiency.

**Cell lysis** - Approximately 24 hours post-transfection, cells were collected with trypsin and pelleted by centrifugation at 1,500 rpm for two minutes. After one wash with 1× PBS, cells were lysed in IP/lysis buffer supplemented with PIC. Lysates were incubated on ice for approximately 20 minutes with intermittent vortexing. Lysates were then cleared by high-speed centrifugation at 4°C for 15 minutes. The supernatant fractions were either collected and boiled in sample buffer for five minutes or utilized in subsequent GST pull-down assays.

**GST Pull-down Assay** - Cleared cell lysates were incubated with glutathione sepharose 4B beads for 2 hours at 4°C. Subsequently, reactions were centrifuged to pellet beads, which were then washed 4 times with two volumes IP/lysis buffer supplemented with PIC. Following the final wash, protein was eluted by boiling in sample buffer.

**Immunoblotting and Antibodies** - Approximately equal amounts of denatured protein lysate was separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted. Mouse monoclonal anti-GSK-3β pY216 (BD Transduction Laboratories) primary antibody, which also recognized GSK-3α pY279, was used at a 1:1000 dilution in conjunction with secondary antibody HRP-linked anti-mouse at a 1:5000 dilution. Detection was facilitated using ECL Western Blotting Substrate.

**Densitometry** - Chemiluminescence was quantified by measuring area density using an UltraLum documentation system and software (UltraLum Inc., Claremont, CA).
Densitometry values represent the ratio of quantified immunoreactivity of phospho-Tyr\textsuperscript{279/216} GSK-3α/β:total GSK-3α/β.

5.2.3 Results

To examine mutant GSK-3α/β Tyr\textsuperscript{279/216} phosphorylation, HEK 293T cells were transfected with a GST-tagged GSK-3α construct or a GST-tagged GSK-3β construct. Cell lysates were collected, purified on glutathione sepharose, and analyzed by immunoblot. Antibodies against GST demonstrated successful purification of GSK-3α (Figure 5.1A) and GSK-3β (Figure 5.1B) deletion and point mutants. Analysis with a GSK-3α/β phospho-Tyr\textsuperscript{279/216} antibody revealed WT GSK-3α (Figure 5.1A) and WT GSK-3β (Figure 5.1B) were phosphorylated on Tyr\textsuperscript{279} and Tyr\textsuperscript{216} respectively. Similarly, GSK-3α ΔNT, GSK-3α ΔCT-1, GSK-3β ΔNT, and GSK-3β ΔCT-1 were also phosphorylated on respective Tyr\textsuperscript{279} and Tyr\textsuperscript{216} (Figure 5.1A-B). However, subsequent C-terminal deletion mutants, GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3β ΔCT-2, and GSK-3β ΔCT-3 exhibited diminished Tyr\textsuperscript{279/216} phosphorylation (Figure 5.1A-B). Phosphorylation of Tyr\textsuperscript{279/216} progressed to complete abolishment in GSK-3α ΔCT-4 and GSK-3β ΔCT-4 deletion mutants (Figure 5.1A-B).
Figure 5.1 C-terminal deletion of GSK-3 isoforms abolishes phosphorylation of GSK-3α Tyr279 and GSK-3β Tyr216 – Deletion and point mutants of GST-tagged GSK-3α (A.) and GSK-3β (B.) were expressed in HEK 293T cells, affinity-purified on glutathione sepharose, separated by tricine-SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies that recognize GSK-3 specifically phosphorylated at Tyr279/216 (pTyr279 and pTyr216, top panels) and GSK-3 (GST, bottom panels). Results are representative of three independent experiments. Numerical values were derived from densitometry experiments. Error represents standard deviation. Untransfected (Un), vector (Vec) alone transfection, GSK-3α K148R, GSK-3β K85R, GSK-3α Y279F, and GSK-3β Y216F serve as negative controls as indicated. GSK-3α WT and GSK-3β WT serve as positive controls as indicated. Arrows denote GSK-3α/β mutants that exhibited impaired phosphorylation of Tyr279/216 (WT, wild type; Δ, deleted; NT, N-terminus; CT, C-terminus)
GSK-3α phosphorylation of Tyr\textsuperscript{279} was not notably effected in GSK-3α P442/443A or GSK-3α R159A point mutants (Figure 5.1A). Likewise, GSK-3β Tyr\textsuperscript{216} phosphorylation was not obviously reduced in GSK-3β P379/380A or GSK-3β R96A point mutants (Figure 5.1B). Phosphorylation of GSK-3α Ser\textsuperscript{21}, GSK-3β Ser\textsuperscript{9}, GSK-3β Thr\textsuperscript{300} was not induced under our conditions and therefore, potential effects of GSK-3α S21A, GSK-3β S9A, and GSK-3β T390A were not evident (Figure 5.1A-B). Tyr\textsuperscript{279/216} phosphorylation was not evident in GSK-3α K148R and GSK-3β K85R, nor was it evident in negative control mutants GSK-3α Y279F or GSK-3β Y216F (Figure 5.1A-B). Densitometry values, which represent the ratio of Tyr\textsuperscript{279/216} phosphorylated GSK-3α/β to total GSK-3α/β, verified our observations.

5.2.4 Discussion

Tyr\textsuperscript{279/216} phosphorylation was progressively lost from C-terminal deletion mutants, indicating these mutants are unable to properly fold and thus, unable to autophosphorylate Tyr\textsuperscript{279/216}. The reduction in Tyr\textsuperscript{279/216} phosphorylation of C-terminal deletion mutants correlates well with the diminished interaction of GSK-3 C-terminal deletion mutants with Axin GID and even better with the reduction in GSK-3 activity exhibited by these mutants. Tyr\textsuperscript{279/216} phosphorylation has been implicated in potentiating GSK-3 activity by others [68, 70-77], but in our assays the ability of GSK-3α Y279F and GSK-3β Y216F to function similar to WT GSK-3α and WT GSK-3β suggests Tyr\textsuperscript{279/216} phosphorylation is dispensable for activity. Thus, under our experimental conditions, the reduction in Tyr\textsuperscript{279/216} phosphorylation in our C-terminal deletion mutants appears to be a better indicator of misfolding than actual activity.
The lack of Tyr\(^{216}\) phosphorylation in catalytically inactive point mutant GSK-3β K85R supports the previous report [72, 231] that an intramolecular autophosphorylation event mediates Tyr\(^{216}\) phosphorylation. Only GSK-3β, and not GSK-3α, was examined in these reports. Thus, our observation that GSK-3α K148R lacks Tyr\(^{279}\) phosphorylation verifies that an analogous intramolecular autophosphorylation mechanism mediates Tyr\(^{279}\) phosphorylation of GSK-3α as well.

5.3 **Protein Stability of GSK-3α and GSK-3β Mutants**

5.3.1 Introduction

The lack of Tyr\(^{279/216}\) phosphorylation suggests C-terminal deletion of GSK-3 isoforms results in protein misfolding, which accounts for the loss of both activity and the interaction with axin GID. We reasoned that if GSK-3 C-terminal deletion mutants are misfolding, then they may exhibit reduced stability reflected by a reduction in protein half-life. Presently, we examined protein half-lives of C-terminal deletion mutants by pulse-chase analysis. With the exception of GSK-3α ΔCT-3, we observed a reduction in protein half-lives when the C-termini were deleted from GSK-3 isoforms.

5.3.2 Materials and Methods

*Plasmids* – Human GSK-3α and GSK-3β WT and mutant constructs were generated as described in section 3.1.2. *pMAX EGFP* was obtained from Amaxa. Empty vector *pcDNA3.1* was obtained from Invitrogen.
Cell Culture and Transient Transfections – HEK 293T cells were maintained in DMEM supplemented with 10% BGS and 1% penicillin-streptomycin solution at 37°C and 5% CO₂. Cells were plated under reduced (2.5%) serum conditions at 1.0 x 10⁶ in 6-well plates 24 hours prior to transfection. Subconfluent HEK 293T cells were maintained in reduced (2.5%) serum conditions during transient transfection with PEI dissolved in 50 mM HEPES buffer, pH 7.05. Each transfection included 0.2 µg pMAX EGFP and 1 µg pcDNA3.1 or GSK-3 construct as indicated. Co-transfection with vector encoding EGFP allowed for visual assessment of transfection efficiency.

Pulse-chase and cell lysis - Twenty-four hours after transfection, cells were pulsed with 85 µCi ³⁵S-methionine-labeled media (PerkinElmer) per well for 15 minutes and subsequently chased with unlabeled media for 0 minutes, 6 hours, 12 hours, and 24 hours. Cells were then collected with trypsin and pelleted by centrifugation at 1,500 rpm for two minutes. After one wash with 1× PBS, cells were lysed in IP/lysis buffer supplemented with PIC. Lysate was incubated on ice for approximately 20 minutes with intermittent vortexing. Lysate was then cleared by high-speed centrifugation at 4°C for 15 minutes. The supernatant fraction was collected and utilized in subsequent GST pull-down assays.

GST Pull-down Assay - Equal amounts of cleared cell lysates were incubated with glutathione sepharose 4B beads for 2 hours at 4°C. Subsequently, reactions were centrifuged to pellet beads, which were then washed 4 times with two volumes IP/lysis buffer supplemented with PIC. Following the final wash, protein was eluted by boiling in sample buffer.

Electrophoresis and protein detection – Glutathione sepharose-purified protein lysates were separated by tricine-SDS-PAGE. The gel was dried and exposed to a phosphor
screen (Amersham Biosciences) for 24 - 48 hours. Images were collected from a Typhoon 9400 variable mode imager (Amersham Biosciences) and analyzed using ImageQuant software (Amersham Biosciences).

5.3.3 Results

Pulse-chase analysis in transfected HEK 293T cells demonstrated that overexpressed GST-tagged WT GSK-3α exhibited a half-life of approximately 290 minutes (Figure 5.2A). By comparison GSK-3α ΔCT-1, GSK-3α ΔCT-2, and GSK-3α ΔCT-4 all displayed reduced half-lives of approximately 225 minutes, 170 minutes, and 175 minutes, respectively (Figure 5.2A). Conversely, GSK-3α ΔCT-3 retained a half-life stability of 315 minutes, which was comparable to WT GSK-3α (Figure 5.2A).
Figure 5.2  GSK-3 C-terminal deletion mutants exhibit reduced half-lives – HEK 239T cells were transiently transfected with GST-tagged GSK-3 constructs. Twenty-four hours after transfection, cells were pulsed with 85 µCi $^{35}$S-methionine-labeled media per well for 15 minutes and subsequently chased for 0 minutes, 6 hours, 12 hours, and 24 hours. Protein lysates from each time point were purified on glutathione sepharose and separated by tricine-SDS-PAGE. After electrophoresis, the gel was dried, exposed to a phosphor screen, and images were collected and analyzed. Empty vector (Vec) and WT GSK-3α/β serve as negative and positive controls, respectively. Arrows denote GSK-3 mutants that exhibit reduced protein half-lives. (WT, wild type; Δ, deleted; CT, C-terminus)
In turn, overexpressed GST-tagged WT GSK-3β exhibited a half-life of about 630 minutes (Figure 5.2B). Half-lives of GSK-3β ΔCT-1, GSK-3β ΔCT-2, GSK-3β ΔCT-3, and GSK-3β ΔCT-4 progressively decreased and these mutants displayed approximate half-lives of 350 minutes, 290 minutes, 225 minutes, and 210 minutes, respectively (Figure 5.2B).

5.3.4 Discussion

The progressive reduction in the half-lives of GSK-3β C-terminal deletion mutants was reminiscent of the progressive reduction in Tyr²¹⁶ phosphorylation. The half-lives of GSK-3α C-terminal deletion mutants, however, were more variable and lacked such progression. Interestingly, GSK-3α ΔCT-3 exhibited stability similar to WT GSK-3α, suggesting that GSK-3α ΔCT3 may acquire a conformational stability analogous to WT GSK-3α that is further abolished in GSK-3α ΔCT4. This apparent stability of GSK-3α ΔCT-3 was not reflected by retention of activity or interaction with axin GID. Since this data represents only a single assay, experimental error may also account for the discrepancy.

In our assays measuring GSK-3 activity, GSK-3 protein levels were approximately equivalent as detected by immunoblot analysis, demonstrating steady-state protein expression under the constitutive cytomegalovirus (CMV) promoter. Thus, the observed reduction in protein half-lives did not cause the reduction in activity we observed with C-terminal deletion mutants. Rather, the reduced half-lives are likely concomitant with reduced activity of C-terminal deletion mutants, both resulting from protein misfolding caused by deletion of the C-terminus.
5.4 Localization of GSK-3α and GSK-3β Mutants

5.4.1 Introduction

To further support our hypothesis that C-terminal deletion results in protein misfolding, we investigated the cellular localization of GSK-3 mutants. We rationalized that a misfolded protein might abnormally accumulate and exhibit altered cellular distribution. We examined localization of fluorescently tagged cherry-GSK-3 mutants in HeLa cells and noted, contrary to WT GSK-3α and WT GSK-3β, an increased propensity of GSK-3 C-terminal deletion mutants to form aggregate-like inclusions.

5.4.2 Materials and Methods

Plasmids – GSK-3α and GSK-3β were directionally cloned into Gateway destination vector pCSCherry DEST (obtained from Dr. Nathan Lawson, University of Massachusetts Medical School), which generated N-terminal Cherry-tagged proteins.

Cell Culture and Transient Transfections – HeLa cells were maintained in DMEM supplemented with 10% BGS and 1% penicillin-streptomycin solution at 37°C and 5% CO₂. Cells were plated at 6.0 x 10⁵ in 6-well plates 24 hours prior to transfection. Subconfluent HeLa cells were maintained in reduced (2.5%) serum conditions during transient transfection with PEI dissolved in 50 mM HEPES buffer, pH 7.05. Each transfection included 1 μg pCSCherry DEST or GSK-3 pCSCherry DEST as indicated. Twenty-four hours post-transfection, cells were transfected to coverslips in a 24-well plate and allowed to adhere an additional 24 hours.
Cell fixation and permeabilization - Transfected cells were fixed with 4% paraformaldehyde (Polysciences, Inc.) for 15 minutes followed by permeabilization with 0.1% Triton-X100 (Sigma) for 10 minutes. Coverslips were mounted using VECTASHIELD mounting media with DAPI (Vector Laboratories). Localization was visualized under oil immersion at 63X magnification with a Leica DMIRB microscope (Leica Microsystems).

Characterization of localization – Cellular localization of overexpressed cherry-GSK-3 was characterized according to the following criteria: 1) nuclei must be intact and cell cannot be dividing, 2) nuclei must not be overlapping, 3) cytoplasm must not be overlapping, and 4) fluorescence must be strong enough to capture with less than or equal to a 30 second exposure under default settings. Localization was classified into one of three categories: cytoplasmic, aggregated, or other. Cytoplasmic localization was generally of a diffuse pattern but may also include punctate localization patterns in addition to diffuse localization patterns. Aggregated localization specifically refers to unilateral, perinuclear, aggregated localization but may also include diffuse or punctate cytoplasmic localization in addition to the aggregated localization. Other includes anything other than that described for cytoplasmic or aggregated patterns of localization. Examples in the other category include punctate, cytoplasmic localization in the absence of diffuse cytoplasmic localization patterns, nuclear localization, localization encompassing the rim of the nucleus, and non-perinuclear aggregate localization, which usually completely overlapped with the nucleus, but was occasionally noted in the cytoplasm as well.
5.4.3 Results

WT GSK-3α (Figure 5.3A) and WT GSK-3β (Figure 5.3B) localized largely in a diffuse pattern in the cytoplasm. However, C-terminal deletion mutants GSK-3α ΔCT-2, GSK-3α ΔCT-4, GSK-3β ΔCT-2, GSK-3β ΔCT-3 and GSK-3β ΔCT-4 frequently localized as aggregate-like inclusions, distributed unilaterally in the perinuclear region (Figure 5.3, Table 5.1). GSK-3α ΔNT, GSK-3α ΔCT-3, GSK-3β ΔNT also exhibited an aggregated localization though to a lesser extent (Figure 5.3A-B, Table 5.1).
A.

GSK-3α WT  GSK-3α ΔCT-4  GSK-3α R159A

B.

GSK-3β WT  GSK-3β ΔCT-4  pCSCherry

**Figure 5.3** GSK-3 C-terminal deletion mutants mislocalize – Fluorescently-tagged cherry-GSK-3α and GSK-3β constructs were transiently transfected into HeLa cells. Transfected cells were transferred to coverslips in a 24-well plate and were subsequently fixed, permeabilization, and mounted with DAPI staining. Representative images of the diffuse pattern of cytoplasmic localization are shown, evidenced with WT GSK-3α (A.) and WT GSK-3β (B.), versus the unilateral perinuclear aggregated localization, demonstrated by GSK-3α ΔCT-4 and GSK-3β ΔCT-4. GSK-3α R159A and empty vector pCSCherry represent the other category. Representative cells are indicated with white arrows. Cherry fluorescence is indicated in red. Nuclear DAPI staining is shown in blue. Images were acquired under oil immersion at 63X magnification. (WT, wild type; Δ, deleted; CT, C-terminus)
Table 5.1 Summary of mutant GSK-3 localization – Localization patterns of fluorescently tagged cherry-GSK-3α (A.) and cherry-GSK-3β (B.) constructs transiently transfected into HeLa cells were classified into one of three categories. Cytoplasmic localization includes diffuse cytoplasmic localization. Aggregated localization includes unilateral, perinuclear, aggregated localization. Other phenotypes, although less frequently noted, includes patterns such as punctate cytoplasmic localization, nuclear rim localization, and non-perinuclear aggregated localization. Percentage represents the percent of scored cells and was derived from the fraction in parentheses in which the denominator represents n. Empty vector pCSCCherry represents negative controls. WT GSK-3α (A.) or WT GSK-3β (B.) serve as positive controls. Arrows denote GSK-3 mutants that formed aggregated patterns of localization in greater than 10% of examined cells. (WT, wild type; Δ, deleted; NT, amino terminus; CT, carboxyl terminus)
Point mutants GSK-3α K148R and GSK-3α R159A occasionally displayed an aggregated localization pattern, as did GSK-3β K148R but not GSK-3β R96A (Table 5.1A-B). GSK-3β P370/380A, but not GSK-3α P442/443A, exhibited a slightly increased tendency to mislocalize (Table 5.1A-B). Other point mutants GSK-3α S21A, GSK-3α Y279F, GSK-3β S9A, GSK-3β Y216F, and GSK-3β T390A did not exhibit alterations in localization and generally localized in a diffuse, cytoplasmic manner, similar to their WT counterparts (Table 5.1A-B).

5.4.4 Discussion

Deletion of both the N- and C-termini resulted in varying degrees of aggregated localization, suggestive of improper protein folding. Though we did not characterize the specific subcellular localization, the perinuclear distribution of the aggregated protein suggests that it may be accumulating in the endoplasmic reticulum (ER), which might be expected for a misfolded protein. C-terminal deletion produced a more pronounced effect on aggregated localization than N-terminal deletion, which is consistent with the reduced activity, inability to interact with Axin GID, and the reduction in Tyr\(^{279/216}\) phosphorylation of the C-terminal deletion mutants. Interestingly, aggregation of GSK-3α ΔCT-3 was not as obvious as GSK-3α ΔCT-2 or GSK-3α ΔCT-4, which may relate to the WT-like stability of GSK-3α ΔCT-3 we observed by pulse-chase analysis.

Interestingly, point mutant GSK-3α R159A exhibited a greater propensity to localize in an aggregated manner than GSK-3β R96A, which support our hypothesis that GSK-3α is more sensitive than GSK-3β to structural deformations as first evidenced by the reduced ability of GSK-3α ΔCT-2 and GSK-3α ΔCT-3 to suppress the β-catenin-
dependent reporter. The detection of aggregated point mutants GSK-3α K148R and GSK-3β K148R, suggests that localization may be dependent on catalytic activity. It does not appear such required catalytic activity is imparted through autophosphorylation of Tyr^{279/216}, as GSK-3α Y279F and GSK-3β Y216F localize normally. Alternatively, if aggregated localization is considered a product of protein misfolding, then GSK-3α K148R and GSK-3β K148R may exhibit impaired structural integrity that accounts for their occasional aggregated localization. Indeed, such impairments may also account for the inability of GSK-3α K148R and GSK-3β K148R to interact with axin GID.

Aggregated localization might explain the reduction in activity and inability to interact with axin GID demonstrated by C-terminal deletion mutants and catalytically inactive point mutants. However, these mutants did not exclusively exhibit aggregated localization. In fact, with the exception of GSK-3β ΔCT-3, aggregated localization represented less than half of the characterized localization patterns of these mutants. Thus, it would appear ample GSK-3 was appropriately localized to sufficiently measure functional characteristics, such as activity and protein interactions. Further, if aggregated protein is indeed localized to the ER as we speculate, phosphorylation of Tyr^{279/216} should also represent an accurate measurement since this event occurs during protein folding.
CHAPTER 6

GENERAL DISCUSSION

The data presented herein provide evidence that the C-termini of GSK-3 isoforms are structurally important for GSK-3 function. Deletion of the C-terminus resulted in reduced ability of mutants to interact with axin GID, impaired tyrosine autophosphorylation, decreased protein stability, and subcellular mislocalization, suggesting that C-terminal deletion mutants do not fold properly. Our observations imply misfolding occurs concomitantly with reduced autophosphorylation, protein destabilization, and mislocalization, ablating activity in the most extensive C-terminal deletion mtants, GSK-3α ΔCT-4 and GSK-3β ΔCT-4. Conversely, N-terminal deletion does not negatively affect GSK-3 activity or the interaction with axin GID and had only a modest effect on localization. Cleavage of GSK-3 isoforms at the N-terminus by calcium-induced calpain increases GSK-3β activity, while GSK-3α activity is unaffected [232]. Our data, however, do not suggest GSK-3 activity is altered with N-terminal deletion and deletions affect GSK-3α and GSK-3β similarly. Thus, the N-termini of GSK-3α and GSK-3β are functionally dispensable under basal conditions in HEK 293T cells.
We initiated the structure-function analysis of GSK-3 isoforms to identify isoform-specific structural requirements. Indeed, several lines of evidence imply that GSK-3α and GSK-3β possess unique structural requirements related to function. First, GSK-3α and GSK-3β exhibit distinct requirements for tau priming. In contrast to GSK-3β R96A, GSK-3α R159A was unable to induce maximal tau phosphorylation at Ser\textsuperscript{396/404}. Additionally, GSK-3α R159A exhibits greater mislocalization into aggregated inclusions than GSK-3β R96A. Together, these data suggest that GSK-3α possesses a more rigid requirement for a functional Arg\textsuperscript{159} than the GSK-3β requirement for the analogous Arg\textsuperscript{96}. Secondly, while both GSK-3α and GSK-3β require an intact C-terminus for optimal activity, subtle isoform-specific differences were noted with C-terminal deletion mutants. GSK-3α ΔCT-2 and GSK-3α ΔCT-3 are slightly impaired in their ability to suppress the TOPFlash reporter, while analogous GSK-3β deletion mutants functioned similarly to WT GSK-3β. Furthermore, close inspection of Tyr\textsuperscript{279/216} autophosphorylation data show an abrupt reduction in GSK-3α autophosphorylation when progressing from GSK-3α ΔCT-1 to GSK-3α ΔCT-2, while autophosphorylation of GSK-3β C-terminal deletion diminishes more gradually. While deletion of the GSK-3β C-terminus progressively reduces protein stability, stability of GSK-3α C-terminal deletion mutants is more variable. In fact, the stability of GSK-3α ΔCT-3 resembles that of WT GSK-3α. GSK-3α ΔCT-3 also exhibits a reduced propensity to localize in an aggregated manner, suggesting a link between protein stability and formation of aggregated protein. Resolution of the GSK-3α crystal structure may provide further insight into the distinct structural requirements of GSK-3 isoforms for priming.
However, analysis of GSK-3β crystal structure indicates that the most distal C-terminal region of GSK-3β is disordered and unstructured [59-62]. Thus, alternative approaches will be required for deciphering the precise structure of GSK-3 isoforms within the C-terminus.

One isoform-specific difference we note in our analysis is the distinct interaction of GSK-3α with RACK1 WD4-7. This interaction requires both the N- and C-termini of GSK-3α. Conversely, the GSK-3α interaction with axin GID requires only the C-terminus. Thus, the GSK-3α structural requirements for the interaction with RACK1 WD4-7 are distinct from those for axin GID. Based on our data, it is likely that the reduced ability of GSK-3α C-terminal deletion mutants to interact with RACK1 WD4-7 results from the compromised structural integrity of GSK-3α. Alternatively, we did not detect structural deformation with deletion of the N-terminus. The N-terminus of GSK-3α therefore represents a valid region for mediating the interaction of GSK-3α with RACK1 WD4-7. It is not clear whether this interaction is direct, requires adaptor proteins, or extracellular stimuli. Further, the functional significance of the GSK-3α-RACK1 interaction is not known but is under investigation in our lab.

Point mutants GSK-3α S21A, GSK-3β S9A, GSK-3β T390A did not display any obvious differences in our assays compared to WT counterparts. We attribute these observations to the absence of stimuli capable of inducing phosphorylation at these sites in WT GSK-3α and WT GSK-3β, which masks the effect by comparison. Future examination of the effect of GSK-3α Ser^{21} phosphorylation on the interaction of GSK-3α with RACK WD4-7 may be particularly worthwhile. The N-terminus of GSK-3α is
important for mediating the GSK-3α interaction with RACK WD4-7. Thus, phosphorylation of N-terminal Ser\(^{21}\) in WT GSK-3α may modulate this interaction. Though GSK-3α S21A interacted with RACK1 WD4-7 similarly to WT GSK-3α, Ser\(^{21}\) phosphorylation is not induced under the basal cell culture conditions employed in which GSK-3 exhibits constitutive activity. Thus, the effect of inhibitory Ser\(^{21}\) phosphorylation is not evident. Re-examining this interaction under conditions that inhibit WT GSK-3α through Ser\(^{21}\) phosphorylation and comparison with GSK-3α S21A may shed light on a physiological mechanism. Furthermore, GSK-3β Thr\(^{390}\) represents an isoform-specific site of modulation by p38 MAPK [135]. Phosphorylation of Thr\(^{390}\) inhibits GSK-3β in a tissue-specific manner and elevates β-catenin levels through a Wnt-independent mechanism [135]. An effect of Thr\(^{390}\) phosphorylation on GSK-3β activity towards tau has not been examined. p38 MAPK is highly activate in brain [135] and may represent a potent mechanism for regulating GSK-3β activity towards tau. Thus, evaluating the effect of Thr\(^{390}\) phosphorylation on GSK-3β activity towards tau may provide physiologically relevant insights to isoform-specific modulation of GSK-3β activity towards tau. Insights into such a mechanism may lead to the identification of p38 MAPK as a therapeutic target for amelioration of tauopathies.

The similarity of GSK-3α Y279F and GSK-3β Y279F to respective WT GSK-3α and WT GSK-3β in our assays consistently challenged the conventional belief that phosphorylation of GSK-3α Tyr\(^{279}\) and GSK-3β Tyr\(^{216}\) is required for maximal activity. Contrary to numerous previous reports [68, 71-78, 96, 220], GSK-3α Y279F and GSK-3β Y279F retained both activity and the ability to interact with axin GID. The
discrepancy between our data and previous reports is unclear. A possible explanation is that, under our experimental conditions, a site nearby GSK-3α Tyr^279^ and GSK-3β Tyr^216^ substitutes as a phosphate acceptor to rescue activity when Tyr^279/216^ are mutated. However, such a mechanism has been previously evaluated in GSK-3β with no evidence of a phosphorylation rescue event [72]. Alternatively, subtle alterations in GSK-3α Y279F and GSK-3β Y279F function may have been overshadowed by overexpression and/or the end-point assays employed. Indeed, our inability to successfully implement *in vitro* kinase assays prevented us from thoroughly analyzing possible functional kinetic deficits of GSK-3α Y279F and GSK-3β Y279F. Nonetheless, our data indicate that phosphorylation of Tyr^279/216^ during protein folding is not an absolute requirement for acquisition of a functional conformation, and Tyr^279/216^ phosphorylation may play a less significant role than originally proposed.

The inability of GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3α ΔCT-4, GSK-3β ΔCT-2, GSK-3β ΔCT-3, and GSK-3β ΔCT-4 to bind axin GID can be attributed to compromised structural integrity of the hydrophobic groove. Similarly, indications of compromised structural integrity of GSK-3α K149R and GSK-3β K85R may also explain the inability of these mutants to bind axin GID. For GSK-3α K149R and GSK-3β K85R, Tyr^279/216^ phosphorylation was not a valid indicator of protein folding because Tyr^279/216^ phosphorylation occurs as an intramolecular autophosphorylation event and GSK-3α K149R and GSK-3β K85R lack catalytic activity. The aggregated localization of GSK-3α K149R and GSK-3β K85R suggests these mutants may fail to fold properly. Aggregated localization was not detected frequently but occurred at a frequency similar to some of the C-terminal deletion mutants. Additionally, molecular dynamic simulation
of GSK-3β K85R also indicated structural abnormalities, although these occurred in the phosphate-binding pocket [233] and alterations of the hydrophobic groove were not reported. Deformations of the phosphate-binding pocket by mutation of GSK-3α Arg^{159} and GSK-3β Arg^{96} apparently do not alter the integrity of the hydrophobic groove, as axin GID binding properties of GSK-3α R159A and GSK-3β R96A were not obviously different from WT GSK-3α and WT GSK-3β, respectively. Variations of the phosphate-binding groove by GSK-3α K149R and GSK-3β K85R mutations may involve distinct alterations of intramolecular interactions that could potentially affect other regions of the GSK-3 molecule. Thus, aberrant protein folding may partially explain the inability of GSK-3α K149R and GSK-3β K85R to interact with axin GID, but further investigation is required.

Compromised structural integrity of GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3β ΔCT-2, and GSK-3β ΔCT-3 and possible structural impairment of GSK-3α K149R and GSK-3β K85R did not prevent these mutants from functioning in the TOPFlash reporter assay. Indeed, GSK-3α ΔCT-2, GSK-3α ΔCT-3, GSK-3β ΔCT-2, and GSK-3β ΔCT-3 retained the ability to suppress reporter gene expression and GSK-3α K149R and GSK-3β K85R exhibited potent dominant-negative activity. Axin-independent mechanisms to account for these observations are unclear. A potential mechanism for GSK-3α K149R and GSK-3β K85R dominant-negative activity may involve re-localization of the cadherin-associated pool of β-catenin to the nucleus. Cadherin-associated β-catenin normally localizes to adherens junctions where it functions in cell adhesion [234, 235]. Dissociation of adherens junctions results in translocation of β-catenin to the nucleus.
Future investigation into a role for GSK-3 isoforms in modulating cadherin-associated β-catenin would provide insight into a potential axin-independent mechanism of dominant-negative activity of GSK-3α K149R and GSK-3β K85R. Though our data suggest that the interaction of GSK-3 with axin is not essential for GSK-3 activity in Wnt signaling, our evaluation of the interaction of GSK-3 with axin GID does not represent an endogenous GSK-3 interaction with axin. To evaluate this further, the interaction of GSK-3 with full-length axin should be examined to verify the potential axin-independence of GSK-3 activity in Wnt signaling. Additionally, reporter expression serves as an indirect measure of β-catenin levels. Since we did not directly β-catenin levels, we cannot discount an indirect activation of reporter expression.

Similar to GSK-3, dual-specificity tyrosine-phosphorylation regulated kinase 2 (DYRK2) also undergoes autophosphorylation of an activation loop tyrosine residue during protein folding. Recently, deletion analysis of DYRK2 revealed the non-catalytic N-terminus is essential for autophosphorylation [237]. N-terminal deletion of DYRK2 removes a conserved region that provides an essential chaperone-like function. Interestingly, the isolated N-terminus rescues tyrosine autophosphorylation of the N-terminal deletion mutant in trans. Thus, future pursuits will examine whether the C-terminus can rescue GSK-3 autophosphorylation in trans to determine whether the C-termini of GSK-3 isoforms play similar chaperone-like role.

The detrimental folding effect of C-terminal deletion on GSK-3 structure precluded us from testing our original hypothesis that the C-terminal of GSK-3 isoforms mediate distinct activities. A more suitable approach to testing this hypothesis may employ chimeric GSK-3 constructs generated by replacing the C-terminus of GSK-3α with that
of GSK-3β and vice versa. Such fusion proteins would facilitate analysis of isoform specificities mediated by the C-terminus in a full-length protein not susceptible to misfolding.

Our data provide a comprehensive analysis of the structure-function relationship of the divergent regions of GSK-3 isoforms and have examined the functional significance of conserved regulatory residues in GSK-3α and GSK-3β. We have characterized the structural significance of the N- and C-termini and propose that therapeutic targeting of the C-termini of GSK-3 isoforms may lead to isoform-specific GSK-3 inhibition through destabilization of GSK-3 structure. Indeed, accumulating evidence demonstrates that GSK-3 isoforms mediate distinct activities [158, 166-172] and suggests isoform-specific GSK-3 inhibition may be therapeutically beneficial. Future studies directed at in vivo evaluation and validation of our work will be necessary for therapeutic initiatives.
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