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Phosphoproteomic Characterization Of Glycogen Synthase Kinase-3

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Abstract
Glycogen Synthase Kinase-3 (GSK-3) is a constitutively active, ubiquitously expressed kinase that acts as a critical regulator of many signaling pathways. These pathways, when dysregulated, have been implicated in many human diseases, including bipolar disorder (BD), cancer, and diabetes. Over 100 putative GSK-3 substrates have been reported, based on direct kinase assays or genetic and pharmacological manipulation of GSK-3, in diverse cell types. Many more have been predicted based upon on the prevalence of the GSK-3 consensus sequence. As a result, there remains an unclear picture of the complete GSK-3 phosphoproteome. We have therefore used a large-scale mass spectrometry approach to analyze global changes in phosphorylation and describe the repertoire of GSK-3 substrates in a single cell type. For our studies, we used stable isotope labeling of amino acids in culture (SILAC) to compare the phosphoproteome of wild-type mouse embryonic stem cells (ESCs) to ESCs completely lacking Gsk3a and Gsk3b expression (Gsk3 DKO). We used titanium oxide chromatography to enrich for phosphorylated peptides. From our analysis, we selected 65 phosphoproteins that exhibited significantly reduced phosphorylation in Gsk3 DKO ESCs as high-confidence candidate substrates of GSK-3. Our findings indicate that these candidate GSK-3 substrates can influence all levels of gene expression including chromatin modulators, transcription factors, RNA binding proteins, splicing factors, translational initiators and cell cycle regulators. Analysis of protein-protein interaction networks revealed enrichment of a cluster of proteins involved in alternative splicing. Our study is the first to discover a function for GSK-3 in alternative splicing. To further validate our top hits, we conducted in vitro kinase assays with recombinant proteins and identified the splicing factor RBM8A and an RNA processing protein NPM1 to be direct targets of GSK-3. Preliminary RNA sequencing results point to an overall increase in alternative splicing events when Gsk3 is deleted. Taken together, the research in this dissertation represents the first unbiased analysis of GSK-3 phosphorylation substrates in a single cell type and provides the first evidence of GSK-3 as a general regulator of alternative splicing.

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Mansi Y. Shinde

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PHOSPHOPROTEOMIC CHARACTERIZATION OF GLYCOGEN SYNTHASE KINASE-3

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ABSTRACT

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Mansi Y. Shinde

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Glycogen Synthase Kinase-3 (GSK-3) is a constitutively active, ubiquitously expressed kinase that acts as a critical regulator of many signaling pathways. These pathways, when dysregulated, have been implicated in many human diseases, including bipolar disorder (BD), cancer, and diabetes. Over 100 putative GSK-3 substrates have been reported, based on direct kinase assays or genetic and pharmacological manipulation of GSK-3, in diverse cell types. Many more have been predicted based upon on the prevalence of the GSK-3 consensus sequence. As a result, there remains an unclear picture of the complete GSK-3 phosphoproteome. We have therefore used a large-scale mass spectrometry approach to analyze global changes in phosphorylation and describe the repertoire of GSK-3 substrates in a single cell type. For our studies, we used stable isotope labeling of amino acids in culture (SILAC) to compare the phosphoproteome of wild-type mouse embryonic stem cells (ESCs) to ESCs completely lacking *Gsk3a* and *Gsk3b* expression (*Gsk3* DKO). We used titanium oxide chromatography to enrich for phosphorylated peptides. From our analysis, we selected 65 phosphoproteins that exhibited significantly reduced phosphorylation in *Gsk3* DKO ESCs as high-confidence candidate substrates of GSK-3. Our findings indicate that these candidate GSK-3 substrates can influence all levels of gene expression including chromatin modulators, transcription factors, RNA binding proteins, splicing factors, translational initiators and cell cycle regulators. Analysis of protein-protein interaction networks revealed enrichment of a cluster of proteins involved in alternative splicing. Our study is the first to discover a function for GSK-3 in alternative splicing. To further validate our top hits, we conducted *in vitro* kinase assays with recombinant proteins and identified the splicing factor RBM8A and an RNA processing protein NPM1 to be direct targets of GSK-3. Preliminary RNA sequencing results point to an overall increase in alternative splicing events

when *Gsk3* is deleted. Taken together, the research in this dissertation represents the first unbiased analysis of GSK-3 phosphorylation substrates in a single cell type and provides the first evidence of GSK-3 as a general regulator of alternative splicing.

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CHAPTER 1

Introduction

1.1 Introduction to Glycogen Synthase Kinase-3

Signal transduction pathways regulate every basic cell process ranging from metabolism to immunity. Protein phosphorylation is a widespread and reversible post-translational modification used in signaling pathways to alter protein function. Protein kinases catalyze the transfer of the gamma phosphate from adenosine triphosphate (ATP), thus mediating signaling events in the cell. Not surprisingly, kinases make up 2% of the entire genome, and how and what they regulate has been studied extensively (Ubersax and Ferrell, 2007). Dysregulated signaling at the level of the kinase can be the underlying cause of many diseases, thus making these proteins attractive drug targets for pharmacological agents. We are specifically interested in exploring signaling mechanisms of Glycogen Synthase Kinase-3 (GSK-3).

GSK-3 is a serine/threonine kinase and is part of the CMGC (CDK, MAPK, GSK-3 and CLK) family of proline directed kinases. It is most closely related to Cyclin dependent kinases (CDKs), Mitogen-activated protein kinases (MAPKs) and CDK-like kinases (CLKs). Unlike most kinases that are activated in response to cellular signals, GSK-3 is constitutively active in resting cells, and in most cases regulates downstream pathways by suppressing the activity of its direct targets. Upstream signaling cues generally inhibit GSK-3 and permit signaling through its diverse range of effectors. With such wide-ranging functions, dysregulation of GSK-3 and its substrates have been implicated in many diseases including diabetes, bipolar disorder and cancer. Thus, GSK-3 has become an attractive target for pharmacological agents and drug development.

As the name suggests, GSK-3 was discovered as an enzyme that can phosphorylate glycogen synthase, the rate-limiting enzyme in glycogen metabolism (Embi et al., 1980; Rylatt et al., 1980). Follow-up studies using *in vitro* kinase assays and measurements of glycogen

synthase activity indicated that GSK-3 phosphorylates glycogen synthase at 3 serine residues, which leads to robust inhibition of enzyme activity (Parker et al., 1982; Wang and Roach, 1993; Welsh and Proud, 1993). Insulin can activate glycogen synthase by inhibiting GSK-3 through activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Cross, 1995). GSK-3 is also well characterized for its role in the Wnt signaling pathway, where it phosphorylates β-catenin and targets it for ubiquitin-mediated proteosomal degradation. Activation of Wnt signaling inhibits GSK-3 leading to β-catenin stabilization and transcription of Wnt target genes (MacDonald et al., 2009).

It was discovered early on that lithium, a drug that has been used as the primary treatment for bipolar disorder for half a century, can inhibit GSK-3 and activate Wnt signaling in multiple contexts, including the mouse brain (Gurvich and Klein, 2002; Huang et al., 2009a; Klein and Melton, 1996; O'Brien and Klein, 2009; Stambolic and Woodgett, 1994). As a result of this finding, many following studies have focused on characterizing the role of GSK-3 in the brain and bipolar disorder (Gould and Manji, 2005; O'Brien and Klein, 2009). Moreover, the identification of a potent, pharmacological inhibitor of GSK-3 enabled further elucidation of functions regulated by this kinase.

Since these studies, over 100 substrates of GSK-3 have been identified and the role of GSK-3 has expanded to many cellular processes, including metabolism, developmental processes, gene expression, immune processes, cell growth and survival, neurobiological processes, cytoskeletal and synaptogenesis (Kaidanovich-Beilin and Woodgett, 2011; Sutherland, 2011). With such a myriad of functions and effectors, precise regulation of GSK-3 is essential. Not surprisingly, the cell has evolved elegant mechanisms for pathway specific regulation of GSK-3 and insulation of one pathway from another.

1.2 Characterization of GSK-3

GSK-3 is ubiquitously expressed and highly conserved, with orthologs in plants, fungi, worms, flies, sea squirts, and vertebrates (Kaidanovich-Beilin and Woodgett, 2011). A screen of a rat brain cDNA library led to identification of two forms of GSK-3, alpha and beta, that are encoded by two independent genes (named *Gsk3a* and *Gsk3b*, respectively) and have a molecular weight of 51 kDa and 47 kDa, respectively (Woodgett, 1990). The overall sequence homology between the two isoforms is 85%, which increases to 98% within the catalytic domains. The most striking difference between GSK-3α and GSK-3β is the glycine rich N-terminal extension of unknown function in the alpha isoform (Woodgett, 1990). Both isoforms are ubiquitously expressed in all mammalian tissues and found in most organisms, except birds, which only express GSK-3β (Kaidanovich-Beilin and Woodgett, 2011).

The high degree of similarity between the two isoforms makes it challenging to develop isoform-specific inhibitors. However, studies have been conducted with isoform specific knockouts to segregate functions of each isoform. *Gsk3b* knockout in mice is embryonic-lethal and embryos die in late gestation due to severe liver degeneration (Hoeflich et al., 2000). Remarkably, *Gsk3a* knockout mice are viable and appear mostly normal. One seminal study that provided insight into isoform specific function looked at the effects of GSK-3 gene dosage on embryonic stem cells where either GSK-3α or GSK-3β or both were knocked out (Doble et al., 2007). This work indicated that the isoforms have redundant roles in Wnt signaling, as deletion of 3 out of 4 alleles was required to stabilize β-catenin (Doble et al., 2007). Functional redundancy of the isoforms in Wnt signaling is a possible explanation for the phenotype of late onset lethality reported in *Gsk3b null* embryos; these mice progress through early development, where Wnt signaling plays a major role(Logan and Nusse, 2004). Most of the attention in the field has focused on GSK-3β and the unique roles for GSK-3α have not been fully investigated. This dissertation will not focus on isoform-specific functions of GSK-3 and for the rest of this dissertation, the two isoforms will be referred to collectively as GSK-3.

Another characteristic that makes GSK-3 distinct from other kinases is the requirement for a prior, priming phosphorylation on most of its substrates. Soon after GSK-3 was discovered, Fiol *et al*. demonstrated the requirement for glycogen synthase to be phosphorylated by Casein Kinase II (CKII) prior to phosphorylation by GSK-3. Using a peptide with the proline/serine rich region of glycogen synthase, they showed CKII phosphorylation on serine 657 led to successive phosphorylation events by GSK-3 on serines at 653, 649, 645, and 641 (Figure 1-1a). They identified GSK-3's target consensus sequence to be SxxxS(p), where the S(p) is phosphorylated by the priming kinase and x's can be any residue. These observations were confirmed using recombinant glycogen synthase and have been since demonstrated in many other GSK-3 substrates (Cohen and Frame, 2001; Kaidanovich-Beilin and Woodgett, 2011; Zhang et al., 1993). Follow-up studies on glycogen synthase phosphorylation by GSK-3 showed that multisite phosphorylation occurs in an obligate order and sequentially forms new recognition sequences (Fiol et al., 1990). Processive phosphorylation by GSK-3 has since been demonstrated in another substrate, β-catenin, where the priming kinase is Casein Kinase Iα (Ikeda et al., 1998; Liu et al., 2002) (Figure 1-1b).

An analysis of known GSK-3 substrates from two comprehensive reviews was performed to estimate how many substrates of GSK-3 require a priming phosphorylation. Out of about 100 identified substrates of GSK-3, approximately 30% (27 substrates) are phosphorylated by GSK-3 at the consensus motif and the kinase that catalyzes the priming phosphorylation has been identified (Kaidanovich-Beilin and Woodgett, 2011; Sutherland, 2011) (Table 1). In addition, there are 17 that have the consensus motif and require the priming phosphorylation, but kinase has not been identified. Interestingly, there are 25 substrates that have the consensus sequence but the requirement for the priming phosphorylation has not been tested. There are also 21 substrates that do not have a consensus sequence, most notably Cyclin D1 and phosphatase inhibitor 2. This analysis was performed using two comprehensive reviews, but is by no means a complete

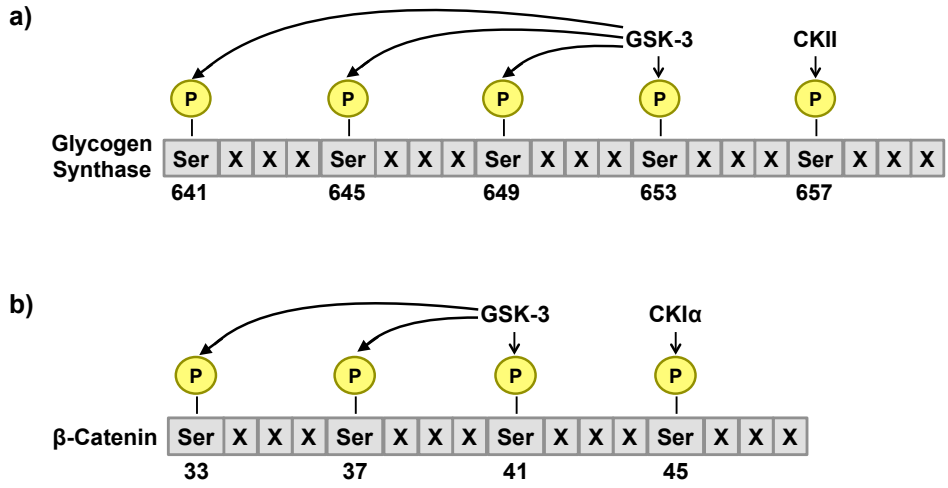


Figure 1-1: Processive phosphorylation by GSK-3. Some GSK-3 substrates are primed by another kinase 4 residues C-terminus of the first GSK-3 phosphorylation site. Priming subsequently allows for sequential phosphorylation events by GSK-3. a) Glycogen synthase phosphorylation by Casein Kinase II allows for GSK-3 phosphorylation sequentially starting at Ser653. b) β-Catenin is first phosphorylated by Casein Kinase Iα, which allows for sequential phosphorylation by GSK-3. X can be any residue

Consensus, known priming kinase	Consensus, priming phosphorylated required	Consensus sequence only	None	Site Unknown
HSF1	hnRNPD	HIF1a	Histone H1.5	κ-casein
MAP1B	IRS1	KRP	Lrp6	MafA
Mcl1	c-Jun	MAP2C	MAP1B	Notch1c
Mdm1	NFAT	MLK3	MARK2/PAR1	Nrf2
c-Myc/l-Myc	p130Rb	c-Myb	MITF	p27Kip
NDRG1	polycystin-2	neurofilament M	alphaNAC	Pyruvate dehydrogenase
p53	RCN1	neurofilament H	PSF	SC35
PTK	SKN-1	neurofilament L	Presenilin-1	Axil
PPM1G	VDAC	Ngn2	Presenilin-1 (Second site)	CLASP
PITK	Axil	p53	C/EBPα (Second site)	CTP
IRS2	Axin	p65, RelA	C/EBPβ (Second site)	Dystrophin
PTEN	BCL-3	SMAD3	Cyclin D1	SMAD1
Tau	CRY2	Snail	Glucocorticoid receptor	Per2
VHL	FAK	SREBP1c	Nascent polypeptide	Nucleophorin p62
APC	CTPS	Stathmin	Cyclin E	GATA4
β-catenin	CRPM2	ZCCHC8	Myelin basic protein	
C/EBPα	Tau	APP	p21 CIP1	
C/EBPβ		δ-catenin	ppp1r2	
Ci-155		Gephyrin	pretein 2C	
CLASP2		BCLAF1	STM1	
CRMP4		MUC1/DF3	OMA1	
CREB		KLCs		
Dynamin I		BMAL1		
eIF2B		Rev-erbα		
CDC25A		Myocardin		
GYS1				
Paxillin				

Table 1-1: Analysis of phosphorylation residues of known GSK-3 substrates. Based on two comprehensive reviews (Kaidanovich-Beilin and Woodgett, 2011; Sutherland, 2011). Consensus sequence only = requirement for priming phosphorylation has not been tested.

representation of all possible GSK-3 substrates. However, it is apparent that although there are many primed substrates of GSK-3, it is clearly not a requirement. The consensus sequence is often used to predict GSK-3 phosphorylation sites (Xu et al., 2009). In fact, there is a report that estimates that 20% of the human proteome contains the consensus sequence and can potentially be regulated by GSK-3 (Taelman et al., 2010; Xu et al., 2009). A comprehensive analysis of the GSK-3 phosphoproteome would be necessary to address how many targets that have the consensus sequence can be phosphorylated by GSK-3.

Two groups, ter Haar *et. al.* and Dajani *et. al.* reported the crystal structure for GSK-3, which provided a structural basis for the observed requirement of the priming phosphorylation. The overall structure of the activated GSK-3 kinase is similar to other kinases in their activated, substrate-bound confirmation, such as CDK or MAPK (Dajani et al., 2001; Haar et al., 2001). GSK-3 has a typical two-domain kinase fold, with a β-strand domain at the N-terminal and α-helix domain at the C-terminal. These two domains converge at the ATP binding site and must align in order to achieve the active conformation. In a typical kinase, such as MAPK, polar residues (arginine/lysine) from the β-strand and α-helix domains bind the phosphate groups of the phosphorylated amino acids in the activation loop to form the proper alignment (Haar et al., 2001). Similarly, there are three basic residues (R96, R180, K205) found on GSK-3 that can potentially interact with the phosphate on the priming residue to correctly align GSK-3 into the active conformation. The structure of GSK-3 shows a binding pocket for the priming phosphorylation and supports a mechanism for activation similar to other kinases.

Interestingly, there is a phosphorylated residue in the activation loop of GSK-3 at Y216 in the beta or Y279 in the alpha isoform. Phosphorylation at this residue increases activity of GSK-3 by 200 fold (Hughes et al., 1993). GSK-3 is constitutively phosphorylated at Y216 in resting cells, but there are conflicting reports on whether this phosphorylation is catalyzed by a tyrosine kinase or autophosphorylated by GSK-3 itself. In *Dictyostelium*, ZAK1, a novel tyrosine kinase, was

proposed to phosphorylate GSK-3 *in vitro* and *zak1*-null cells were reported to be defective in GSK-3 regulated early developmental events (Kim et al., 1999). However, orthologs of ZAK1 or functional equivalents have not been identified. Multiple studies support that GSK-3 is autophosphorylated at the tyrosine residue (Cole et al., 2004; Hughes et al., 1993; Lochhead et al., 2006). Lochhead *et. al.* showed that GSK-3 autophosphorylates the Y216 residue soon after it is synthesized, during chaperone-mediated folding and maturation(Lochhead et al., 2006) . Despite being important for enzymatic activity, there is no data that links specific cellular signaling pathways to the phosphorylation of this site (Kaidanovich-Beilin and Woodgett, 2011).

1.3 Regulation of GSK-3

GSK-3 is key regulatory node that responds to a host of cell signals and translates these cues into a diverse and often complex network of downstream pathways. To prevent unwanted crosstalk between its downstream signaling functions, GSK-3 is separated into distinct sub-populations that are regulated by different mechanisms (Figure 1-2).

In one of these subpopulations, GSK-3 is inhibited by N-terminal phosphorylation found at Serine-9 in GSK-3β and Serine-21 in GSK-3α. Upon treatment of cells with serum, insulin or growth factors, GSK-3 activity decreases by ~50% within 10 minutes (Cross et al., 1995; McManus et al., 2005). Welsh *et. al.* first reported that CHO cells treated with insulin rapidly inactivated GSK-3 and this effect could be reversed by treatment with a phosphatase (Welsh and Proud, 1993). It was later identified that insulin leads to GSK-3 inhibition by insulin receptor substrate-1 (IRS-1) dependent induction of the PI3K pathway, which stimulates Akt (also known as protein kinase B) phosphorylation of Ser9/21 (Cross et al., 1995) (Figure 1-3). Growth factors, such as EGF, can inhibit GSK-3 through activation of the P13K pathway or the MAPK cascade (Saito et al., 1994; Stambolic and Woodgett, 1994). There have been subsequent reports demonstrating this inhibitory mechanism can be induced by agonists and growth factors that activate protein kinases to phosphorylate GSK-3, such as p90RSk, p70 S6 kinase, and protein

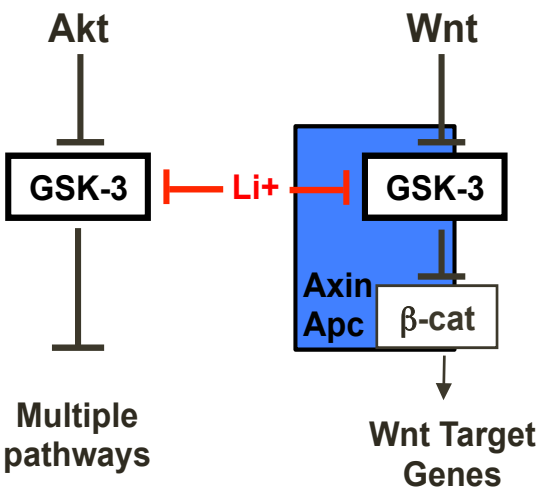


Figure 1-2: Multiple pools of GSK-3.

There are multiple pools of GSK-3 in the cell, regulated by distinct mechanisms.

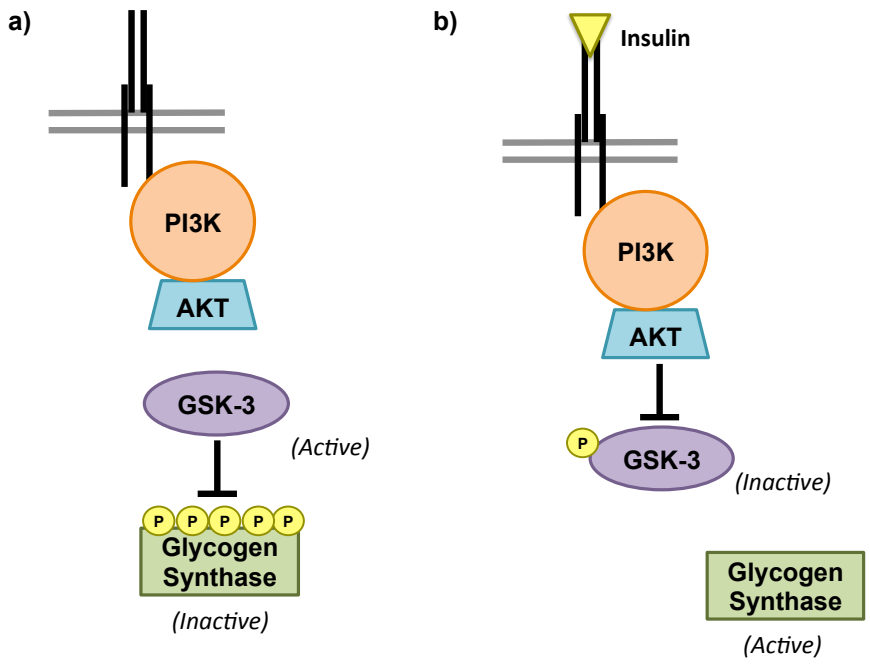


Figure 1-3: Akt-mediated inhibition of GSK-3. Akt phosphorylates the N-terminus of GSK-3 at Ser9/21 (for β/α) to inhibit GSK-3. a) In resting cells, GSK-3 is constitutively active and inhibits glycogen synthase by direct phosphorylation. b) Insulin receptor activation leads to Akt-mediated inhibition of GSK-3 by N-terminal phosphorylation.

kinase A (Eldar-Finkelman et al., 1995; Fang et al., 2000; Li et al., 2000; Saito et al., 1994; Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994). However, the best-characterized, *in vivo* mechanism for GSK-3 inhibition is by Akt phosphorylation of the N-terminal serine residue. Analysis of the GSK-3 structure revealed that phosphorylation of the inhibitory serine creates a pseudosubstrate that mimics a primed substrate and can thereby competitively inhibit interactions with the substrate (Dajani et al., 2001). Other phosphorylation sites on GSK-3 have been reported including phosphorylation at Thr43 by p44/42 MAPK (MAPK) and at Thr390 and Ser389 by p38 MAPK (Ding et al., 2005; Thornton et al., 2008).

It is noteworthy that mice with knock-in mutations in *Gsk3a* and *Gsk3b* that convert these N-terminal serines to alanines appear surprisingly normal, with only subtle defects in glycogen synthase regulation by insulin in skeletal muscle (McManus et al., 2005). The mild phenotype is surprising given the many pathways in developing and adult mice that have been reported to function through phosphorylation and inhibition of GSK-3 (Kaidanovich-Beilin and Woodgett, 2011). Therefore, although Ser9/21 is the best-characterized mechanism for GSK-3 inhibition by phosphorylation, alternative mechanisms may also be involved in GSK-3 inhibition.

A second pool of GSK-3 is involved in Wnt/ β-catenin signaling and is independent of the N-terminal serine phosphorylation (Ding et al., 2000; McManus et al., 2005). Canonical Wnt signaling has a variety of functions in early embryonic development and stem cell homeostasis (Clevers, 2006). In unstimulated cells, about 5-10% of the GSK-3 in the cell is bound to the scaffolding protein, Axin, as part of a cytoplasmic complex (Benchabane et al., 2008; Lee et al., 2003). This complex, termed the β-catenin destruction complex, is made up of GSK-3, β-catenin and Adenomatous polyposis coli (APC) all directly bound to Axin (Figure 1-4). Wnt ligands, which are secreted glycoproteins, activate signaling by binding to Frizzled, a 7-transmembrane receptor, and induce dimerization with co-receptor low-density lipoprotein receptor-related protein 6

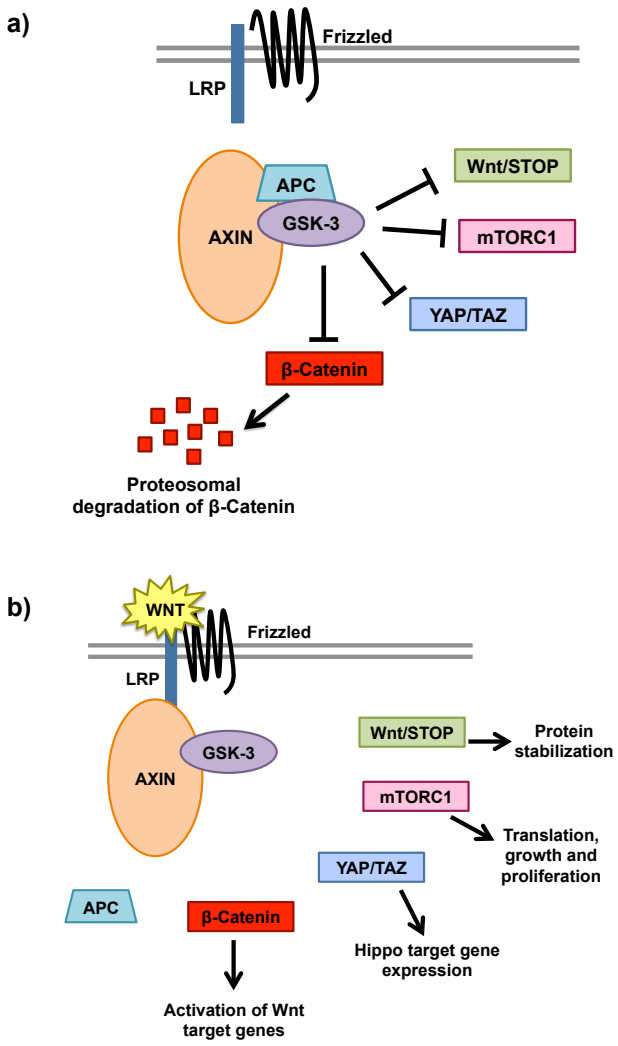


Figure 1-4: The Wnt Signaling Pathways. These include the traditional Wnt/ β-catenin pathways (shown above) but also β-catenin-independent pathways. a) In resting cells, GSK-3 is constitutively active and phosphorylates β-catenin resulting in its degradation. b) In the presence of Wnt, Axin and GSK-3 are recruited to the membrane, which leads to β-catenin accumulation, translocation to the nucleus and activation of Wnt target genes. Other Wnt signaling pathways are also activated. The exact mechanism for GSK-3 inhibition is not known, but shown here is the mechanism proposed by Valvezan *et al.* (Valvezan et al., 2012).

(LRP6). Phosphorylation of LRP6 promotes interaction with Axin and recruits the destruction complex to the membrane. GSK-3 is then inhibited, so β-catenin can accumulate and translocate to the nucleus to activate transcription of Wnt target genes (MacDonald et al., 2009). There are reports that Casein Kinase 1α (CKIα), the priming kinase for β-catenin, may also be part of the complex (Amit et al., 2002; Liu et al., 2002). CKIα adds a priming phosphorylation on β-catenin at Ser45 so GSK-3 can then processively phosphorylate it at Thr41, Ser37 and Ser33, which leads to its ubiquitin-mediated proteosomal degradation (Figure 1-1a) (Liu et al., 2002).

The mechanism of how GSK-3 is inactivated in the complex is not known. Hypotheses for GSK-3 inhibition include degradation of Axin, so the destruction complex does not form, and the sequestration of GSK-3 into multivesicular endosomes (Taelman et al., 2010). Since these occur several hours after Wnt stimulation and β-catenin accumulation is seen within minutes, they likely do not explain the initial stabilization of β-catenin (Ding et al., 2000).

A number of studies have reported that Wnt dependent phosphorylation of the LRP6 intracellular domain directly inhibits GSK-3 activity (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). This phosphorylated LRP6 domain was able to stabilize β-catenin, independent of Axin, in *Xenopus* egg extract, and reduce phosphorylation of β-catenin in *in vitro* kinase assays. These effects were blocked when the phosphorylation sites were mutated to alanines. Interestingly, there was no change in the phosphorylation of Tau, a GSK-3 substrate independent of Wnt signaling (Cselenyi et al., 2008). This indicates that LRP6 is not acting as a global GSK-3 inhibitor (Cselenyi et al., 2008). Since the intracellular domain of LRP6 has been shown to be a substrate for GSK-3, the reduced phosphorylation of β-catenin may be due to substrate competition (Zeng et al., 2008). Further experiments need to be conducted to elucidate the role of LRP6 phosphorylation and GSK-3 in Wnt signaling.

The Klein lab has published another mechanism for regulation of GSK-3 by APC (Valvezan et al., 2012). Using a fragment of APC that binds to Axin, they found that it increases

phosphorylation of GSK-3 substrates, glycogen synthase and β-catenin. Follow-up experiments demonstrated that upon Wnt stimulation APC dissociates from the Axin complex, without affecting GSK-3 association, and provides a mechanism for GSK-3 inhibition in Wnt signaling. Moreover, APC regulation of Wnt-independent GSK-3 substrates such as glycogen synthase and Tau indicates that modulation of APC enhancement of GSK-3 activity may be another mechanism to inhibit GSK-3 activity in the cell. There are also examples that show changes in GSK-3 intracellular localization as a way to regulate GSK-3 activity (summarized in (Kaidanovich-Beilin and Woodgett, 2011)). As GSK-3 has so many different functions in the cell, it is probably regulated by a combination of the mechanisms above. More studies need to be conducted to further define the intricacies of GSK-3 regulation in the cell.

These distinct mechanisms to regulate GSK-3 underscore the importance of insulating multiple pathways that share GSK-3 as an intermediate. There is overwhelming evidence that GSK-3 downstream of PI3K/Akt signaling and Axin-bound GSK-3, which responds to Wnt signaling, are independent pools of the kinase. Specifically, cells that are stimulated with insulin do not exhibit any change in β-catenin stability or GSK-3 association within the Axin-complex, and the Axin-associated pool of GSK-3 is not phosphorylated at the N-terminus in response to AKT activation (Ding et al., 2000; McManus et al., 2005). Alternatively, Wnt ligands do not affect GSK-3 inhibition of glycogen synthase and do not increase GSK-3 phosphorylation at Ser9/21. Cells in culture or mice expressing GSK-3 with serine 9/21 mutated to alanine show no response to insulin, but respond normally to Wnt signaling (McManus et al., 2005). Moreover, cancers with mutations in PI3K do not display β-catenin stabilization, and vice-versa (Ng et al., 2009). Although there is strong evidence against crosstalk between PI3K and Wnt signaling at the level of GSK-3 phosphorylation, it is important to consider that chemical inhibitors of GSK-3, such as lithium, act on both pathways.

1.4 Pathways and cell processes regulated by GSK-3

Since the discovery of GSK-3, over 100 putative substrates have been identified using genetic studies, *in vitro* kinase assays or small molecule GSK-3 inhibitors in diverse cell types. Through the phosphorylation of its substrates, GSK-3 regulates many signaling pathways and cellular processes. A majority of GSK-3 substrates are transcription factors but GSK-3 also phosphorylates signaling molecules, other kinases and structural proteins. GSK-3 phosphorylation can have a multitude of effects on proteins including modulation of catalytic activity, stability, localization, protein-protein interactions or DNA binding capacity. To provide an overview, a selection of substrates will be discussed that are categorized into two groups: those regulated by growth factor signaling and those regulated by Wnt signaling. It is important to note that the downstream substrates or functions can overlap between the two groups.

GSK-3 has many functions in growth factor signaling

GSK-3 phosphorylation of signaling molecules.

GSK-3 is well characterized for its regulation of its eponymous substrate, glycogen synthase. Insulin activates PI3K/Akt, which in turn inhibits GSK-3 to activate glycogen synthase and glycogen synthesis. Insulin, specifically, binds to the insulin receptor (IR) tyrosine kinase, which phosphorylates tyrosine residues on insulin receptor substrate 1 (IRS-1). IRS-1 can subsequently recruit proteins with SH2 domains to activate downstream signaling. Interestingly, GSK-3 has another role in insulin signaling as a negative feedback regulator. IRS-1 can be phosphorylated by GSK-3 at Ser332, which lies in the GSK-3 consensus motif, and results in reduced tyrosyl phosphorylation by IR (Eldar-Finkelman and Krebs, 1997; Liberman and Eldar-Finkelman, 2005). Serine to alanine mutations of Ser332 or Ser336, the priming site, causes enhanced tyrosine phosphorylation of IRS-1 in resting and stimulated cells (Liberman and Eldar-Finkelman, 2005). Moreover, high glucose levels can cause proteosomal degradation of IRS-1

that is mediated through GSK-3 phosphorylation of IRS-1(Leng et al., 2010). Thus, GSK-3 phosphorylation of IRS-1 interferes with its ability to respond to insulin activation.

Protein phosphatase 1 (PP1), another key component of the insulin signaling pathway, can be regulated by GSK-3 activity. Dephosphorylation activity of PP-1 is stimulated in response to insulin signaling and leads to activation of glycogen synthase and glycogen synthesis (Ragolia and Begum, 1998). GSK-3 phosphorylates the inhibitory subunit, protein phosphatase inhibitor 2 (I-2), thereby inhibiting the inhibitor and activating PP1 (DePaoli-Roach, 1984; Hemmings et al., 1982; Park et al., 1994). This provides another example of how GSK-3 can attenuate insulin signaling. Additionally, GSK-3 activation of PP1 has been proposed as a mechanism for autoregulation of GSK-3 (Zhang et al., 2003). Several small molecule inhibitors of GSK-3, including lithium, can induce an increase in GSK-3 N-terminal phosphorylation (Zhang et al., 2003). This increase has been attributed to PP1 inhibition as *in vitro* assay showed that when GSK-3 was added in combination with PP1, I-2 and ATP, GSK-3 N-terminal phosphorylation was reduced compared to no ATP control where GSK-3 is inactive (Zhang et al., 2003).

GSK-3 can inhibit translation initiation by phosphorylating the catalytic epsilon-subunit of eukaryotic initiation factor 2B (eIF2Bε). eIF2B is a five subunit, guanine nucleotide exchange factor (GEF) that catalyzes removal of GDP from eIF2 to regenerate eIF2-GTP and initiate translation. GSK-3 phosphorylation of eIF2Bε inhibits GEF activity. Upon insulin activation, GSK-3 is inhibited by Ser9 phosphorylation and GEF activity is restored (Wang et al., 2001; Welsh et al., 1998). Additionally, phosphorylation of eIF2Bε at the GSK-3 site requires a priming phosphorylation by dual-specificity tyrosine phosphorylated and regulated kinase (DYRK) (Woods et al., 2001).

Cyclin D1, a member of the cyclin family that promotes G1 progression during the cell cycle, is a known substrate of GSK-3 (Pestell, 2013). Cyclin D1 accumulates in the nucleus during the G1 phase and relocalizes to the cytoplasm during interphase (Pestell, 2013). GSK-3

phosphorylation at Thr286 redirects cyclin D1 to the cytoplasm, by promoting its association with nuclear exportin, CRM1, and triggers rapid turnover (Alt et al., 2000; Diehl et al., 1998). Overexpression of GSK-3 redirects most of the cyclin D1 to the cytoplasm, where as the T286A mutant remains in the nucleus throughout the cell cycle (Alt et al., 2000). Mitogen stimulation involving Ras, PI3K and AKT inactivates GSK-3 by N-terminal phosphorylation to allow cyclin D1 to accumulate in the nucleus. This provides an example of GSK-3 substrate where phosphorylation regulates cellular localization and protein stability. GSK-3 inhibition by Wnt signaling can also stabilize cyclin D1 protein (Acebron et al., 2014).

GSK-3 phosphorylation of transcription factors.

The stability of c-Myc, a proto-oncogene and master transcriptional regulator, is modulated by GSK-3 phosphorylation. c-Myc levels are high during cellular proliferation and differentiation, but decrease once the cell stops cycling (Dang et al., 2006). Extracellular signal-related protein kinase 2 (ERK2) primes c-Myc at Ser62 and GSK-3 phosphorylates c-Myc at Thr58 (Sears et al., 2000). GSK-3 phosphorylation increases Myc ubiquitination and localizes Myc to nuclear bodies (Gregory et al., 2003; Lutterbach and Hann, 1994; Sears et al., 2000). Activation of PI3K or MAPK by serum inhibits GSK-3 by N-terminal Ser9/21 phosphorylation and leads to stabilization of Myc protein (Sears et al., 2000; 1999). Over-expression of GSK-3 in HEK293 cells increases Thr58 phosphorylation and causes a reduction in Myc protein levels that can be rescued by GSK-3 inhibition (Soutar et al., 2010). The Myc protein has also been found to be elevated in Gsk3 KO brain, further supporting the notion that GSK-3 phosphorylation regulates Myc stability (Soutar et al., 2010). In addition, GSK-3 suppresses c-Myc transcription, a downstream target of Wnt signaling. Therefore, inhibition of GSK-3 increases c-Myc levels both by stabilizing the protein and by enhancing gene expression.

Transcriptional responses by NFκB, which are a group of closely related protein dimers that regulate immune response, differentiation, proliferation and apoptosis, can be regulated by

GSK-3 (Buss et al., 2004; Christian et al., 2016; Hoeflich et al., 2000). In the resting state, GSK-3 phosphorylation of the NFκB1 subunit, p105, promotes its to stabilization. Upon tumor necrosis factor alpha (TNFα) stimulation, however, this phosphorylation primes it for degradation (Demarchi et al., 2011). This highlights a cell state specific role for GSK-3 regulation of its substrate.

Hypoxia-inducible factor 1 alpha (HIF1α) is a master transcriptional regulator that is activated during hypoxic conditions and is additionally regulated by the PI3K/Akt pathway. HIF1α plays a major role in development and HIF signaling is dysregulated in many cancers (Shay and Simon, 2012). GSK-3 phosphorylation of HIF1α leads to proteosomal mediated degradation, which can be inhibited by N-terminal GSK-3 phosphorylation by Akt (Flügel et al., 2007; Mottet et al., 2003). Therefore signaling pathways that activate Akt may regulate HIF1 function through inhibition of GSK-3. Furthermore, hypoxia regulates embryonic and adult stem cells through HIF1α-dependent activation of Wnt signaling (Mazumdar et al., 2010). Hypoxia promotes neurogenesis through activation of Wnt target genes in embryonic and adult stem cells that is inhibited once the cells are differentiated (Mazumdar et al., 2010).

GSK-3 can phosphorylate cyclic AMP (cAMP) response element binding protein (CREB) and inhibit its DNA binding activity (Bullock and Habener, 1998; Fiol et al., 1994; Grimes and Jope, 2001). CREB is a transcription factor that regulates cyclic AMP responsive genes and affects a wide array of cell processes including immune response, cell survival, synaptic plasticity and neural development (Martin et al., 2005; Shaywitz and Greenberg, 1999; Silva et al., 1998; Walton and Dragunow, 2000). Specifically, CREB phosphorylation at Ser133 by several kinases including PKA and MAPK activates CREB and creates a consensus site for inhibitory GSK-3 phosphorylation at Ser129. GSK-3 inhibition by lithium increases CREB DNA binding and activates CREB target genes in neurons (Grimes and Jope, 2001; Ozaki and Chuang, 2002). Lithium treatment has a neuroprotective effect that may be due to activation of CREB

transcription of brain-derived neurotrophic factor (BDNF) (Mai et al., 2002; Walton and Dragunow, 2000).

GSK-3 also phosphorylates and reduces DNA binding capacity of the transcription factor, Heat Shock Factor 1 (HSF-1), which promotes survival in response to cell stressors by activating expression of heat shock proteins (Chu et al., 1996). Overexpression of GSK-3 in HeLa cells causes rapid HSF-1 inactivation after heat shock that is reversed with an MAPK inhibitor (He et al., 1998). In contrast, in a neuronal model, PI3K/Akt inactivation of GSK-3 leads to HSF-1 activation (Bijur, 2000). This highlights cell type specific upstream signals that inactivate GSK-3 and inhibit phosphoryaltion of HSF-1. Additionally, lithium has neuroprotective actions that could function by activating HSF-1 in neurons to inhibit pro-apoptotic activity (Bijur, 2000).

The proto-oncogene c-Jun is also regulated by GSK-3 phosphorylation. c-Jun is one component of the activator protein (AP-1), a transcription factor complex of c-Jun and Fos, that regulates cell proliferation, survival and death. GSK-3 phosphorylation of c-Jun inhibits DNA binding that can be rapidly reversed by Akt activation or by lithium treatment (Boyle et al., 1991; Hedgepeth et al., 1997; Troussard et al., 1999).

GSK-3 can phosphorylate structural proteins and proteins involved in neurobiology

Since the discovery that the mood stabilizer, lithium, can inhibit GSK-3, there have been many studies describing brain-specific roles of GSK-3. GSK-3 regulates substrates involved in cytoskeletal reorganization and neurobiology, some of which have been implicated in Alzheimer's disease. A role for GSK-3 in neural progenitor homeostasis was identified by studies in mouse neural progenitors, where GSK-3 can regulate multiple signaling pathways, including Notch and Hedgehog (Kim and Snider, 2011). Deletion of *Gska* and *Gskb* in mouse neural progenitors leads to a marked increase in progenitor proliferation and inhibits differentiation. Further analysis

demonstrated that this effect was mediated through aberrant Wnt, Sonic Hedgehog, Notch and fibroblast growth factor signaling (Kim and Snider, 2011).

GSK-3 regulates microtubule stability in growing axons by phosphorylating MAP1B, a microtubule-associated phosphoprotein that regulates microtubule dynamics (Goold et al., 1999; Scales et al., 2009; Trivedi et al., 2005). GSK-3 phosphorylates MAP1B at S1388, after priming phosphorylation by DYRK and at other non-primed sites, Ser1260 and Thr 1265 (Scales et al., 2009; Trivedi et al., 2005). Phosphorylation by GSK-3 destabilizes the microtubules and is important for proper axonal growth (Scales et al., 2009). GSK-3 can also phosphorylate Adenomatous polyposis coli (APC) and cytoplasmic linker-associated proteins (CLASPs) to inhibit microtubule binding and therefore, destabilize microtubules (Kim and Snider, 2011).

GSK-3 is thought to be the predominant phosphorylator of Tau, a protein that associates with and promotes polymerization of microtubules (Hanger et al., 1992; Yang et al., 1993). GSK-3 has multiple phosphorylation sites on Tau that can be inhibited by lithium (Hanger et al., 1998),(Lovestone et al., 1994; Muñoz-Montaño et al., 1997). GSK-3 also phosphorylates amyloid precursor protein (APP) and lithium inhibition or over-expression of a dominant negative GSK-3 can inhibit β-amyloid production (Aplin et al., 1996; Santos et al., 2011; Su et al., 2004). Aberrant phosphorylation and regulation of Tau and APP are implicated in pathogenesis of Alzheimer's disease (Beurel et al., 2015).

GSK-3 can regulate proteins with functions in the circadian clock

GSK-3 is expressed in the primary center of circadian rhythm regulation, the suprachiasmatic nucleus (SCN) of the hypothalamus, and is involved in regulation of the circadian clock, which is crucial for organisms to respond to and anticipate environmental changes (Iitaka et al., 2005). GSK-3 inhibition lengthens the period of circadian rhythms in a wide range of experimental systems (Kaidanovich-Beilin and Woodgett, 2011). GSK-3 has been shown

to directly phosphorylate and promote degradation of two critical transcription factors for mammalian clock machinery, CLOCK and BMAL1 (Sahar and Sassone-Corsi, 2009; Spengler et al., 2009). Contrastingly, GSK-3 phosphorylates and stabilizes Rev-erbα, the orphan nuclear receptor responsible for repression of Bmal1 gene transcription (Yin et al., 2006)

These examples highlight the broad range of substrates that can be directly phosphorylated by GSK-3 and influence a wide range of gene targets and cellular functions. As these substrates have been identified in a diverse range of cell types, using a variety of assays, the spectrum of GSK-3 substrates in a single cell type remains unclear.

GSK-3 regulates multiple targets in Wnt signaling

The function of GSK-3 in the Wnt pathway was originally identified by genetic and developmental studies in Dictyoselium, Drosophila and Xenopus (Bourouis et al., 1990; Dominguez et al., 1995; Harwood et al., 1995; Siegfried et al., 1990). Expression of dominant negative GSK-3 in Xenopus causes dorsal differentiation in the embryo and this was mediated through the Wnt/β-catenin signaling pathway (He et al., 1995; Yost et al., 1996). Since this initial discovery, the role of GSK-3 and Wnt/β-catenin signaling in cell patterning and cell specification in early embryonic development and adult homeostasis has been extensively studied (Logan and Nusse, 2004). Wnt target genes are varied based on cellular context but usually regulate proliferation, survival and inhibition of differentiation (Clevers, 2006). Aberrant Wnt pathway activation has been linked to many diseases including colon cancer and leukemia (MacDonald et al., 2009).

Traditionally, the Wnt/β-catenin signaling pathway has been thought of as a linear signaling pathway where the downstream effect is the direct result of the transcriptional program activated by β-catenin. It is now appreciated in the field that Wnt signaling diverges at multiple points and has functions independent of β-catenin (Figure 1-4). Consequently, this has also

expanded the breadth of cell processes that are potentially affected by GSK-3 activity. These divergent functions include regulation of the microtubule associated proteins, YAP/TAZ and mTOR signaling pathways and Wnt-dependent stabilization proteins (Wnt/STOP) (Acebron and Niehrs, 2016). YAP/TAZ are transcriptional co-activators that are downstream targets of the Hippo signaling pathway, a well-known regulator of organ growth. YAP/TAZ can be bound to the β-catenin destruction complex in resting cells (Azzolin et al., 2014). Upon Wnt activation, YAP/TAZ are released from the complex and accumulate in the nucleus to activate transcriptional targets. This may be partially due to inhibition of GSK-3 phosphorylation of TAZ, which targets it for ubiquitin-mediated degradation (Azzolin et al., 2014; Finch-Edmondson et al., 2015; Huang et al., 2012b).

mTOR signaling

Mechanistic Target of Rapamycin (mTOR) signaling is a nutrient sensing pathway that integrates extracellular and intracellular signals to regulate cell processes, such as proliferation, protein translation, and autophagy. Inoki et al. found that Wnt signaling activates the mTOR effectors, ribosomal protein S6 (rpS6) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), by inhibition of GSK-3, independent of β-catenin (Inoki et al., 2006). Specifically, GSK-3 phosphorylates Tuber Sclerosis Complex 2 (TSC2), a negative regulator of mTOR, only after a priming phosphorylation by AMPK (Inoki et al., 2006).

In support of these observations, another group found that TSC2 can be co-immunoprecipitated with both Axin and GSK-3 and this interaction was sensitive to Wnt treatment (Mak et al., 2005; 2003). Of additional interest, another study reported that transcription factor Smad3 that can be degraded in response to GSK-3 phosphorylation can also associate with the Axin-GSK-3 degradation complex (Guo et al., 2008). Taken together, these data support that Axin has other binding partners that can be regulated by GSK-3 phosphorylation and thus, further increase the possible GSK-3 substrates in the cell.

Wnt/STOP

Recent studies have found that many proteins in addition to β-catenin are stabilized upon Wnt activation and are substrates of GSK-3 (Acebron and Niehrs, 2016). This process, termed Wnt/STOP, is critical during mitosis to stabilize protein levels as the cells prepare to divide (Acebron et al., 2014; Huang et al., 2015). In HeLa cells, Wnt signaling activation stabilizes GSK-3 targets, such as c-Myc and Cyclin D1 (Acebron et al., 2014). A microarray analysis of polyubiquitinated proteins showed that GSK-3 inhibition reduces polyubiquitination of 1.4% of all the proteins detected (Acebron et al., 2014). Of these, five proteins were confirmed to be stabilized by Wnt activation. Microarray analysis of polyubiquitination in Xenopus egg extracts treated with lithium showed GSK-3 inhibition reduces polyubiquitination of 9.6% of all proteins detected (Huang et al., 2015). Further analysis confirmed GSK-3 stabilization of five mitotic effectors (UBE2C, PLK1, AURKB, CYCE, and CDK7). Wnt/STOP signaling has also been proposed to play a role in sperm maturation and specifically stabilize BRD3 and BUB1 (Koch et al., 2015). Cumulatively across all cell types, about 35 proteins are reported to be stabilized by Wnt/STOP signaling (Acebron and Niehrs, 2016). However, it has been speculated that GSK-3 can target many more proteins, and this has led to the general belief that GSK-3 inhibition stabilizes proteins (Acebron and Niehrs, 2016; Taelman et al., 2010; Xu et al., 2009). A global analysis of protein abundance changes in the cell would be required to determine how many proteins are stabilized in response to GSK-3 inhibition.

GSK-3 regulates diverse stem cell populations

GSK-3 in the Wnt signaling pathway is a well-characterized regulator of diverse stem cell populations, including embryonic, hematopoietic, intestinal, and neural stem cells (Kim and Snider, 2011; Reya and Clevers, 2005; Wray and Hartmann, 2012). GSK-3 inhibition in embryonic stem cells (ESCs) supports self-renewal and is used in combination with an MEK inhibitor to maintain ESCs in a pluripotent state (Sato et al., 2004; Smith et al., 2012; Ying et al.,

2008). Double knockout of Gsk3a and Gsk3b in embryonic stem cells (ESCs) hyperactivates Wnt signaling and leads to a restricted differentiation potential that can be reversed by reintroducing GSK-3 (Doble et al., 2007). A similar result is observed in ESCs completely deficient in APC (Kielman et al., 2002). The effects of GSK-3 inhibition have been attributed to Wnt/β-catenin signaling, specifically by derepression of pluripotency genes bound by repressor T cell factor 3 (Tcf3) (Lyashenko et al., 2011; Wray et al., 2011). These studies support a role for GSK-3 inhibition in promoting pluripotency by activating Wnt signaling, but the role of other effectors of GSK-3 have not been addressed in this setting.

In neural progenitors, deletion of Gsk3a and Gsk3b leads to a marked increase in proliferation while inhibiting differentiation (Ka et al., 2014). This effect can be reverse by mTOR inhibition (Ka et al., 2014). Similarly in hematopoietic stem cells (HSCs), GSK-3 is a key regulator of stem cell homeostasis. Loss of GSK-3 leads to expansion and eventual depletion of HSCs, in a β-catenin independent manner, which can be rescued by inhibiting mTOR (Huang et al., 2009a). Wnt signaling has been shown to be important for homeostasis in epidermal and intestinal stem cells as well (Clevers et al., 2014). A balance between self-renewal and differentiation is essential for maintenance of regenerative tissues, such as the hematopoietic system, and GSK-3 acts as a master switch by regulating many of the pathways that dictate self-renewal, differentiation, and proliferative signals.

1.5 Clinical implications and use of GSK-3 inhibitors

As a critical regulator of a diverse array of cellular processes, it is not surprising that GSK-3 dysregulation is associated with a variety of disease pathologies. GSK-3 is not a drug target in the classical sense, due to its promiscuous actions, but its prototypical inhibitor lithium has been used for decades as the first-line therapy for bipolar disorder and has set a precedent for the GSK-3 targeted therapies.

Diabetes

GSK-3 can directly phosphorylate glycogen synthase and IRS-1, as well as indirectly regulate the activity of protein phosphatase-1. All of these are key downstream targets of insulin signaling. GSK-3 is an attractive therapeutic target for type 2 diabetics, whose peripheral tissues have lost the ability to respond to normal levels of insulin in the body. Specifically, GSK-3 activity has been shown to be elevated in diabetic tissues and inhibition of GSK-3 can improve insulin resistance (Eldar-Finkelman, 2002). Overexpression of GSK-3 in mice is sufficient to cause glucose intolerance and several studies have demonstrated that GSK-3 inhibition can improve glucose regulation in diabetic mice models (Amar et al., 2011). Of note, the Eldar-Finkleman lab has designed a series of synthetic phosphorylated peptide inhibitors of GSK-3 that are substrate competitive and take advantage of the GSK-3 consensus sequence (Eldar-Finkelman et al., 2010; Plotkin et al., 2003). Their lead candidate, L803-mts, is able to specifically and potently inhibit GSK-3 in a substrate competitive manner (Plotkin et al., 2003). Moreover, it was able to improve glucose tolerance mice on a high-fat diet and long-term treatment improved glucose homeostasis in obese mice (Kaidanovich-Beilin and Eldar-Finkelman, 2006; Plotkin et al., 2003). To date, no GSK-3 inhibitors have been tested in clinical trials for treatment for diabetes.

Bipolar disorder

Bipolar disorder (BD) is a group of manic-depressive disorders that cause erratic shifts in mood and energy, thus hindering a patient's ability to carry out daily tasks. Lithium has been used as the first-line therapy for BD for decades, but the mechanism for therapeutic response is not well understood (Alda, 2015). Interest in studying the role of GSK-3 in BD only came about when it was discovered that lithium directly inhibits GSK-3 and activates Wnt signaling both *in vitro* and in cells (Klein and Melton, 1996; Stambolic et al., 1996). Lithium inhibits GSK-3 by competing with magnesium, which is a co-factor for GSK-3 (Ryves and Harwood, 2001). GSK-3 has been identified as a direct target of lithium in diverse settings, including the mammalian brain (Beurel et al., 2015; Gurvich and Klein, 2002; Wada, 2009). Lithium can mimic *Gsk3* loss of function

developmental phenotypes in diverse organisms, including *Xenopus* and *Drosophila* (Gurvich and Klein, 2002). Lithium also reduces phosphorylation of many GSK-3 substrates, including Tau, Cyclin D1 and glycogen synthase (Gurvich and Klein, 2002). Several labs, including ours, have identified multiple behaviors in mice that are sensitive to lithium and demonstrated that these can be mimicked by genetic *Gsk3* loss of function or other inhibitors of GSK-3 (Beaulieu et al., 2008; 2004; O'Brien, 2004; O'Brien et al., 2011). Taken together, these studies provide compelling evidence that GSK-3 inhibition may be involved in lithium therapeutic response. However, although lithium is effective in many patients, it is ineffective in some patients and has many side effects. Thus, it is of interest to address to what extent lithium mimics GSK-3 loss of function and potentially develop more targeted therapies with reduced side effects.

Alzheimer's Disease

Tau is a bona fide substrate of GSK-3 and many following studies have implicated GSK-3 in aberrant tau phosphorylation, which leads to neurofibrillary tangles that are characteristic of Alzheimer's disease (Beurel et al., 2015; Kramer et al., 2012). In addition, GSK-3 can regulate amyloid precursor protein, and thus production of amyloid-beta peptides that make up the amyloid plaques, another characteristic of Alzheimer's disease (Pheil, 2003). It is not surprising that GSK-3 inhibition using several inhibitors, including lithium, has been shown to reduce Alzheimer's pathology in animal models (Avrahami et al., 2013; Domínguez et al., 2012; Muñoz-Montaño et al., 1997; Phiel et al., 2003; Serenó et al., 2009). Tideglusib, a non-competitive, irreversible inhibitor of GSK-3 was well tolerated in patients but did not demonstrate any clinical benefit at 26 weeks in a Phase II clinical study (Lovestone et al., 2015). Interestingly, a lower prevalence of dementia was found in patients on long-term lithium treatment, which had mixed results in Alzheimer's trials (Beurel et al., 2015).

Cancer

GSK-3 has also been implicated in a wide range of tumors, including colorectal cancer and hematological malignancies (McCubrey et al., 2014). The role of GSK-3 in cancer is complicated because it can act as a tumor promoter or as a tumor suppressor (McCubrey et al., 2014). GSK-3 is over expressed in a variety of tumors but the mechanism of GSK-3 as a tumor promoter is unclear. GSK-3 acts as a tumor suppressor through inhibition of Wnt signaling, which is aberrantly activated in many cancers (Anastas and Moon, 2013).

It is surprising lithium is one of the only examples of a GSK-3 targeted therapy in the clinic as dysregulation of GSK-3 substrates has been implicated in many diseases. This can likely be attributed to its promiscuous actions and lack of understanding of its overall range of targets. To improve upon lithium and future GSK-3 targeted therapies, a more thorough understanding of GSK-3 effectors is required.

1.6 Scope of the thesis

The focus of this work is on the understanding of the many regulatory roles of GSK-3 in the cell. Characterization of pharmacologic inhibition of GSK-3 in embryonic stem cells provided evidence that although GSK-3 suppresses mTOR signaling in ESCs, the dual suppression of these essential signaling nodes is not sufficient to maintain pluripotency. The use of a stable genetic knockdown of *Gsk3a* and *Gsk3b* permitted an unbiased proteomic analysis of the entire spectrum of GSK-3 regulated substrates in a single cell type. We found that GSK-3 substrates spanned a broad range of cell processes and we observed enrichment of three protein networks: transcriptional regulators, cell cycle proteins and splicing factors. To validate these observations, we performed follow-up *in vitro* kinase assays and confirmed that GSK-3 is capable of directly phosphorylating splicing factors. This cluster of substrates involved in alternative splicing represents a new level of cellular regulation previously undescribed for GSK-3. The role of GSK-3 as a regulator of splicing and the implications of this discovery for disease pathology are the subject of the general discussion.

CHAPTER 2

Materials and Methods

2.1 Cell culture

Gsk-3a/b double knock out and wild-type (floxed) E14 ESCs (Doble, 2007) were kindly provided by Drs. Bradley Doble (McMaster Stem Cell and Cancer Institute, Hamilton, ON) and James Woodgett (Lunenfeld-Tanenbaum Research Institute, Toronto, ON). All media and supplements were obtained from GIBCO (Invitrogen) unless otherwise noted. E14 mESCs were cultured at 37°C in 5% CO_2 in DMEM supplemented with 15% FBS (Hyclone Defined), 0.1mM MEM nonessential amino acids, 2mM Glutamax, 0.055mM β-mercaptoethanol, and 1000 units/ml of LIF (Chemicon) on 0.1% gelatin-coated plates with irradiated mouse embryonic fibroblasts (Global Stem). Cells were routinely tested for mycoplasma using the MycoAlert detection kit (Lonza). For SILAC labeling experiments, cells were cultured with DMEM without arginine or lysine supplemented with 0.798 mM isotope heavy L-lysine ($^{13}C_6$, $^{15}N_2$) and 0.398 mM heavy L-arginine ($^{13}C_6$, $^{15}N_4$) (Cambridge Isotope Labs) and 15% KnockOut Serum Replacement with the same media components as standard culture. In case of light media, standard L-lysine and L-arginine were used. During isolation, ES cells were dissociated into a single cell suspension with TrypLE and absorbed twice for 30 minutes, to remove MEFs. Proteomic experiments were conducted in biological triplicate.

2.2 Proteomics and phosphoproteomics analysis using nLC-MS/MS

All chemicals used for preparation of nLC-MS/MS samples were of at least sequencing grade and purchased from Sigma-Aldrich, unless otherwise stated. Light and heavy labeled cells were separately lysed using 6M urea, 2M thiourea, 50 mM ammonium bicarbonate, pH 8.2, phosphatase and protease inhibitors mix (Thermo Fisher Scientific) by vortexing. After protein quantification using Bradford, equal amounts of light and heavy protein lysates were mixed.

Proteins were first digested using endopeptidase Lys-C (Wako, MS grade) for 3 h, after which the solution was diluted 10 times with 20 mM ammonium bicarbonate. Subsequently, samples were reduced using 10 mM DTT for 1 hour at room temperature and alkylated with 20 mM iodoacetamide (IAA) in the dark for 30 minutes at room temperature. Further digestion was performed using trypsin (Promega) at an enzyme-to-substrate ratio of approximately 1:50 overnight at room temperature. After digestion, the samples were concentrated to the volume of ~100 µl by lyophilization. Phosphopeptide enrichment using titanium dioxide (TiO_2) chromatographic resin was performed as previously described (Thingholm and Larsen, 2016). The lyophilized phosphorylated peptide samples were reconstituted in 0.1% trifluoroacetic acid (TFA) and desalted using Poros Oligo R3 RP (PerSeptive Biosystems) P200 columns. Unbound peptides from the TiO_2 flow-through and subsequent TiO_2 washes were combined and lyophilized to produce the non-modified peptide fraction. The non-modified peptide fraction was resuspended in 0.1% TFA and desalted using Sep-Pak tC18 Plus Light Cartridge (Waters). Afterwards, samples were dried to completely remove traces of acetonitrile from stage tip elution.

Dried samples were resuspended in buffer A (0.1% formic acid) and loaded onto an Easy-nLC system (Thermo Fisher Scientific, San Jose, CA, USA), coupled online with a Q-Exactive or an Orbitrap Fusion Tribrid mass spectrometer (both Thermo Scientific). Peptides were loaded into a picofrit fused silica capillary column (75 µm inner diameter) packed in-house with reversed-phase Repro-Sil Pur C18-AQ 3 µm resin of about 18 cm. A gradient of 165 minutes was set for peptide elution from 2-28% buffer-B (100% ACN/0.1% formic acid) at a flow rate of 300 nl/min. Both instruments were programmed in a data-dependent acquisition (DDA) mode. For the Q-Exactive the full MS scan range was 360-1200 m/z in the orbitrap with a resolution of 70,000 (200 *m/z*) and an AGC target of 5x10e5. MS/MS was performed in the orbitrap selecting the top 12 ions, a resolution of 17,500, an AGC target of 5x10e4 and a collision energy of 22. For the Orbitrap Fusion Tribrid the full MS scan was 350-1200 m/z in the orbitrap with a resolution of

120,000 (200 *m/z*) and an AGC target of 5x10e5. MS/MS was performed in the ion trap using the top speed mode (3 secs), an AGC target of 10e4 and an HCD collision energy of 32.

MS raw files were analyzed by the MaxQuant software version 1.5.2.8. MS/MS spectra were searched by the Andromeda search engine against the mouse UniProt FASTA database (version November 2015) (Cox and Mann, 2008; Cox et al., 2011). The search for total proteome included variable modifications of methionine oxidation and N-terminal acetylation, and fixed modification of carbamidomethyl cysteine. Analysis of the phosphoproteome included carbamidomethylation on cysteine residues as a fixed modification, while phosphorylation on serine, threonine and tyrosine residues was set as variable modification. Trypsin was specified as the digestive enzyme. SILAC labeling was used as quantification. Match between runs was enabled and set to 1 min window. All other values were kept as default. Protein tables were filtered to eliminate the identifications from the reverse database, only identified by site and common contaminants.

Statistical analysis and downstream bioinformatics

Each analysis was performed with a number of biological replicates specified elsewhere. Heteroscedastic T-test was used to assess the significant differences in peptide/protein abundance (p-value lower than 0.05). Data distribution was assumed to be normal but this was not formally tested. Protein interaction network was extracted from the STRING database (Szklarczyk et al., 2015) and visualized using Cytoscape (Shannon et al., 2003). GO and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis were performed using DAVID version 6.8. The list of proteins with GSK-3 dependent phosphorylation (Supplementary Table 1) was submitted as the gene list and automatic *Mus Musculus* background was used. Categories with a p-value of <0.05 and DAVID enrichment score >1.0 were selected.

2.3 Western blot analysis

Cells were lysed in buffer containing 20mM Tris pH 7.5, 140 mM NaCl, 1mM EDTA, 10% glycerol, 1% Triton X-100, 1mM DTT, 50mM NaF with protease inhibitor cocktail (Sigma) and Phosphatase inhibitor cocktail 2 & 3 (Sigma) used at 1:100. Supernatants were collected after centrifugation at 14,000 rpm for 10 min. at 4°C. Protein was quantified by Bradford assay. Standard SDS-PAGE and western blot protocol was followed. The following antibodies were used at 1:1000, unless otherwise listed: GAPDH (Cell Signaling), β-catenin (Cell Signaling), Proyl Oligopeptidase (Abcam), phosphorylated ribosomal protein S6 (Cell Signaling), ribosomal protein S6 (Cell Signaling), GSK-3α/β (Cell Signaling).

2.4 Phos-Tag electrophoresis and immunoblotting

Cells were lysed in 6M urea, 2M thiourea, 50 mM ammonium bicarbonate, pH 8.2 with protease inhibitor cocktail (Sigma) and Phosphatase inhibitor cocktail 2 & 3 (Sigma), used at 1:100. Supernatants were collected after centrifugation at 14,000 rpm for 10 min. at 4°C. Protein was quantified by Bradford assay. Samples were diluted in standard 2X Laemmli Sample Buffer and heated at 95°C for 5 minutes before PhosTag analysis. PhosTag Gels were prepared using PhosTag™ acrylamide reagent (Wako Pure Chemical Industries, Ltd., catalog # AAL-107) with ZnCl2 based on manufacturer's protocol (Kinoshita, 2005). The composition for optimal separation of NPM1 was 30 μM PhosTag™ acrylamide reagent and 10% acrylamide using a acrylamide:bis solution (29:1). The composition for optimal separation of RBM8A was 30 μM PhosTag™ acrylamide reagent and 12% acrylamide. Gels were run at constant 70V for 3-4 hours, until dye front was off the gel. PhosTag gels were soaked in transfer buffer with 1mM EDTA for 15 minutes then washed for 15 minutes in transfer buffer without EDTA. Gels were transferred using PVDF membrane, at 150mA for 16 hours. Gels were blocked with 5% non-fat milk. The following antibodies were used at 1:1000: NPM1 (Bethyl Laboratories), RBM8A (Bethyl Laboratories).

2.5 In vitro kinase assays

Recombinant proteins were incubated in kinase reaction buffer (100mM Tris pH 7.5, 5mM DTT, 10mM MgCl2) with 800µm ATP for 60 minutes at 30°C. Reactions were stopped by adding standard 2X Laemmli Sample Buffer and incubating at 95°C for 5 min. Sample were run on PhosTag gels following the reaction. 0.4ug of recombinant protein was used: RBM8A (ProSpecBio) and NPM1 (ProSpecBio). Recombinant GSK-3 was used at 50units/20µL reaction (New England Biolabs). For MS analysis, MS proteolysis methods were followed starting at the 10mM DTT step. Peptides were analyzed following similar methods as indicated in MS section.

2.6 Intracellular Flow Cytometry

Up to 5 million cells were fixed in 1.6% paraformaldehyde for 20 minutes at 37°C and washed in FACS buffer (Cells were permeabilized with 1x saponin buffer (Biolegend 421002). To stain for Oct4 and Nanog, cells for were incubated with primary antibody at 1:200 for 1 hour at room temperature. Antibodies: Oct4 (SantaCruz), Nanog (Abcam). Cells were incubated with secondary antibody at 1:400 for 30 minutes at room temperature. Antibody: goat anti-mouse IgG-PE (SantaCruz), IgG-CFL488 (SantaCruz). Samples were resuspended in FACS buffer (1x PBS + 0.5% BSA, 0.05% Azide). Analysis was performed using FACSCanto flow cytometer (BD) and data were analyzed using FlowJo software (Treestar).

2.7 2i experiments (Chapter 3 only)

2i Serum-free culture: Wild-type E14 and D3 ESCs were maintained in N2B27 medium prepared as described (Ying et al., 2008) supplemented with 3µm CHIR99021 (Cayman Chemical), 1µm PD0325901 (Cayman Chemical) and LIF (Chemicon) on 0.1% gelatin-coated plates. Rapamycin was used at 10nM, unless otherwise listed in figure legend.

Microarray Analysis

Microarray analysis on mouse D3 ESCs was performed using the Affymetrix Mouse Gene 1.0 ST Array. Cells were cultured in serum-free, N2B27 medium with inhibitors for 24 hours. Inhibitor concentrations were as follows: 3μm CHIR99021,1μm PD0325901, 10nm Rapamycin (all from Cayman Chemical). Samples were collected in triplicate from independent biological replicates. Total RNA was isolated using RNAeasy Kit (Qiagen). cDNA was prepared by the University of Pennsylvania Molecular Profiling Facility. Microarray analyses were performed with standard Affymetrix GeneChip Expression protocols. Data analysis was performed using PartekGS software. Multivariate statistical analysis (Mixed ANOVA) was employed to identify significant differences in conditions. The heat map in Figure 3-3b was generated using Multi Experiment Viewer (http://www.tm4.org/). Expression levels of mir302c were validated using quantitative RT-PCR.

Reverse Transcription

RNA was isolated using RNAeasy kit (Qiagen). cDNA was synthesized using SuperScriptIII (Thermo), according to manufacturers protocol, using OligoDT. SYBR Green Master Mix was used for reverse transcription, according to manufacturers protocol. Primers: AGCTGCTGAAGCAGAAGAGGATCA (Oct4, Fwd); TCTCATTGTTGTCGGCTTCCTCCA (Oct4, Rev); CCACGAAGCCAGTGTGTTA (Fgf5, Fwd); CCACTCTCGGCCTGTCTTTT (Fgf5, Rev).

CHAPTER 3

Regulation of Embryonic Stem Cell Pluripotency by GSK-3

3.1 Introduction

Embryonic Stem cells (ESCs) are characterized by their ability to self-renew as well as differentiate into all mature cell types. The ability to efficiently control cell fate decisions and generate a specific cell type possesses great therapeutic potential, and provides a valuable research tool. However, the signaling pathways involved in the regulation of self-renewal versus lineage commitment remain unclear.

The canonical Wnt signaling pathway is critical for cell patterning and polarity in embryonic development, and for homeostasis and tissue maintenance in adult stem cells (Clevers, 2006; Logan and Nusse, 2004). Wnt signaling can promote pluripotency in ESCs (Wray and Hartmann, 2012). Consequently, pharmacological inhibition of Glycogen Synthase Kinase-3 (GSK-3) has been used to activate Wnt signaling and promote self-renewal in ESCs (Sato et al., 2004; Ying et al., 2008).

GSK-3 is a ubiquitously expressed serine/threonine kinase that is encoded by two genes that generate related homologs, GSK-3α and GSK-3β. GSK-3 is constitutively active and is generally inhibited by upstream signals. It has a well-characterized mechanism in Wnt signaling and the GSK-3 α and β isoforms have redundant roles in this pathway. In resting cells, GSK-3, adenomatous polyposis complex (APC), and the transcriptional co-activator, β-catenin, are bound directly to the scaffolding protein, Axin, in a cytoplasmic complex. GSK-3 phosphorylation of β-catenin leads to its ubiquitin-mediated proteosomal degradation. Upon Wnt activation, GSK-3 is inhibited, which leads to β-catenin accumulation and translocation into the nucleus to activate transcription of Wnt target genes.

Although short-term GSK-3 inhibition in mouse ESCs enhances self-renewal through Wnt activation, cells cultured with GSK-3 inhibitors alone eventually succumb to differentiation (Wray et al., 2011; Ying et al., 2008). It has been proposed that GSK-3 inhibition relieves TCF3 repression of pluripotency genes, Oct4, Nanog and Sox2 (Wray et al., 2011). Additional studies have also attributed the positive effects on self-renewal by GSK-3 inhibition to Wnt/β-catenin signaling, and β-catenin independent pathways have not been considered (Bone et al., 2009; Kelly et al., 2011). Since only around 10% of the GSK-3 in the cell is bound to Axin and engaged in Wnt signaling, we proposed that GSK-3 regulates multiple pathways that promote both self-renewal and differentiation in ESCs (Benchabane et al., 2008; Lee et al., 2003)

There exists another pool of GSK-3 in the cell that is engaged in growth factor signaling (Voskas et al., 2010). Insulin and other growth factors engage receptor tyrosine kinases, which lead to activation of the PI3K/Akt signaling pathway. Direct inhibition of GSK-3 by Akt-mediated N-terminal phosphorylation of Ser9/21, in α/β isoforms respectively, promotes activation of multiple substrates, including glycogen synthase. N-terminal serine phosphorylation does not affect the pool of GSK-3 bound to Axin, nor is it required for inhibition in response to Wnt signaling (Ding et al., 2000; Doble et al., 2007; McManus et al., 2005). These data therefore provide strong evidence against crosstalk between PI3K and Wnt signaling at the level of GSK-3 phosphorylation. However, it is important to consider that chemical inhibitors can act on all GSK-3 in the cells and thus, GSK-3 may regulate multiple pathways to promote both self-renewal and differentiation

Our leading candidate for these studies is the Mechanistic Target of Rapamycin Complex 1 (mTOR) signaling pathway. mTOR is a key nutrient sensing pathway that integrates extracellular and intracellular signals to regulate many cell processes, such as protein translation and autophagy. In other cell types, GSK-3 has been shown to directly regulate mTOR activity by phosphorylating TSC2, a well-established negative regulator of mTOR (Inoki et al., 2006).

Previous studies in our lab have identified GSK-3 as a pivotal regulator of stem cell balance in hematopoietic stem cells (HSCs) (Huang et al., 2009b). GSK-3 knockdown leads to an initial expansion of HSCs, mediated through Wnt signaling, and an eventual decline of HSCs through mTOR signaling (Huang et al., 2009a). Moreover, Wnt activation in epidermal stem cell compartment also caused mTOR-mediated stem cell exhaustion (Castilho et al., 2009). Thus, we would like to address if the interplay between Wnt and mTOR pathways is a general mechanism to regulate self-renewal and differentiation of stem cells. These studies aim to provide insight into signaling pathways that dictate cell fate decisions in ESCs.

3-2 Results

GSK-3 suppression of mTOR in ESCs

In order to assess the GSK-3/mTOR axis in ESCs, we first tested if GSK-3 suppresses the mTOR signaling pathway in ESCs. We found that serum removal in ESCs with *Gsk3a* and *Gskb* deletion resulted in a marked increase in mTOR activity compared to wild-type ESCs, as indicated by increased phosphorylation of ribosomal protein S6, a downstream effector of mTOR signaling (Figure 3-1a). This activation was sensitive to mTOR inhibitor, rapamycin. We also observed mTOR activation in wild-type ESCs treated with GSK-3 inhibitor, CHIR99021 (Figure 3-1b). Taken together, these data provide evidence that GSK-3 suppresses mTOR signaling in ESCs.

mTOR is activated during differentiation

Next, we assessed mTOR activity during differentiation. Wild-type ESCs were differentiated by removal of the pluripotency factor, Leukemia Inhibitory Factor (LIF) and spontaneous formation of embryoid bodies (EBs). EB differentiation was performed for 14 days and mTOR activation was assessed during differentiation, as indicated (Figure 3-2). We found that mTOR activity was high in pluripotent cells. Although initially reduced, we observed an

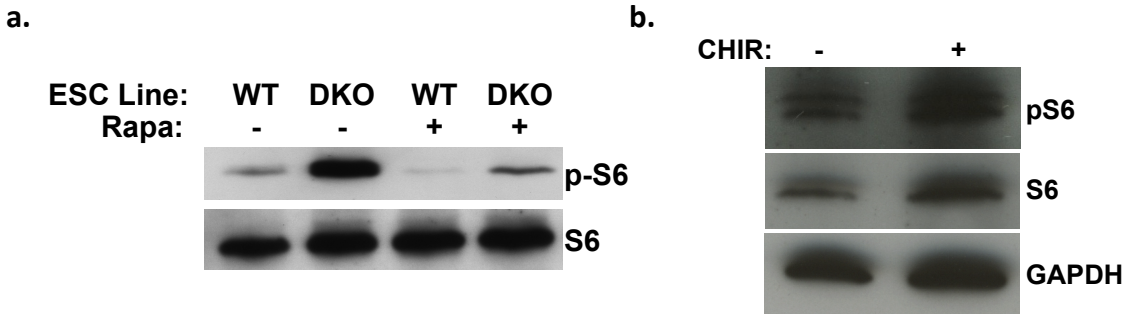


Figure 3-1 GSK-3 suppresses mTOR activity in ESCs. a) Immunoblot of lysates from wild-type (WT) and *Gsk-3α/Gsk-3β* double knockout (DKO) ESCs with or without 100nM rapamycin (Rapa). Cells were serum starved for 2 days prior to isolation. b) Immunoblot of lysates from wild-type ESCs treated with GSK-3 inhibitor, CHIR99021 (3μM). p-S6 = phosphorylated S6

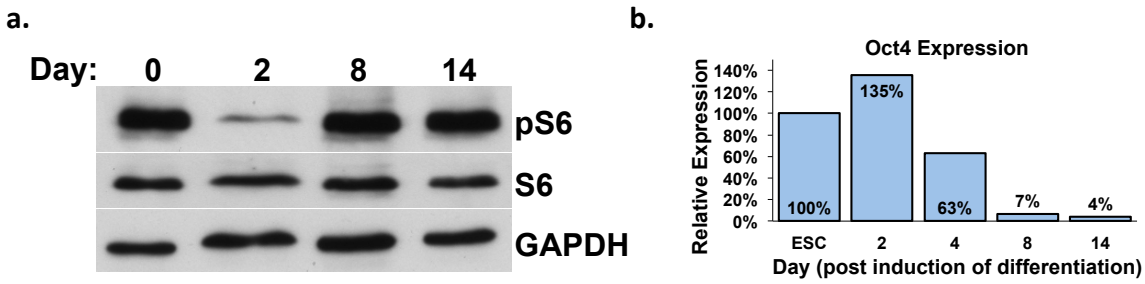


Figure 3-2: mTOR is activated during differentiation. Wild-type ESCs induced to spontaneously differentiate by embryoid body formation. Isolated at 0,2,8, and 14 days post-induction. A) Immunoblot to assess mTOR activation. pS6 = phosphorylated S6. B) Expression of pluripotency marker, Oct4.

eventual increase in mTOR activity during differentiation. These results indicate that mTOR activation could provide signals to promote differentiation.

Microarray Analysis

Having observed that GSK-3 can suppress mTOR signaling in ESCs and mTOR is activated during differentiation (in serum-free medium), we next sought to test if the GSK-3/mTOR axis is involved in ESC maintenance. ESCs can be maintained in serum/feeder-free conditions by addition of a GSK-3 inhibitor, CHIR90021 (CHIR), and a MEK inhibitor, PD0325901 (PD) (2i medium). Thus, we employed this culture system for the rest of our experiments to test whether dual inhibition of GSK-3 and mTOR can maintain pluripotent ESCs in long-term culture.

We began testing our hypothesis by conducting a microarray analysis of WT ESCs cultured overnight in serum/feeder-free medium with CHIR alone (C), Rapamycin alone (R), or both inhibitors in combination (CR). Our goal was to identify changes in gene expression specific to dual inhibition (CR condition) (Figure 3-3a). Out of 34,000 genes, we filtered 300 genes that were significantly ($p \leq 0.05$) different in the CR condition at a false discovery rate of 20%. To further filter our candidate genes, we selected for genes that had an absolute fold change of 1.5x or greater. We identified a total of 15 genes that met these criteria.

Multiple microRNAs (miR) encoded by the miR302/367 cluster were among the genes that were potently induced by the combination treatment (Figure 3-3b). The miR302 family is known to be highly expressed in stem cell populations and recent work has suggested that expression of these miRNAs can reprogram fibroblasts to iPS cells, bypassing the need for the conventional Yamanaka factors (Anokye-Danso, 2011; Jesus, 2009; Lipchina, 2012). These results were verified by rtPCR for the precursor of Mir302c (Figure 3-3c). These results suggest a role for the GSK-3/mTOR axis in ESC maintenance.

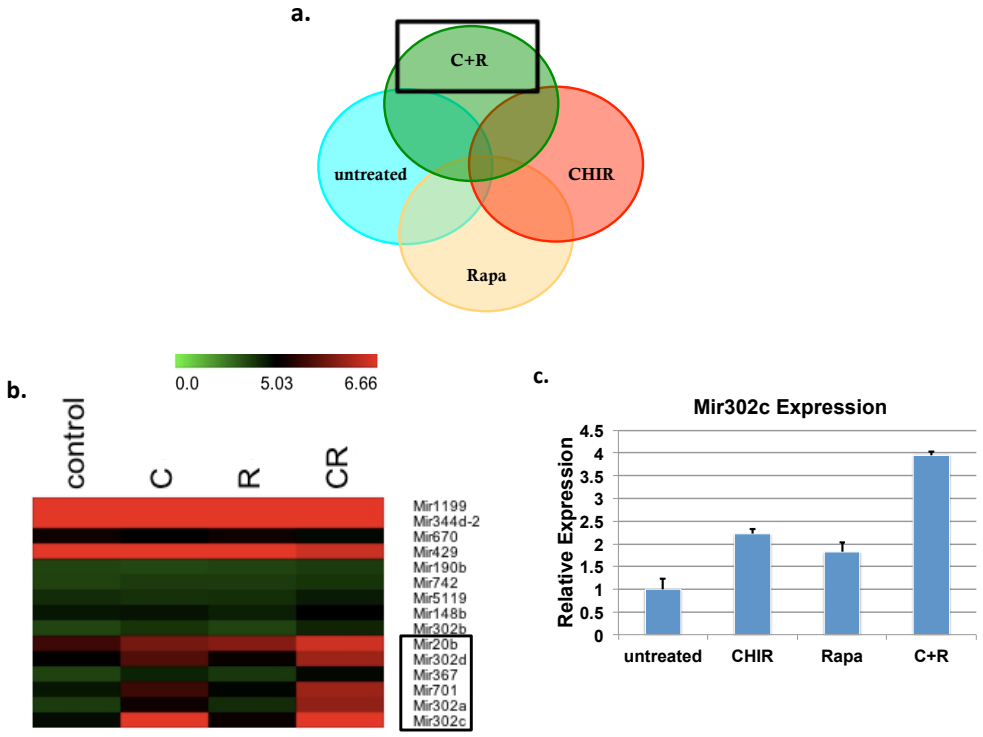


Figure 3-3: Expression of miR302/367 cluster is significantly increased by dual inhibition of GSK-3 and mTOR. a) Selection of genes that were differentially expressed in CR condition. b) Heat map of miRNAs that were screened. Conditions: untreated (control), CHIR (C), rapamycin (R), and CR combination. The black box shows Mir transcripts upregulated in the CR. c) rt-PCR validation of Mir302c precursor expression. Dose: C=3μM, R=10nM

Dual inhibition of GSK-3 and mTOR pathways is not sufficient to maintain pluripotency in ESCs

The enrichment of the miR302/367 cluster prompted us to further test if a combination of GSK-3 and mTOR inhibitors could maintain ESCs in a pluripotent state in culture. Using cells that were adapted to the 2i culture system, we substituted the MEK inhibitor for mTOR inhibitor, rapamycin. The cells were cultured for 4 days and morphology and gene expression were assessed at day 0, 1 and 4. Consistent with our microarray results, Mir302c expression, after 24 hours in culture, was higher in the CR condition than in either inhibitor alone (Figure 3-4a). However, Mir302c expression was not maintained after 4 days in culture (Figure 3-4b). Additionally, the morphology of the colonies in CR appears to be flattened, and more spread out than the colonies found in both the CHIR and 2i conditions (Figure 3-5). Moreover, cells in CR have approximately a 200x fold increase (compared to Day 0 cells) in expression of early differentiation marker, Fgf5 (Figure 3-6a). Oct4 expression was not different compared to day 0 in any of the conditions, except for the untreated samples (Figure 3-6b). These data demonstrate that CR is not sufficient to maintain ESCs in long-term culture. As the CHIR condition appears to have less differentiation and lower Fgf5 expression than in CR, the addition of rapamycin seems to accelerate differentiation. Cells cultured in rapamycin alone were not included in the day 4 analyses as they exhibited marked cell death.

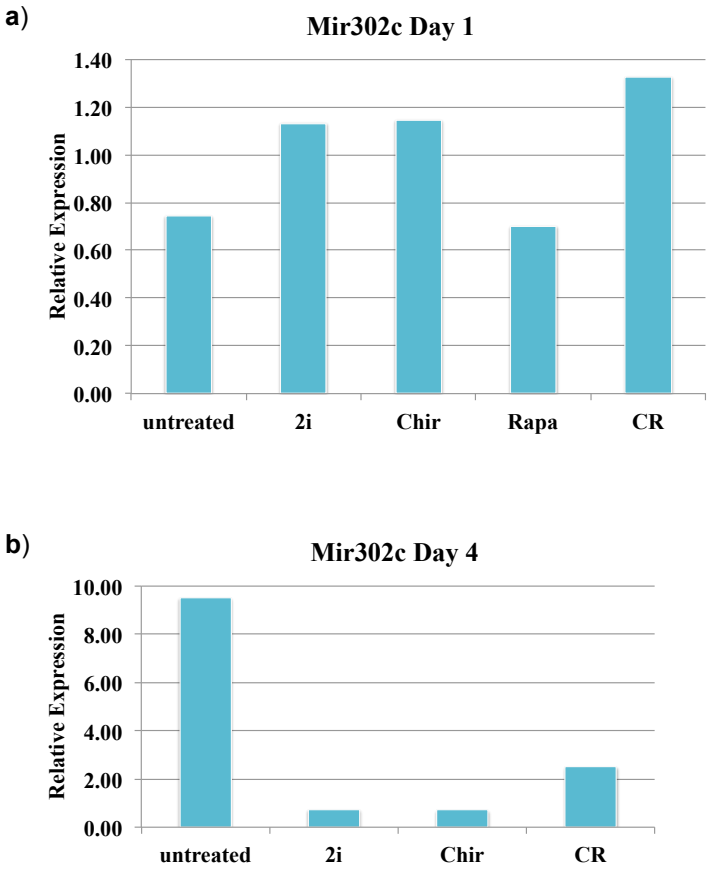


Figure 3-4: Expression of miR302c is not maintained during culture with CR. a) Expression of mir302c after 24 hours in culture. b) Expression of mir302c after 4 days in culture. All values are relative to expression level at Day 0.

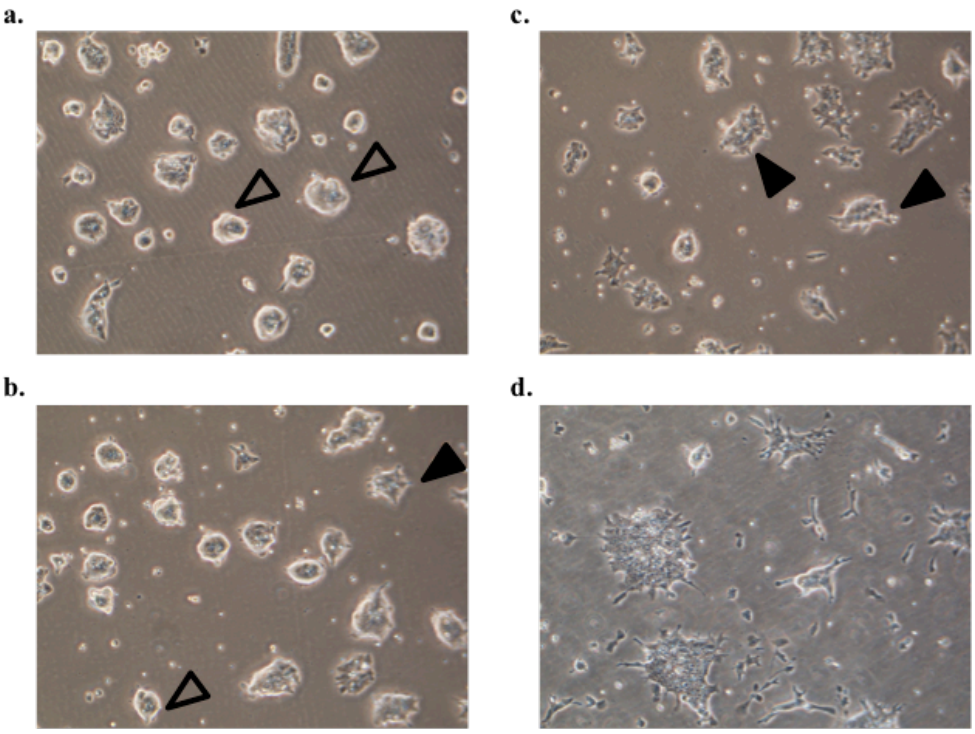


Figure 3-5: Murine embryonic stem cells after 4 days in culture. Phase-contrast image of ES cells in serum-free medium with indicated inhibitors. a) 2i (CHIR and PD). b) CHIR alone c) CHIR + Rapamycin d) Untreated. Open arrows indicate undifferentiated colonies, closed arrows indicate differentiated colonies. Inhibitor concentrations were as follows: CHIR = 3μM; Rapamycin = 10nm; PD = 1μM

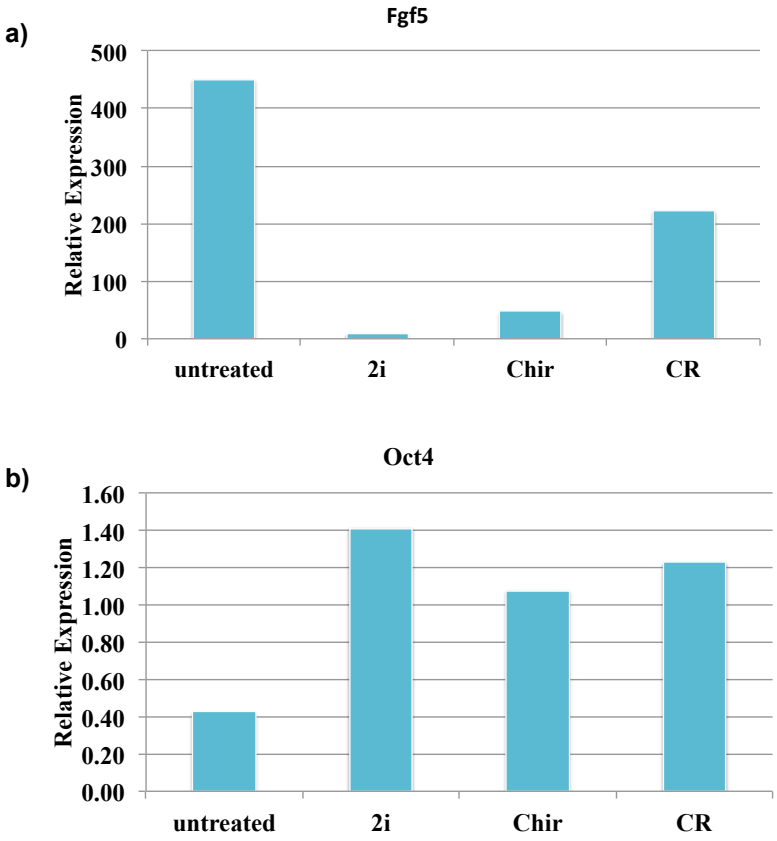


Figure 3-6: Expression analysis of ESCs at 4 days in culture. a) Expression of early differentiation marker, Fgf5. b) Expression of pluripotency marker, Oct4. All values are relative to expression level at Day 0.

3.3 Discussion

The goal of this study was to test if GSK-3 inhibition in ESCs promotes self-renewal through Wnt activation and lineage commitment through mTOR activation. We found that GSK-3 suppresses mTOR signaling in ESCs, consistent with studies in other cell types (Huang et al., 2009b; Inoki et al., 2006). Interestingly, we observe that both *Gsk3a* and *Gsk3b* must be deleted to observe robust activation of mTOR signaling (Supplementary Figure 3-1). This is consistent with published work demonstrating that inhibition of GSK-3 by Wnt activation leads to activation of mTOR and that the two isoforms of GSK-3 are functionally redundant in Wnt signaling. Therefore, we have shown that GSK-3-mediated activation of mTOR in ESCs likely occurs downstream of Wnt signaling.

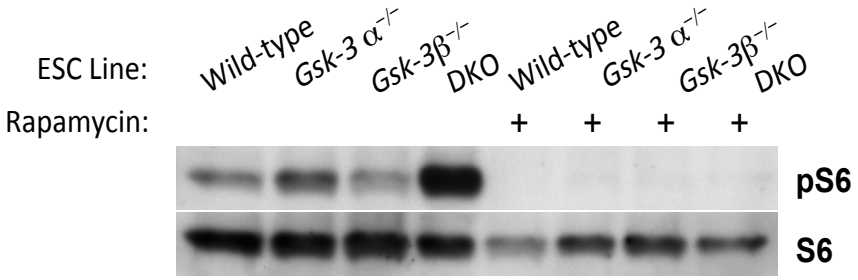
We also observed that the combination of GSK-3 and mTOR inhibitors could significantly increase expression of the mir302/367 cluster more than either inhibitor alone. However, this increased expression is not sustained during culture. Moreover, the combination of GSK-3 and mTOR inhibitors did not maintain ESCs in a pluripotent state as evidenced by the increased expression of an early differentiation marker and lack of undifferentiated colonies in the culture.

Since this study, our lab has discovered that hematopoietic stem cells can be maintained in a quiescent state with serum-free culture medium with only GSK-3 and mTOR inhibitors (Huang et al., 2012a). Another study also demonstrated that GSK-3 deletion in neural progenitors leads to a hyperproliferation, which can be suppressed with the addition of rapamycin (Ka et al., 2014). A similar balance of Wnt and mTOR signaling pathway is proposed to maintain the epidermal stem cell compartment (Castilho et al., 2009). All of these studies have assessed the role of the GSK-3/mTOR axis in maintenance of adult stem cell populations, which are responsible for regenerating tissues and stem cell compartments. Inherently, these stem cell populations require a quiescent state where they do not proliferate and can be activated during tissue injury. Thus, mTOR inhibition likely mimics their quiescent state.

ESCs are highly proliferative and thus alternatively inhibition of mTOR may oppose proliferative activity. ESCs can be maintained in long-term culture in a combination of MEK and GSK-3 inhibitors. It has been suggested that the addition of GSK-3 inhibitor to the culture system relieves inhibition of biosynthetic pathways that allow the ESCs to grow (Ying et al., 2008). A study to identify genes regulating ESC commitment found that ESCs with *Tsc2* deletion, with consequent activation of mTOR signaling, prevents their exit from pluripotency (Betschinger et al., 2013). Consistent with our result, they also found that mTOR activity is increased during differentiation. However, this increase does not dictate cell fate transitions (Betschinger et al., 2013). These data support that mTOR activation by GSK-3 inhibition in ESCs is promoting their proliferative state, rather than cell fate specification.

Taken together, the GSK-3/mTOR axis does not have a similar function in ESCs and HSCs. However, GSK-3 has many other effectors that have not been studied in ESCs that could be the subject of future studies.

3.4 Supplemental Material



Supplementary Figure 3-1 GSK-3a/b are redundant in suppression of mTOR activity. Immunoblot of lysates from wild-type and *Gsk-3a or Gsk-3b* single and double knockout (DKO) ESCs with or without 100nM Rapamycin (Rapa). Cells were serum starved for 2 days prior to isolation. pS6 = phosphorylated S6

CHAPTER 4

Phosphoproteomic analysis of GSK-3

4.1 Introduction

Glycogen synthase kinase-3 is a ubiquitously expressed, highly conserved serine/threonine kinase. Unlike most kinases, GSK-3 is constitutively active and generally regulates downstream pathways by suppressing the activity of its direct substrates. As a result, upstream signals typically inhibit GSK-3 and thus, phosphorylation of its diverse range of effectors. GSK-3 was first identified as one of the enzymes that phosphorylates and inhibits glycogen synthase, the rate-limiting enzyme in glycogen synthesis. However, our knowledge of the role of GSK-3 has since expanded to a broad range of cellular processes, such as developmental processes, cell growth and survival, immune response, cytoskeletal organization and circadian rhythm (Kaidanovich-Beilin and Woodgett, 2011). Dysregulation of GSK-3-regulated signaling pathways is moreover implicated in a broad range of pathological settings, including diabetes, bipolar disorder, Alzheimer's disease and cancer (Beurel et al., 2015). To understand the specific role of GSK-3 in these disease pathologies, it is therefore necessary to characterize the entire spectrum of GSK-3 substrates and downstream signaling functions.

It is not surprising that there are distinct pools of GSK-3, regulated by different mechanisms, to prevent unwanted crosstalk between downstream signaling pathways. Hormones and growth factors, such as insulin, can activate receptor tyrosine kinases that consequently activate the phosphoinositide 3-kinase (P13K)/Akt signaling pathway. Akt can directly phosphorylate the N-terminal of GSK-3 to inhibit its activity (Cross et al., 1995). There exists another pool of GSK-3 that is bound to axin and is involved in Wnt signaling. Contrastingly, the N-terminal phosphorylation of GSK-3 does not affect GSK-3 activity in the canonical Wnt signaling pathway (Ding et al., 2000; McManus et al., 2005).

The Wnt pathway has important roles during development, in cell patterning and specification, and adult stem cell homeostasis (Clevers et al., 2014; Logan and Nusse, 2004). In resting cells, GSK-3 is bound to the scaffold protein, Axin, as part of the of the β-catenin destruction complex, which also includes β-catenin, Casein Kinase Iα and Adenomatous polyposis coli (APC) (Peterson-Nedry et al., 2008). In the absence of Wnt ligands, GSK-3 phosphorylates β-catenin and targets it for ubiquitin-mediated proteosomal degradation. Upon activation, Wnt ligands induce dimerization of coreceptors, Frizzled and LRP5/6, which promotes interaction of LRP6 with Axin and recruits the destruction complex to the membrane. This leads to inhibition of GSK-3 and stabilization of β-catenin, which can then translocate the nucleus to activate transcription of Wnt target genes, which are cell-context dependent and influence proliferation, survival and cell fate (Clevers, 2006; MacDonald et al., 2009).

Recent studies have described that Wnt signaling can stabilize many proteins in addition to β-catenin, in a process termed Wnt-dependent stabilization of proteins (Wnt/STOP) (Acebron and Niehrs, 2016; Kim et al., 2009; Taelman et al., 2010; Xu et al., 2009). Wnt/STOP is critical during mitosis to stabilize protein levels as cells prepare to divide and plays a role during sperm maturation (Acebron et al., 2014; Huang et al., 2015; Koch et al., 2015). Together across all cell types, there are about 35 proteins that have been demonstrated to become stabilized by GSK-3 inhibition. However, it has been speculated that GSK-3 can target many more proteins, and this has led to the general belief that GSK-3 inhibition stabilizes proteins (Acebron and Niehrs, 2016; Taelman et al., 2010; Xu et al., 2009). Thus, a global analysis of changes in protein abundance changes in response to GSK-3 inhibition is required to address exactly how many proteins are stabilized.

To date, over 100 putative substrates of GSK-3 have been identified diverse cell types. A majority of GSK-3 substrates are transcription factors, such as Smad1, CREB and c-Jun, through which GSK-3 can regulate transcription of many genes. Other substrates of GSK-3 include

signaling molecules (e.g. translational initiation factor, eIF2B and the mTOR regulator, TSC2), structural proteins (e.g. Tau and MAP1B) and other kinases (e.g. focal adhesion kinase) (Sutherland, 2011). Phosphorylation by GSK-3 can have a multitude of effects on target proteins including modulation of catalytic activity, stability, localization, protein-protein interactions or DNA binding capacity (Sutherland, 2011). Although many substrates have been proposed to be phosphorylated by GSK-3, these reports have differed in the cell models and experimental approaches used to identify targets. Thus, a complete characterization of the GSK-3 phosphoproteome in one uniform model is needed fully recognize the repertoire of potential substrates.

Many GSK-3 substrates also contain a consensus sequence, S/TxxxS/T(p), where the second S/T residue is pre-phosphorylated by another kinase. The GSK-3 consensus was first demonstrated in glycogen synthase, which is phosphorylated by Casein kinase II at Ser657 and subsequently leads to processive phosphorylation by GSK-3 at Ser653, 649, 645 and 641 (Zhang et al., 1993). The presence of the consensus sequence has since been demonstrated in many other GSK-3 substrates and is often used to predict a GSK-3 phosphorylation site (Cohen and Frame, 2001; Xu et al., 2009). On examining the substrates using two comprehensive reviews, the requirement of the priming phosphorylation has only been tested for 45% of substrates (Kaidanovich-Beilin and Woodgett, 2011; Sutherland, 2011). There are at least 25 substrates that are phosphorylated at the consensus site, but the requirement for priming phosphorylation has not been tested. Interestingly, 20% of GSK-3 substrates do not have the consensus sequence, most notably Cyclin D1 and phosphatase inhibitor 2. Thus, it is apparent that although there are many substrates of GSK-3 that are pre-primed substrate, it is clearly not a requirement. A comprehensive analysis of the GSK-3 phosphoproteome would be required to address how many proteins that have the consensus sequence can be phosphorylated by GSK-3.

It is apparent that GSK-3 is a key regulatory node that responds to a host of cell signals and translates these cues into a diverse and often complex network of downstream pathways. We therefore aimed to use a large-scale proteomic approach to characterize the GSK-3 phosphoproteome and determine the overall range of GSK-3 substrates within a single cell type. Specifically, stable-isotope labeling by amino acids in culture (SILAC) was used to metabolically label the entire proteome of wild-type and *Gsk3a/b* double-knockout (referred to as DKO) mouse embryonic stem cells (ESCs) (Doble et al., 2007). ESCs provide an ideal model as they are a highly proliferative, uniform population of cells. Moreover, GSK-3 inhibition has been shown to promote self-renewal in ESCs (Ying et al., 2008). To our knowledge, this is the first study to conduct a global analysis to characterize GSK-3 substrates.

4.2 Results

Global phosphoproteomic analysis of GSK-3α and GSK-3β Null Embryonic Stem Cells

To characterize the GSK-3 phosphoproteome, we employed a large-scale screening method that allows investigation of global phosphorylation events within a cell population. We performed quantitative mass spectrometry (MS) to compare the phosphoproteome of Gsk3 double knockout (DKO) ESCs to wild-type (Figure 4-1a). Western blotting was used to confirm GSK-3α and GSK-3β deletion and activation of Wnt signaling by β-catenin accumulation (Figure 4-1b). For SILAC labeling, DKO ESCs were cultured in isotopically labeled lysine ($^{13}C_6{}^{15}N_2$) and arginine ($^{13}C_6{}^{15}N_4$) amino acids for at least 4 passages to achieve complete labeling of the proteome (Supplementary Figure 4-1). Wild-type ESCs were cultured in parallel in normal lysine ($^{12}C_6{}^{14}N_2$) and arginine ($^{12}C_6{}^{14}N_4$) amino acid medium. Prior to protein isolation, similar expression of stem cell markers Oct4 and Nanog was confirmed by flow cytometry (Supplementary Figure 4-2).

Whole-cell lysates in biological triplicate were digested with trypsin in order to obtain short (6-20 amino acids) peptides for effective chromatographic separation and MS detection. To

enhance detection of phosphorylated peptides, phosphopeptide enrichment was performed using titanium dioxide (TiO_2) chromatography. The unbound peptides from the TiO_2 flow-through were used for non-modified, proteomic identification. For the analysis we employed EasyLC (Thermo Scientific) nano liquid chromatography (flow rate 300 nl/min), which currently provides the highest sensitivity. The MS analysis was performed using two instruments, the Q-Exactive (Thermo Scientific) and the Orbitrap Fusion Tribrid (Thermo Scientific), both coupled online to the nano chromatographic system. Both instruments provide high mass resolution (>120,000), mass accuracy (<1 ppm) and sensitivity (<fmol). Results were processed using MaxQuant (reference PMID: 19029910), a freely available software.

Within both the DKO and wild-type data sets, we identified 5858 phosphosites belonging to 1939 phosphoproteins. Most phosphosites were identified from peptides carrying a single phosphorylation (Figure 4-1c). The relative frequency of phosphorylation on the three amino acids serine:threonine:tyrosine was highly comparable to the 80:20:1 distribution known from most mammalian reports (Figure 4-1c) (Olsen et al., 2006). We found the overwhelming majority of identified phosphorylation sites could be localized with high confidence to specific positions on the amino acid sequence (Figure 4-1d).

Using a p-value of ≤ 0.05, we found 404 phosphopeptides, representing 269 unique proteins, that were differentially phosphorylated in DKO compared to wild-type ESCs. To isolate the strongest candidates, we filtered our dataset for phosphopeptides with an absolute fold change of 1.5x or greater. We found 89 phosphopeptides from 65 unique proteins (3.4% of all phosphoprotiens identified) that had significantly reduced phosphorylation in DKO compared to wild-type ESCs (Figure 4-2a, Supplemental Table 1). Of these phosphorylation events, 88% were on a serine residue and 12% on threonine, as would be expected for GSK-3, a serine/threonine kinase (Figure 4-2b). We also detected 72 phosphopeptides with increased phosphorylation, from

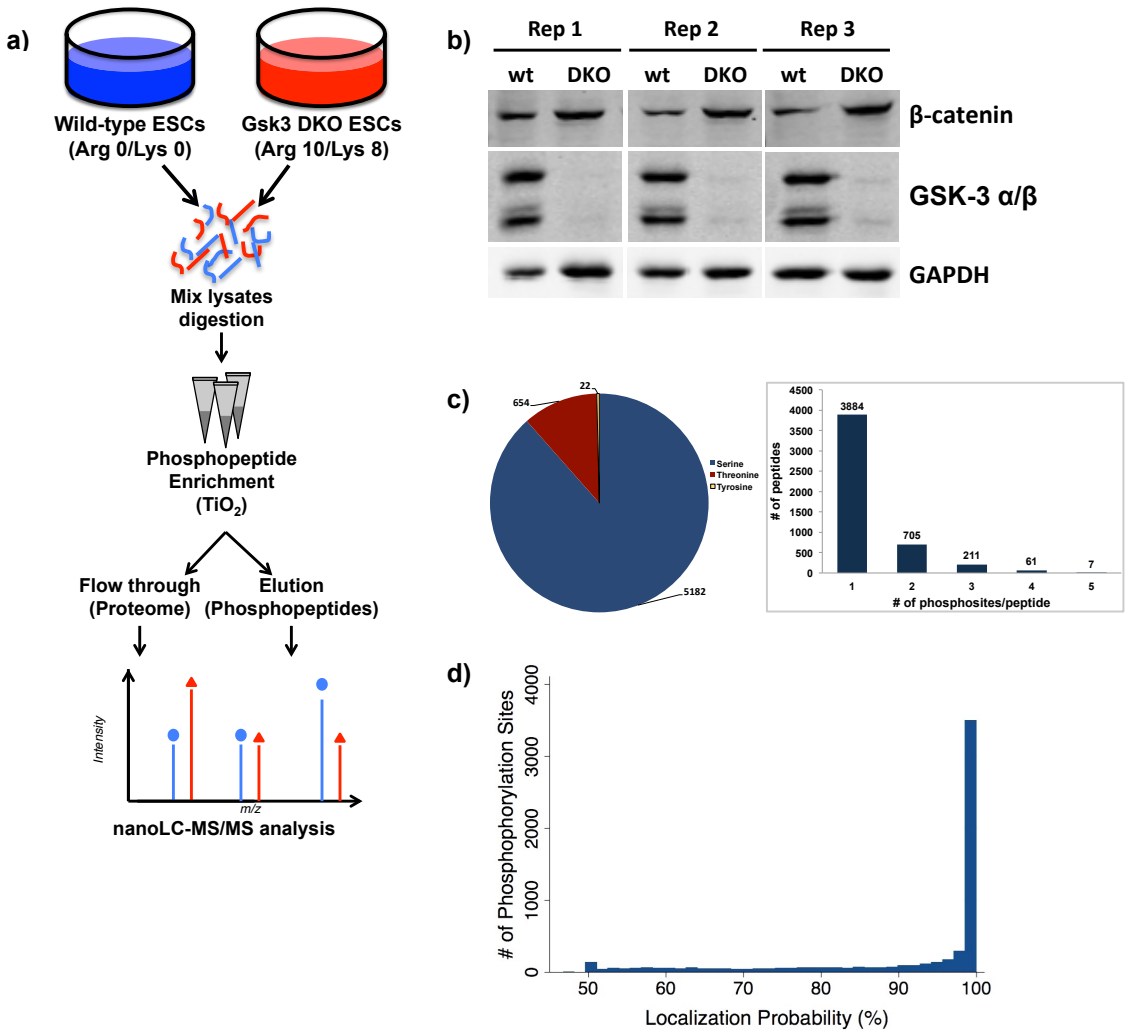


Figure 4-1: Global phosphoproteome analysis using SILAC technique. a) Experimental work flow. Cells were cultured for 4 passages to ensure complete incorporation of heavy isotope. Lysates were digested with trypsin and phosphorylated peptides were enriched using home-made titanium oxide columns. Flow-through was used to identify the proteome. b) Western blot to assess *Gsk3* knockout and Wnt signaling activation by β-catenin accumulation. c) Overview of phosphorylation sites identified in our screen. d) Distribution of site-localization confidence from screen.

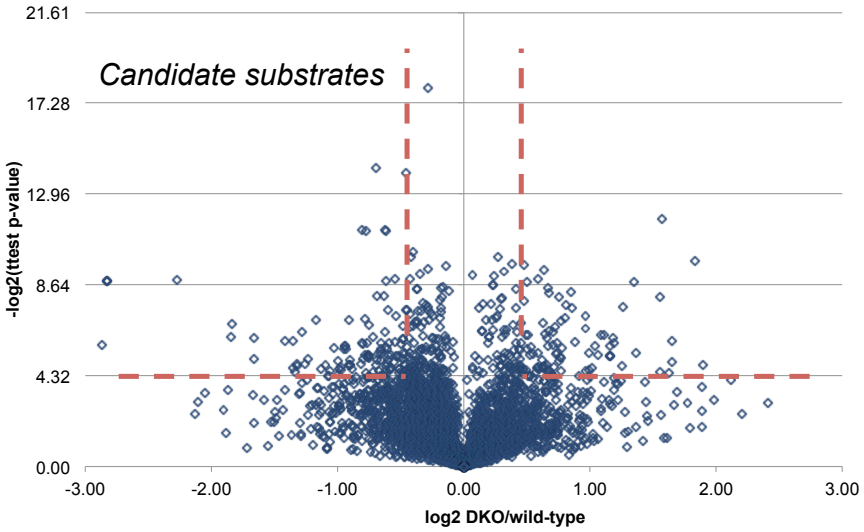
59 unique proteins, in the DKO compared to wild-type cells (Figure 4-2a). Because these phosphorylations were enhanced despite GSK-3 depletion, we consider these phosphosites to be regulated by compensatory mechanisms downstream of GSK-3's target substrates. The indirect effects of long-term GSK-3 deprivation may be an interesting subject for further research.

A substantial number of phosphopeptides (143) were identified only in the wild-type condition, in two or more biological replicates, with no corresponding peptide (phosphorylated or otherwise) detected in the DKO condition (Figure 4-2c, see "wild-type only"). Although p-values and fold change cannot be calculated, these peptides could indicate an absolute requirement for GSK-3 dependent phosphorylation. For future inquiry, these peptides could be compared to short-term GSK-3 inhibition to identify potential substrates of GSK-3.

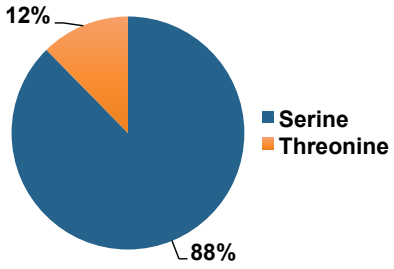
Phosphoproteome analysis identifies novel candidate GSK-3 substrates

As direct substrates of GSK-3 should have reduced phosphorylation in the knockout, we identified 89 phosphosites with reduced phosphorylation in DKO compared to wild-type as high-confidence GSK-3-dependent phosphorylation sites. GSK-3 commonly engages with "primed" substrates that have been pre-phosphorylated at a serine or threonine 4 residues C-terminal to the GSK-3 site, providing the loose consensus S/T-X-X-X-pS/pT in which the 1st ser/thr is a preferred GSK-3 site and the 4th residue is phosphorylated by another protein kinase. However, the prevalence of this consensus has not previously been addressed in a systematic manner in a single cell type. We therefore, we asked how many of the high-confidence sites identified in ESCs contain the putative GSK-3 consensus sequence (Figure 4-3a). Serine is the most common residue at the +4 position, occurring at 20 sites, and a threonine residue is present at an additional 6 sites (Figure 4-3b). These data indicate that 29% of the GSK-3-dependent phosphosites have the potential to be phosphate primed by phosphorylation (Figure 4-3b). We did not observe a similar enrichment at the +4 position when we included all of the identified phosphopeptides, indicating that this enrichment is specific to the high-confidence GSK-3

a)


Candidate substrates
21.61
17.28
12.96
8.64
4.32
0.00
-log2(ttest p-value)
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
log2 DKO/wild-type

b)


12%
88%
Serine
Threonine

c)

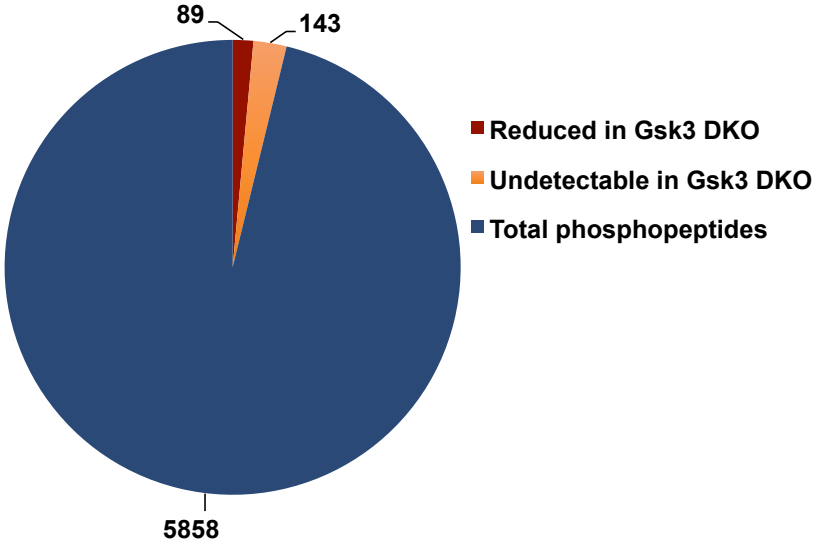

89
143
5858
Reduced in Gsk3 DKO
Undetectable in Gsk3 DKO
Total phosphopeptides

Figure 4-2: GSK-3 null ESC phosphoproteome identifies 65 candidate substrates of GSK-3. a) All phosphopeptide changes in DKO compared to wild-type. Each dot represents a phosphorylation site, and one protein may be represented by multiple dots in this plot. Red box indicates peptides with a absolute fold change of 1.5x or greater, *p*-value<=0.05. b) Significantly reduced phosphorylation events occur at Serine or Threonine residues. c) Distribution of GSK-3-dependent phosphopeptides (red) and phosphopeptides found in wild-type condition only (yellow) compared to background (blue).

phosphorylation sites (Supplemental Figure 4-3a). These results demonstrate that although many GSK-3 substrates have the consensus sequence, it is not a requirement.

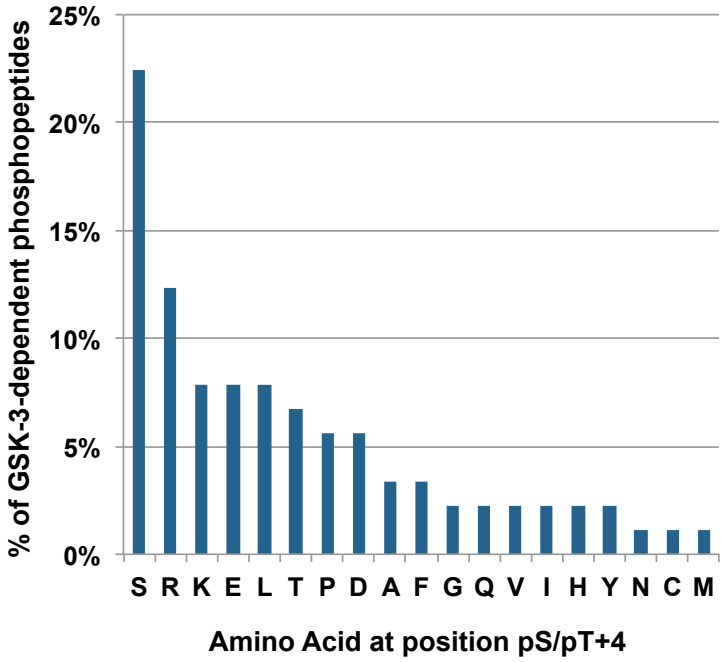
For several established GSK-3 targets, phosphorylation at this consensus sequence targets the protein for proteosomal degradation. As 20% of the human proteome contains putative GSK-3 consensus sites, a large proportion of the proteome has been predicted to include potential substrates of GSK-3 (Taelman et al., 2010; Xu et al., 2009). Thus, we determined the frequency at which the consensus amino acid sequence occurs in our data and wondered how many of these sequences are present in our candidate phosphopeptides. We observed that 1075 of all the detected phosphopeptides have a Serine or Threonine at the pS/pT+4 position, relative to the site of phosphorylation, and thus, have the potential to be phosphate-primed GSK-3 sites (Figure 4-3c). Strikingly, only 4.5% of these sites are found in our list of high-confidence GSK-3-dependent phosphorylation sites (Figure 4-3c). We confirmed our analysis using the pLogo generation tool that employs iterative comparisons of selected peptide sequences to background peptide sequences to extract significantly enriched motifs (O'Shea et al., 2013; Schwartz and Gygi, 2005). Although we did not observe statistically significant enrichment of any residues, the most common residues at the pS/pT+4 position are S and T (Supplementary Figure 4-3b). Our consensus sequence analysis suggests that prior studies are likely over-estimating the number of GSK-3 substrates and that the consensus sequence alone is not sufficient to determine if a substrate can be phosphorylated by GSK-3.

We next conducted a gene ontology (GO) analysis on the 65 candidate GSK-3 substrates using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to categorize proteins by known functions and gain an overall understanding of the cellular processes that these proteins influence (Figure 4-4a) (Dennis et al., 2003). The GO terms with the most substrates include: regulation of transcription (14 genes), mRNA processing (10 genes), RNA splicing (8 genes), cell cycle (7 genes) and DNA repair (6 genes).

a)


S/TxxxS/T
GSK-3 P-site
Priming Site

b)


% of GSK-3-dependent phosphopeptides
25%
20%
15%
10%
5%
0%
S R K E L T P D A F G Q V I H Y N C M
Amino Acid at position pS/pT+4

c)

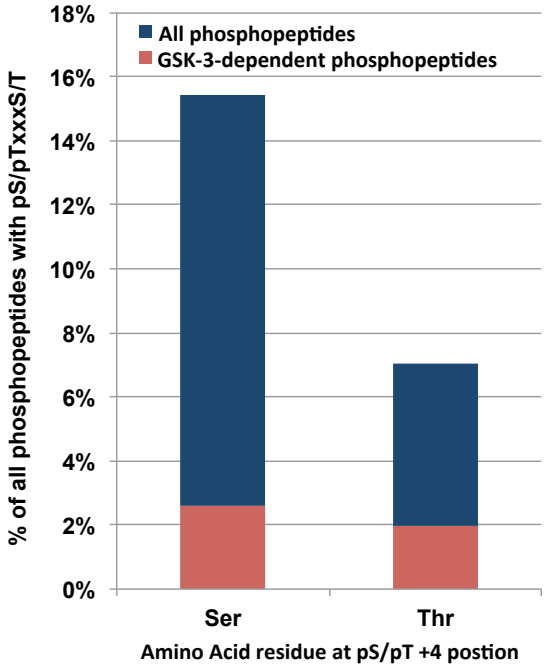

All phosphopeptides
GSK-3-dependent phosphopeptides
% of all phosphopeptides with pS/pTxxxS/T
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
Ser
Thr
Amino Acid residue at pS/pT +4 postion

Figure 4-3: GSK-3 consensus sequence analysis. Threshold used was -1.5 fold change, *p*<=0.05. a) GSK-3 consensus sequence with priming phosphorylation. b) Histogram of amino acid residue 4 positions after the phosphorylation site. C) Frequency of Ser or Thr at the pS/pT+4 position. All candidate peptides (red) compared to all identified peptides (blue). Value is presented as percentage of total peptides. pS/pT+4 = 4 residues C-terminal from phosphorylation site.

Other functional categories the candidate substrates have been associated with include chromatin remodeling, translation initiation, stem cell maintenance, regulation of biosynthetic processes, rRNA processing and DNA replication.

To identify potential relationships between the identified substrates, we analyzed direct and indirect interactions among the proteins enriched in the GO analysis using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (Szklarczyk et al., 2015). This tool provides a confidence score for each interaction based on known experimental data, pathway knowledge from curated databases, text mining and co-expression. The analysis enriched for three networks of proteins with related functions: alternative splicing, transcriptional regulators and cell cycle (Figure 4-4b).

Many known transcription factors are phosphorylated by GSK-3, leading to changes in protein stability, DNA binding and localization (Sutherland, 2011;(Beurel et al., 2015). Our analyses (Figure 4-4b, see “Transcriptional Regulators”) are congruent with prior reports that link GSK-3 to transcriptional regulation. For instance, this cluster includes c-Jun, whose phosphorylation by GSK-3 has previously been found to inhibit its DNA binding activity(Boyle et al., 1991; Troussard et al., 1999). Outside of the known transcription factor identified in this substrate cluster, phosphorylation of the SWI/SNF chromatin remodeling factor ATRX was reduced 1.7-fold in the DKO at a GSK-3 consensus site. Two other candidates can be further linked as regulators of ribosomal RNA synthesis: PHF6 suppresses ribosomal RNA synthesis, and Baz2a is an epigenetic silencer of ribosomal RNA genes (Gu et al., 2014; Wang et al., 2013).

We also identified a network of candidate substrates with functions related to the cell cycle (Figure 4-4b, see “Cell cycle”). We also identified a network of candidate substrates with functions related to the cell cycle that formed around cyclin dependent kinase 1 (CDK-1). CDK-1 phosphorylates the adaptor protein Ajuba (also known as Jub) at Ser119 and Ser135 during the

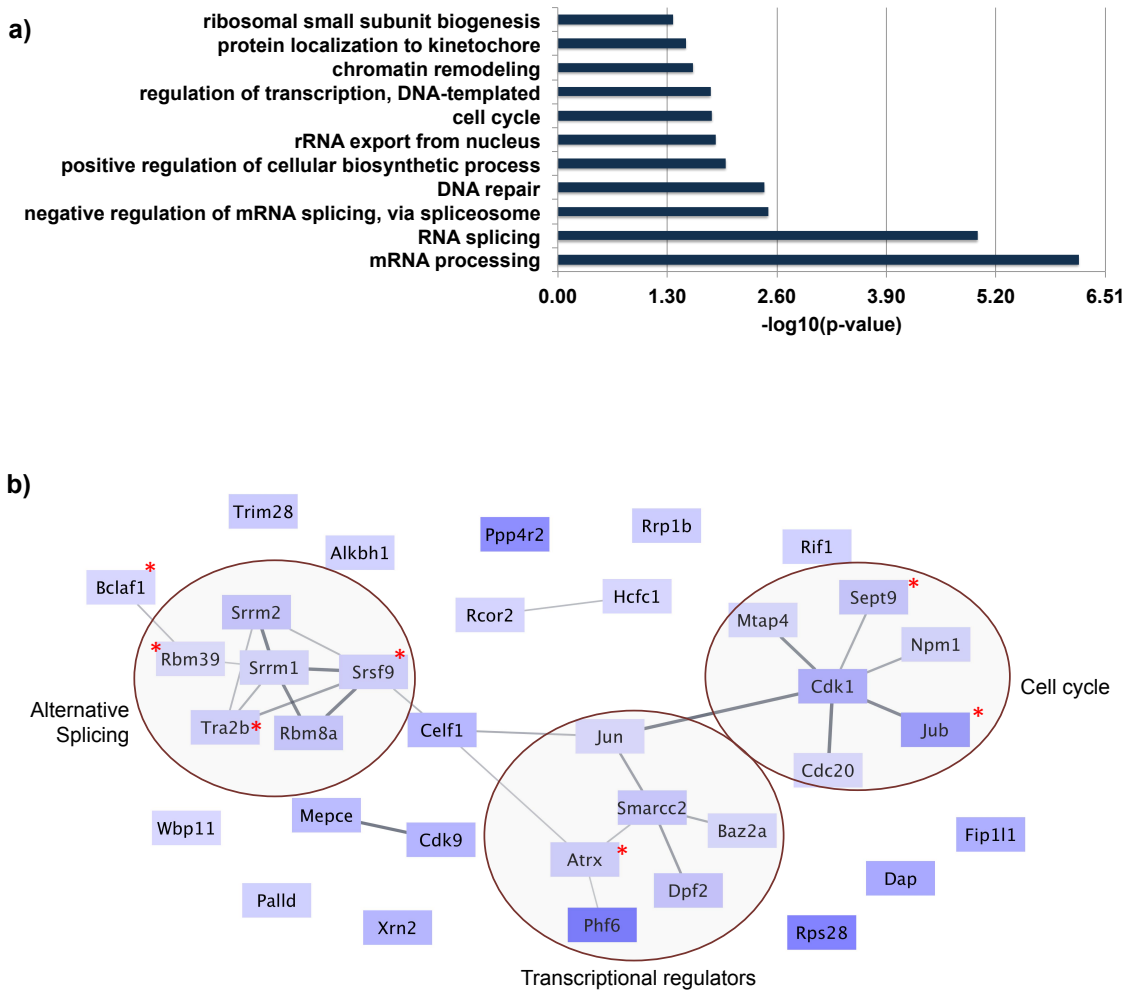


Figure 4-4: Analysis of the GSK-3 null ESC phosphoproteome identifies substrates with a broad range of functions. a) Gene Ontology analysis of high-confidence candidate GSK-3 substrates using Database for Annotation, Visualization and Integrated Discovery database (DAVID). b) Protein interactions of the proteins enriched in the GO analysis generates three networks of proteins with related functions. Red star indicates phosphorylation sites with GSK-3 consensus motif. Darker shade of blue indicates higher absolute fold change. All proteins in these analysis have fold change <-1.5; *p-value* <= 0.05.

G2/M phase of the cell cycle. This in turn controls expression of other cell cycle regulators (Chen et al., 2016). To regulate mitosis, CDK-1 also phosphorylates SEPT9, a GTP binding protein involved in cytokinesis and exocytosis, at Thr24 (Estey et al., 2013). We found a 3-fold reduction in Ajuba phosphorylation at Ser129, a GSK-3 consensus site, in DKO compared to wild-type cells. SEPT9 phosphorylation at Ser85, also a GSK-3 consensus site, was reduced by almost 2-fold in DKO.

Phosphoproteome analysis identifies a role for GSK-3 in regulation of RNA splicing

GSK-3's role as a global cellular regulator has to date been attributed to its direct inactivation of protein function, modulation of transcription factors that control the production of new proteins, or targeting of proteins for degradation. By modulating a network of splicing factors, identified as the third cluster of enriched proteins in our data set (Figure 4-4b, see "Alternative Splicing"), GSK-3 appears to exert an additional level of cellular control by regulating the splice variants transcribed from mRNA. We were most interested in this group, as RNA splicing and mRNA processing were also the most significantly enriched functions in our GO analysis (Figure 4-4a). We also observed significant enrichment of the spliceosome KEGG pathway. This is the first study to implicate GSK-3 in the general regulation of alternative splicing through a network of splicing factors, although one previously-published study demonstrated that GSK-3 phosphorylates a proline- and glutamine-rich splicing factor, PSF, to inhibit alternative splicing of CD45 in T cells (Heyd and Lynch, 2010).

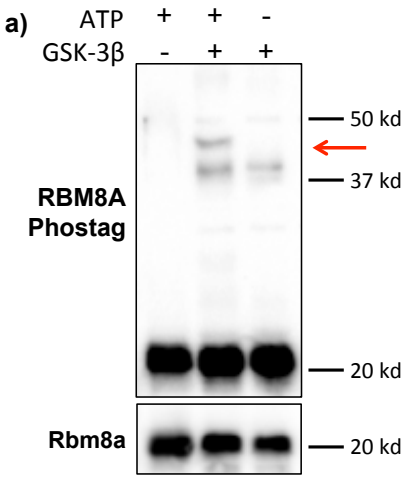
Many of the identified splicing factors contain arginine-serine dipeptide motifs (RS domains), including multiple SR family members, SR family-related factors, and other RS containing splicing factors (Long and Caceres, 2009). These include RNA binding protein 8A (RBM8A), RBM39, SRSF9, and TRA2B (SRSF10). Our screen identified candidate GSK-3 phosphorylation sites for 2 of 3 proteins in the RS domain (Figure 4-b, Table 4-1). The phosphorylation status of serine residues in this domain determines cellular localization, protein-

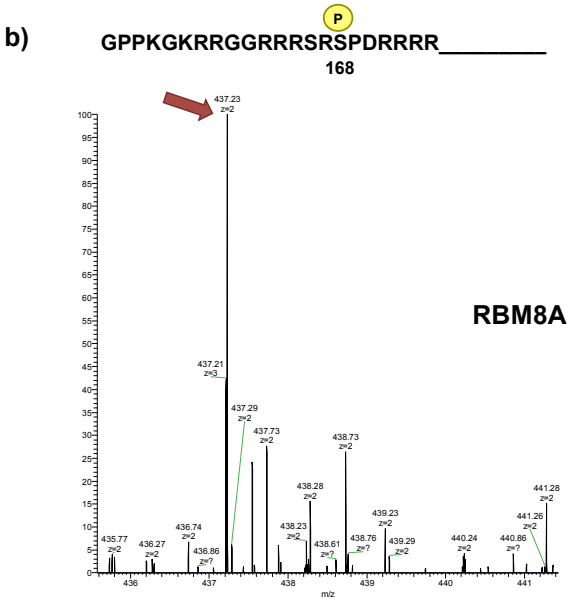
protein interactions, and overall activity of these proteins (Zhong et al., 2009). Thus, phosphorylation of these proteins by GSK-3 could modulate their activity and subsequently influence alternative splicing events.

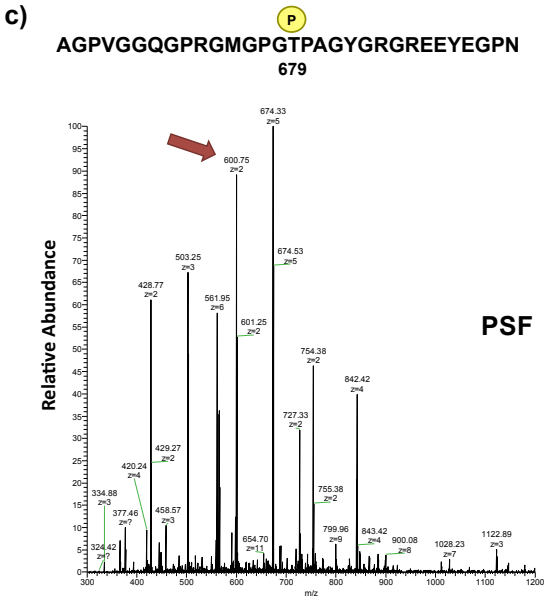
We selected RBM8A, a component of the exon-junction complex (EJC), for further characterization. The SILAC/MS data identified GSK-3-dependent phosphorylation of RBM8A at Ser166 and Ser168, which lie within adjacent RS motifs; prior work has shown that phosphorylation of these residues inhibits RBM8A interaction with other EJC components (Hsu et al., 2005; Ishigaki et al., 2015) (Table 4-1). As these sites do not appear to have a consensus priming site, GSK-3 should be able to phosphorylate these sites in an *in vitro* protein kinase assay using recombinant RBM8A and GSK-3.To further validate RBM8A, we employed Phos-tag acrylamide gels, which take advantage of a phosphate binding tag to separate phospho-species from non-phosphorylated forms (Kinoshita, 2005). We used recombinant RBM8A and GSK-3 protein to conduct *in vitro* kinase assays. We observed an ATP dependent increase in RBM8A phosphorylation (Figure 4-5a, indicated by the red arrow). Mass spectrometry analysis of this reaction confirmed Ser168 to be the site of phosphorylation by GSK-3 (Figure 4-5b). In parallel, we also tested whether GSK-3 phosphorylates the splicing factor PSF (Heyd and Lynch, 2010). Prior work had shown that PSF function is sensitive to a small molecule GSK-3 inhibitor and suggested that Threonine-679 is a critical site of phosphorylation. Mass spectrometry after an *in vitro* kinase reaction with recombinant PSF and GSK-3 confirmed that Thr679 is phosphorylated by GSK-3 (Figure 4-5c). We attempted to assess the phosphorylation status of the protein in wild-type and DKO cell lysates using Phos-tag gels but were unable to detect any significant difference in RBM8A phosphorylation between wild-type and DKO cell lysates (Figure 4-5d). This is not entirely unexpected, because the removal of a single phosphate group from a highly phosphorylated protein would be expected to have a much more subtle shift using this Phos-tag method. Collectively, we demonstrate GSK-3 directly phosphorylates two splicing factors, RBM8A at Ser168 and PSF at Thr679.

Splicing Factor	Site	Sequence
Tra2b	S83	PETRYERSRSREHLRpSRSPSPESRSRHEHKG
Rbm39	S125	GKIGLPHSIKLSRRRpSRSKSPFRKDKSPVRE
Srsf9	S190	EGETSYIRVYPERSTpSYGYSRSRSGSRGRDS
Rbm8a	S168	GPPKGKRRGGRRRSRpSPDRRRR_____

Table 4-1: Potential GSK-3 phosphorylation site on splicing factors. RS dipeptide is shown in red. Phosphorylation site indicated by “pS”.


a)
ATP + + -
GSK-3β - + +
50 kd
37 kd
RBM8A
Phostag
20 kd
Rbm8a
20 kd


b)
P
GPPKGKRRGGRRRSRSPDRRRR_____
168
RBM8A


c)
P
AGPVGGQGPRGMGPGTPAGYGRGREEYEGPN
679
Relative Abundance
PSF

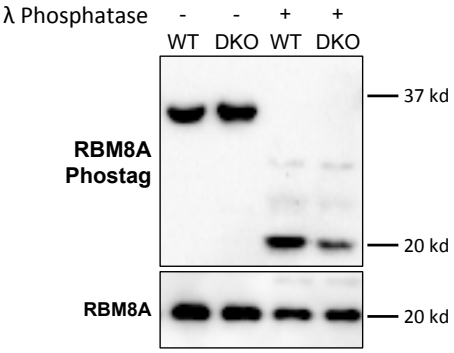

d)
λ Phosphatase - - + +
WT DKO WT DKO
37 kd
RBM8A
Phostag
20 kd
RBM8A
20 kd

Figure 4-5: GSK-3 can phosphorylate splicing regulators, RBM8A and PSF. a) In-vitro kinase assay with recombinant RBM8A protein indicates ATP-dependent GSK-3 phosphorylation of RBM8A (red arrow). In-Vitro Reaction included 0.4µg recombinant RBM8A, 500 units GSK-3 (Neb), 800µM ATP and was incubated for 1 hour at 30 C. b) MS/MS analysis of in-vitro kinase reaction with recombinant RBM8A indicates GSK-3 phosphorylates RBM8A at Ser 168 and c) PSF at 679 (indicated by red arrow). d) Phos-tag gel is not sensitive enough to resolve differential phosphorylation pattern in wild-type and *Gsk3* DKO cell lysates, 12.5% Acrylamide gel plus/minus 30um phos-tag reagent (Wako) was used for all RBM8A gels.

GSK-3 directly phosphorylates nucleolar protein, NPM1

We also selected Nucleophosmin 1 (NPM1) for further validation as this protein is over-expressed in many solid tumors and is one of the commonly mutated genes in acute myeloid leukemia (Cancer Genome Atlas Research Network, 2013; Di Matteo et al., 2016). Moreover, NPM1 acts as a negative regulator of alternative splicing (Tarapore et al., 2005). NPM1 is an abundant nucleolar protein that has many protein interaction partners and diverse functions in the cell, including ribosome biogenesis, chromatin remodeling, and mRNA processing (Box et al., 2016; Di Matteo et al., 2016). We observed 11 NPM1 phosphopeptides in our data set that have also been identified by others (Grosstessner-Hain et al., 2011; Humphrey et al., 2013; Santamaria et al., 2011). Furthermore, phosphorylation at one of these sites, Ser225, was significantly reduced in *Gsk3* DKO. We confirmed a reduction in NPM1 phosphorylation in DKO compared to wild-type cell lysates using Phos-tag gels and immunoblotting (Figure 4-6a). The appearance of multiple bands in the phos-tag gel above the non-phosphorylated band, at 37kd, indicates there are several phosphorylated species of NPM1 in ESCs. We next performed *in vitro* kinase assays using DKO cell lysates to demonstrate a GSK-3 specific change. We detected a GSK-3 dependent and ATP dependent increase in a phosphorylated form of NPM1 (Figure 4-6b, indicated by red arrow, compare lanes 1-4). This phosphorylation event was sensitive to high urea concentrations, which likely denatures the kinase (Figure 4-6b, compare lanes 1,2,5,6). We also observed ATP-dependent phosphorylation of recombinant NPM1 in an *in vitro* kinase assay, confirming that NPM1 can be directly phosphorylated by GSK-3 (Figure 4-6c). Future experiments using site-specific mutagenesis may help elucidate a functional role for GSK-3 phosphorylation of NPM1.

GSK-3 regulates alternative splicing of multiple genes

Prior work showed that GSK-3 regulates splicing of CD45 in human T cells and data using a small molecule GSK-3 inhibitor suggested that the splicing of multiple mRNAs is similarly

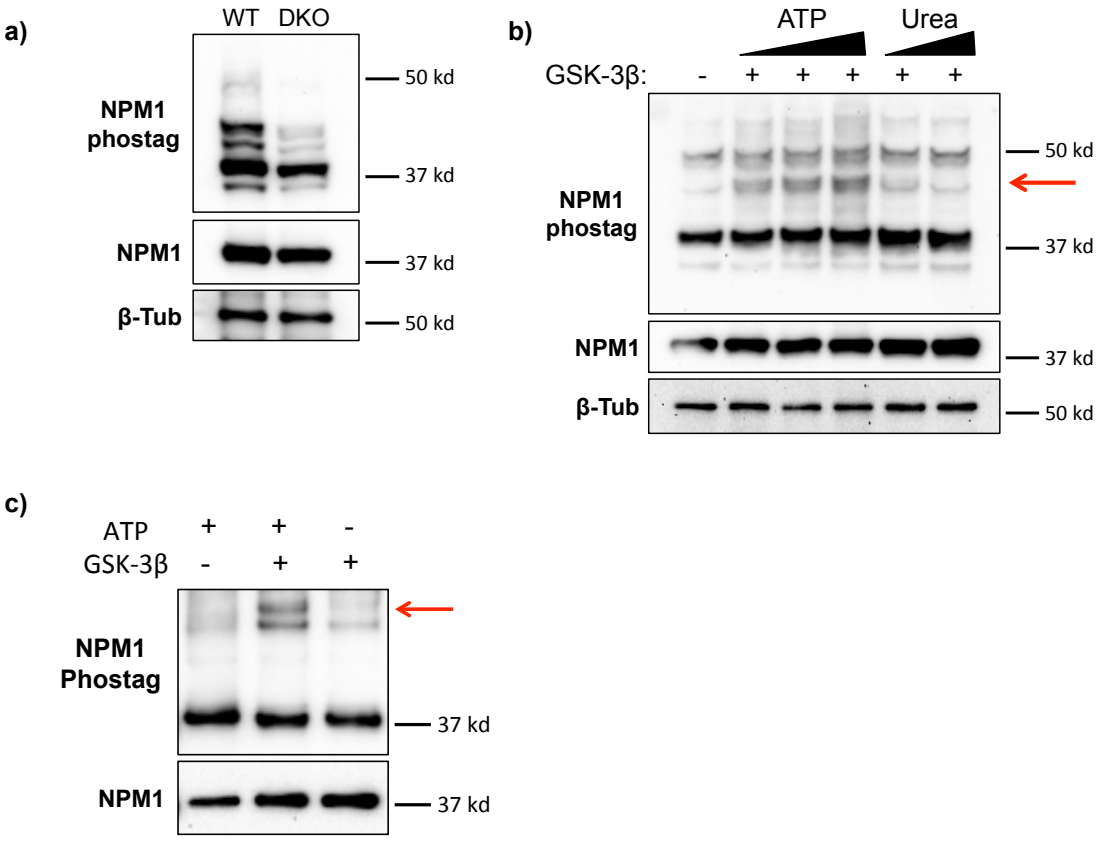


Figure 4-6: GSK-3 directly phoshorylates NPM1. a) Phos-tag gel to shows differential phosphorylation pattern for wild-type and *Gsk3* DKO cell lysates. b) In vitro kinase assay with Gsk3 DKO cell lysates shows ATP dependent increase in NPM1 phosphorylation. Arrow indicates band of interest. ATP concentrations = 200μM, 400μM, 800μM. Urea concentrations = 0.8M, 1.2M. c) In vitro kinase assay with recombinant NPM1 protein indicates a GSK-3 phosphorylates NPM1 and this requires ATP. In vitro reaction included 0.4μg recombinant NPM1, 500 units GSK-3 (Neb), 800μM ATP and was incubated for 1 hour at 30°C. 10% Acrylamide gel plus/minus 30μM phos-tag reagent (Wako) was used for all NPM1 gels.

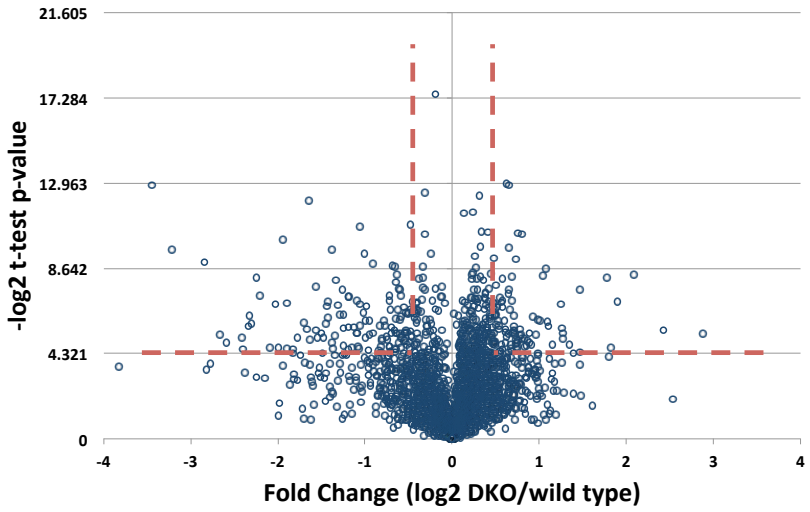
regulated in primary T cells (Heyd and Lynch, 2010; Martinez et al., 2015). However, a small molecule inhibitor could have off-target effects and may not inhibit the intended target completely. To address whether complete loss of GSK-3 modulates alternative splicing, we conducted deep sequencing of RNA from wild-type and DKO ESCs and then used MAJIQ software to identify and quantify changes in splicing between these two groups. We identified 258 genes that exhibit a significant change in spliced isoform expression (20% difference in relative isoform expression, > 95% confidence) between wild-type versus *Gsk3* DKO cells. These data confirm that GSK-3 regulates alternative splicing in mouse ES cells, as observed previously with pharmacological inhibition of GSK-3 in human T cells.

Proteome of Gsk3 DKO ESCs is distinct from wild-type ESCs

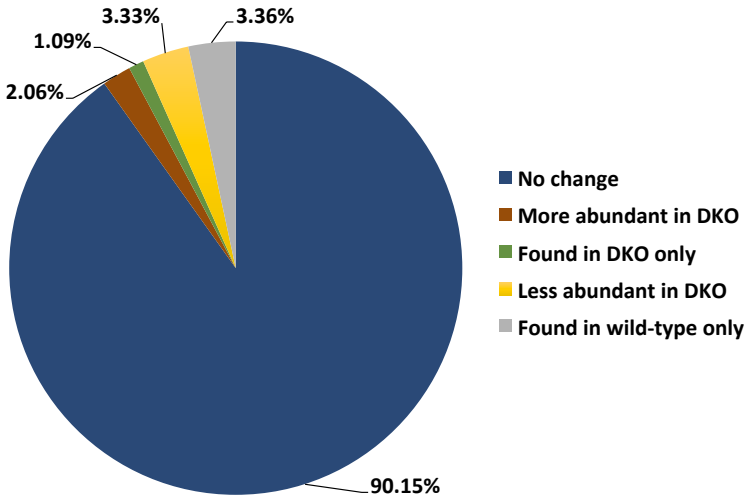
As GSK-3 regulates the stability of many of its target proteins, we also analyzed changes in total protein abundance between the wild-type and DKO ESCs. Our analysis identified 459 of 3299 total proteins that had significantly ($p \leq 0.05$) different abundance when comparing wild-type and DKO. Unexpectedly, we found the number of proteins with reduced abundance in DKO was similar to the number of proteins that increased in abundance (Figure 4-7a). Although destabilization of proteins by GSK-3 phosphorylation has been more extensively characterized, GSK-3 phosphorylation can also promote stabilization of some targets, including the nuclear hormone Rev-erbα and the NFkB1 subunit p105 (Acebron and Niehrs, 2016; Demarchi et al., 2011; Kim et al., 2009; Yin et al., 2006).

To select for the strongest candidates, we filtered for proteins with an absolute fold change of 1.5x or greater and identified 69 proteins (2.1%) with greater abundance in the DKO and 110 proteins (3.3%) with reduced abundance in DKO, compared to wild-type (Figure 4-7a). Furthermore, peptides representing 36 proteins were detected only in the DKO set in two or more biological replicates, and similarly 111 proteins were identified only in wild-type cells (Figure 4-7b). These data will be compared to the RNA sequencing results to identify the fraction of

a)


21.605
17.284
12.963
8.642
4.321
0
-log2 t-test p-value
-4
-3
-2
-1
0
1
2
3
4
Fold Change (log2 DKO/wild type)

b)


3.33%
3.36%
1.09%
2.06%
90.15%
No change
More abundant in DKO
Found in DKO only
Less abundant in DKO
Found in wild-type only

c)

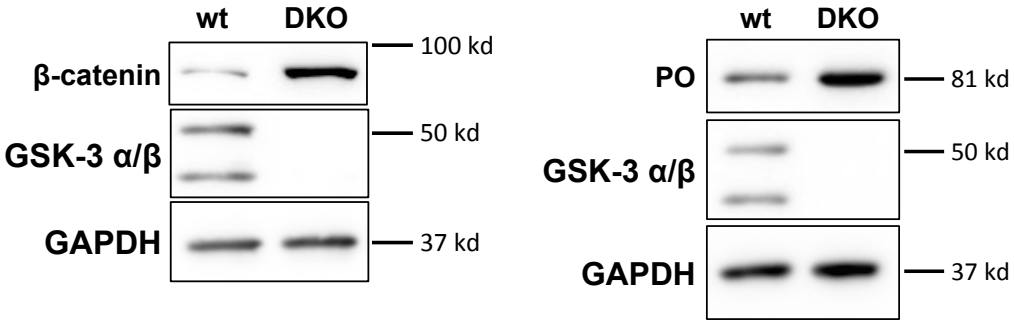

wt
DKO
β-catenin
100 kd
GSK-3 α/β
50 kd
GAPDH
37 kd
wt
DKO
PO
81 kd
GSK-3 α/β
50 kd
GAPDH
37 kd

Figure 4-7: Protein abundance changes in DKO compared to wild-type. a) Volcano Plot indicates similar number of stabilized and destablized proteins in the DKO compared to wild-type. Each dot is represented by the protein abundance in DKO compared to WT. b) Percentage of proteins that are destabilized by GSK-3 (increased in DKO) or stabilized (reduced in DKO). Threshold of destabilization or stabilization was positive or negative 1.5 fold change, respectively, $p<=0.05$. c) β-catenin and PO total protein levels are increased in DKO compared to wild-type.

proteins with transcriptional changes. Proteins with changes in abundance that have no change in RNA transcription may be influenced by GSK-3 at the level of protein stability.

Of the proteins stabilized by *Gsk3* deletion, we detected β-catenin, a well-known substrate of GSK-3 (Figure 4-7c). We conducted further validation by western blot analysis of Prolyl Oligopeptidase (PO), which displayed increased abundance in DKO compared to wild-type (Figure 4-7c).

4.3 Discussion

Our knowledge of GSK-3 phosphorylation substrates is based on a diverse range of assays performed in different experimental models leaving an incomplete picture of the overall phosphoproteome. We have used a quantitative, proteomic method to characterize the GSK-3 phosphoproteome in a single cell type. To identify global changes in phosphorylation, we compared the phosphorylation state of proteins in ESCs lacking *Gsk3a* and *Gsk3b* genes (DKO) to wild-type ESCs. This approach provides an unbiased picture of the spectrum of GSK-3 substrates. We found 65 phosphoproteins that have significantly reduced phosphorylation in DKO compared to wild-type cells that we selected as high-confidence candidate substrates of GSK-3. Analysis of overall proteomic changes between wild-type and DKO revealed a total of 69 proteins with significantly increased abundance and 110 proteins with significantly decreased in abundance in the DKO condition.

The candidate substrates of GSK-3 that were identified span a wide array of functions and are organized into three main functional groups: splicing factors, transcriptional regulators and cell cycle. Although GSK-3's modulation of transcription and cell cycle checkpoints have long been appreciated, the third cluster represents a novel mechanism by which GSK-3 can control alternative splicing events by phosphorylation of alternative splicing factors. Additional validation is required to demonstrate that these proteins are physiological substrates and to determine the functional consequence of GSK-3 phosphorylation.

Even among known functions for GSK-3, new substrates were identified that provide insight into the breadth of targets by which GSK-3 can exert regulatory control.

The SWI/SNF chromatin remodeler ATRX, found in the cluster of transcriptional regulators, represents an interesting candidate for further studies. Although ATRX has been identified in other phosphoproteomic screens, no kinase has been implicated in direct phosphorylation of ATRX (Humphrey et al., 2013). Mutations in the *Atrx* gene are the underlying cause of a rare genetic disorder that causes mental retardation and facial abnormalities (Clynes et al., 2013). Validation of ATRX as a direct target of GSK-3 and functional consequences may provide insight into the pathogenesis of this rare disease.

A novel candidate from the cell cycle cluster, Ajuba, is another interesting target for further validation. The LIM-domain containing protein Ajuba is part of a family of cytosolic adapter proteins involved in several cell processes, including mitosis/cytokinesis (Chen et al., 2016). Ajuba is a negative regulator of the Hippo pathway and has been shown to directly associate with the protein kinases, LATS1/2 and SAV1 to inhibit phosphorylation of YAP (Rauskolb et al., 2014; Thakur et al., 2010). YAP is an oncogene involved in growth factor-independent growth, epithelial-to-mesenchymal transition and suppression of apoptosis (Overholtzer et al., 2006). The Hippo pathway, a well-established regulator of organ size, functions through a kinase cascade to phosphorylate and inhibit the transcriptional co-activators, YAP/TAZ. GSK-3 can directly phosphorylate TAZ to promote degradation (Huang et al., 2012b). Thus, further validation of Ajuba as a direct substrate would identify another level of regulation of the Hippo pathway by GSK-3.

We also detected a number of phosphoproteins that had increased phosphorylation in the DKO condition compared to wild-type, which are likely a result of indirect regulation of phosphorylation. As these cells have a long-term deletion of GSK-3, it is likely that a portion of

these phosphoproteomic changes may be due to compensatory mechanisms rather than GSK-3 signaling.

GSK-3 substrates often have a prior priming phosphorylation four residues C-terminal to the GSK-3 phosphorylation site. Our unbiased phosphoproteome analysis revealed that 26 out of 68 of our high-confidence candidate substrates contain the consensus sequence and have the potential to be phosphorylated at the priming site. It is now evident that the GSK-3 consensus sequence is not a requirement. Moreover, of all the phosphopeptides identified in our analysis, 18% had the consensus sequence and only 4.5% of these satisfied the criteria to be selected as candidate substrates of GSK-3. This provides evidence that there are other attributes, such as phosphorylation by the priming kinase, that direct phosphorylation by GSK-3. As we cannot determine the phosphorylation state of the priming residue, we cannot comment on how many substrates that were primed were indeed phosphorylated by GSK-3. Our consensus sequence analysis suggests that prior studies are likely over-estimating the number of GSK-3 substrates and that the consensus sequence alone is not sufficient to determine if a substrate can be phosphorylated by GSK-3.

Another finding from our analysis was that *Gsk3* deletion increases in abundance of 68 proteins. Previous analyses that tested proteins stabilized by GSK-3, including by Wnt/STOP signaling, have confirmed stabilization of around 35 proteins cumulatively, across all cell types (Acebron and Niehrs, 2016; Kim et al., 2009). Surprisingly, we also found 110 proteins that were reduced in abundance by *Gsk3* deletion. GSK-3 is well characterized to phosphorylate substrates, like β-catenin and Smad1, that leads to their ubiquitin-mediated proteosomal degradation, which has given rise to the general notion that GSK-3 inhibition leads to stabilization of many proteins (Acebron and Niehrs, 2016; Fuentealba et al., 2007; Taelman et al., 2010; Xu et al., 2009). In addition, most of these studies have only looked at changes in ubiquitination levels in response to GSK-3 inhibition, which does not provide any information about changes in protein

abundance (Acebron et al., 2014; Huang et al., 2015; Kim et al., 2015)). Our study is the first to provide an unbiased view of overall changes in protein abundance in response to *Gsk3* deletion. In support of our findings, there are published examples of GSK-3 substrates that are stabilized by GSK-3 phosphorylation, such the nuclear receptor Rev-erbα and the NFkB1 subunit p105 (Demarchi et al., 2011; Yin et al., 2006). Direct validation of targets identified and functional consequences will be the subject of future studies.

One of the proteins we found to be stabilized in the knockout was Prolyl Oligopeptidase, a serine peptidase that has been shown to be involved in inositol monophosphatase (IP) signaling (King et al., 2010; Williams et al., 1999). Lithium, a GSK-3 inhibitor and first line therapy for bipolar disorder, can directly inhibit inositol monophosphatase (IMP) and inositol polyphosphate 1-phosphatase (1PP) to cause inositol depletion, which has been implicated in its therapeutic response (Harwood, 2011; Teo et al., 2009). Loss of PO or PO inhibition increase IP3 levels, therefore counteracting the decrease caused by lithium treatment (Williams et al., 1999). Our finding that *Gsk3* deletion increases PO protein abundance links GSK-3 inhibition by lithium to the inositol depletion hypothesis and further supports the claim that lithium's therapeutic response is mediated through GSK-3 inhibition. Future studies are required to test whether GSK-3 regulates PO protein stability or gene expression and to investigate whether the GSK-3 mediated increase in PO abundance is sufficient to modulate sensitivity to lithium.

The most compelling discovery in our phosphoproteomic data set was the enrichment of proteins involved in RNA splicing and the spliceosome. We discovered that GSK-3 directly phosphorylates the splicing factor, RBM8A, and potentially TRA2B, RBM39 and SRSF9. There is only one example in the literature that describes a direct role of GSK-3 in regulation of splicing (Heyd and Lynch, 2010). In resting T cells, GSK-3 phosphorylates the splicing factor PSF and promotes PSF interaction with its binding partner, TRAP150. When T cells are activated, PSF phosphorylation by GSK-3 and binding to TRAP150 are inhibited, thus allowing PSF to enter the

nucleus and initiate CD45 mRNA alternative splicing (Heyd and Lynch, 2010). RBM8A is one part of the multicomponent exon junction complex (EJC) and its phosphorylation has been described previously to modulate interaction with proteins in this complex (Hsu et al., 2005). Therefore, we hypothesize that GSK-3 phosphorylation may be influencing RBM8A, in a similar manner as PSF, by altering its interaction with proteins that make up the EJC. Although further validation is needed to determine the functional effect of phosphorylation, we provide strong evidence to implicate RBM8A as a novel substrate of GSK-3.

The finding that GSK-3 phosphorylates splicing factors led us to ask whether GSK-3 regulates alternative splicing events. Pharmacological inhibition of GSK-3 in primary T cells mimicked 34% of the splicing events that occur in stimulated T cells (Martinez et al., 2015). Preliminary RNA sequencing findings suggest ESCs with *Gsk3a/b* deletion exhibit alternative splicing events in 250 genes compared to the wild-type condition. These findings provide additional evidence that GSK-3 acts as a regulator of alternative splicing. It remains to be tested if GSK-3 can influence these splicing events by direct phosphorylation of RBM8A or the other splicing factors identified in our screen.

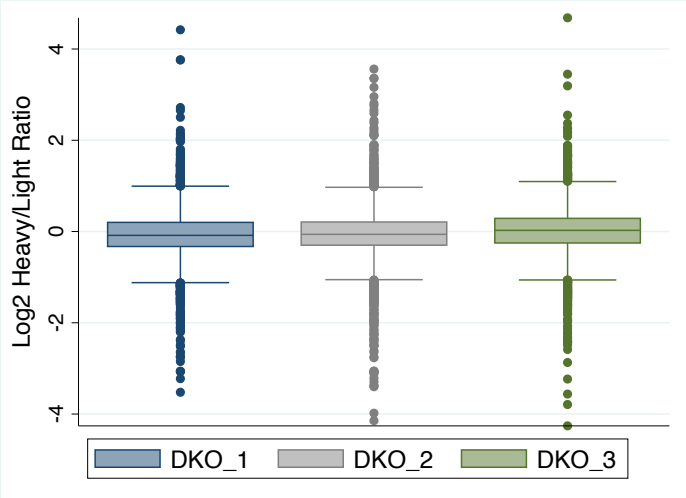
GSK-3 regulation of splicing could be important in several contexts. Alternative splicing in ESCs has been implicated as an essential part of the signal that directs a cell to self-renew or differentiate (Ye and Blelloch, 2014). GSK-3 inhibition promotes self-renewal in ESCs, which has mostly been attributed to activation of Wnt signaling (Wray and Hartmann, 2012). Thus, GSK-3 regulation of splicing could be a critical, unaddressed function for determining cell fate in ESCs. Additionally, there are reports that describe alternative splicing of TCF/LEF transcription factors, which are well-known mediators of the transcriptional response to Wnt signaling (Cadigan and Waterman, 2012; Van de Wetering et al., 1996). Alternative splicing of TCF/LEFs produces modified functional domains, which can alter DNA binding specificity or protein interactions. Consequently, this would affect the transcriptional response to Wnt signaling and adds another

level of regulation of the pathway. The specific signals that regulate these alternative-splicing events are not fully understood, but our studies point to the involvement of GSK-3.

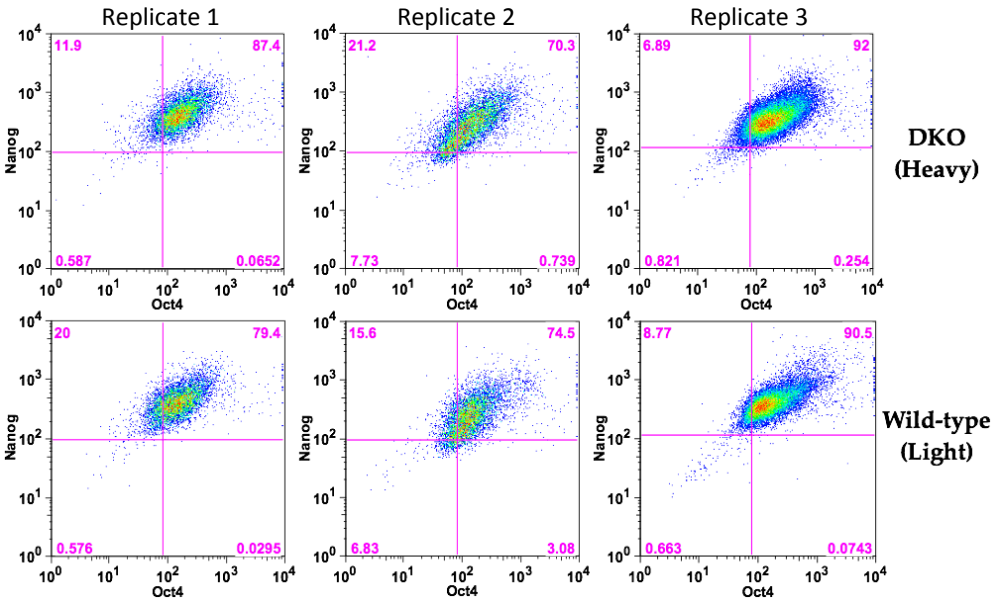
Aberrant alternative splicing is a wide-spread phenotype across myeloid malignancies (Lee and Abdel-Wahab, 2016). Mutations in splicing factors are common in patients with myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) (Cancer Genome Atlas Research Network, 2013; Lee and Abdel-Wahab, 2016; Yoshida et al., 2011). As a regulator of hematopoietic stem cell (HSC) homeostasis, it has been suggested that GSK-3 may also be involved in leukemic pathogenesis (Huang et al., 2009b; Takahashi-Yanaga, 2013; Takebe et al., 2010; Wang et al., 2008). Conditional knockout of *Gsk3* in murine HSCs leads to a myelodysplastic-like state in mice (Guezguez et al., 2016). Therefore, GSK-3 regulation of splicing events could provide a mechanistic link between GSK-3 and AML pathogenesis. Our finding that the nucleolar protein NPM1 is a direct substrate of GSK-3 provides additional support that GSK-3 is involved in AML pathogenesis. Heterozygous mutations in the terminal exon of the NPM1 are found in one third of AMLs (Cancer Genome Atlas Research Network, 2013; Di Matteo et al., 2016; Falini et al., 2011). The mutation arises from a duplication or insertion that causes an altered reading frame but how this leads to transformation is not known (Di Matteo et al., 2016). Our data links GSK-3 directly to the regulation of NPM1, but also implicates GSK-3 regulation of alternate splicing as a potential mechanism by which NPM1 mutations arise.

In conclusion, we have conducted a global analysis to provide an unbiased overview of the GSK-3 phosphoproteome. Our findings suggest a role for GSK-3 in alternative splicing. These data provide evidence to link leukemic pathogenesis caused by GSK-3 and common mutations found in leukemia. Further studies are needed to investigate the mechanism by which GSK-3 phosphorylation of splicing factors modulates splicing events and determine if this plays a role in leukemogenesis.

4.4 Supplementary Material

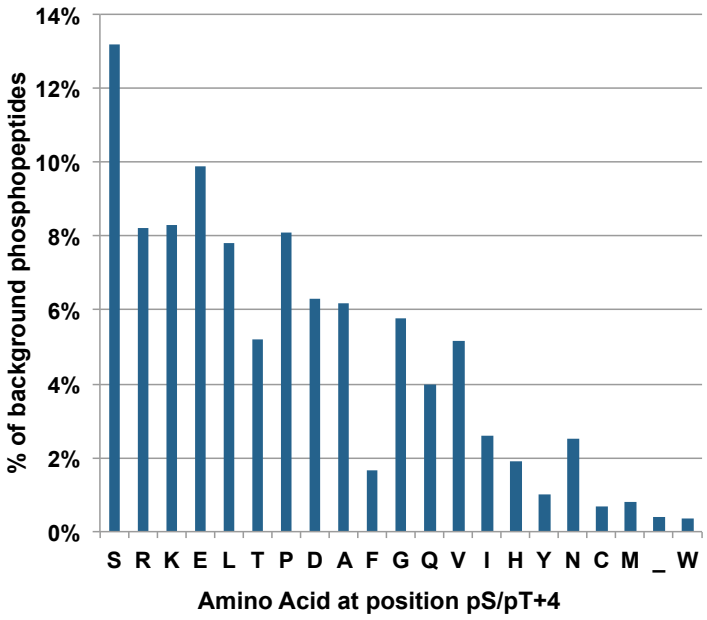


Supplementary Figure 4-1: Pre-normalization ratio of all heavy and light peptides. Heavy:light is close to 1, which indicates complete labeling of proteome.

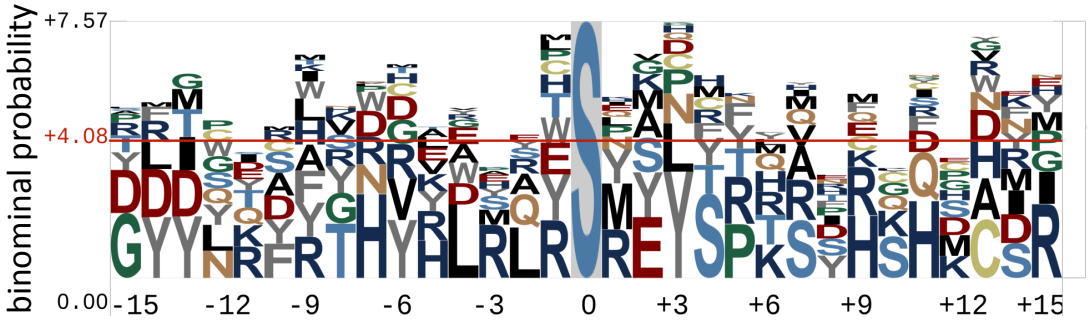


Supplementary Figure 4-2: Staining with pluripotency markers indicates high homogeneity between biological replicates. Intracellular flow cytometry for ES markers, Oct4 and Nanog, to assess homogeneity between wild-type and DKO cell populations.

a)



b)



Supplementary Figure 4-3: Further consensus sequence analysis. a) Background peptides are not enriched for consensus sequence. Background consists of all peptides that do not exhibit significantly reduced phosphorylation in response to GSK-3 deletion. b) Kinase motif enrichment analysis using Plogo generator (https://plogo.uconn.edu). Most common peptides are the +4 position are S and T.

Supplementary Table 4-1: List of candidate substrates of GSK-3 identified in our phosphoproteomic screen. Yellow row indicates phosphorylation at a GSK-3 consensus site.

Protein names	Gene names	Phos-Site	Average all	t-test
Septin-9	Sept9	85	-0.94527	0.021192
LIM domain-containing protein ajuba	Ajuba	129	-1.66111	0.029149
Alkylated DNA repair protein alkB homolog 1	Alkbh1	378	-0.66465	0.049628
Transcriptional regulator ATRX	Atrx	1088	-0.80667	0.000412
Barrier-to-autointegration factor	Banf1	2	-1.02824	0.020485
Barrier-to-autointegration factor	Banf1	4	-0.99766	0.03131
Bromodomain adjacent to zinc finger domain protein 2A	Baz2a	695	-0.63829	0.00351
Bcl-2-associated transcription factor 1	Bclaf1	748	-0.60657	0.020859
Cell division cycle protein 20 homolog	Cdc20	106	-0.61744	0.019501
Cyclin-dependent kinase 1	Cdk1	14	-1.36034	0.015935
Cyclin-dependent kinase 17	Cdk17	137	-0.58597	0.041583
Cyclin-dependent kinase 9	Cdk9	186	-1.09852	0.034685
CUGBP Elav-like family member 1	Celf1	28	-1.05776	0.043114
Chromosome alignment-maintaining phosphoprotein 1	Champ1	405	-1.35714	0.038454
Death-associated protein 1	Dap	51	-1.26239	0.034072
Drebrin	Dbn1	142	-1.23783	0.029291
ATP-dependent RNA helicase DDX24	Ddx24	80	-0.64975	0.017141
Zinc finger protein ubi-d4	Dpf2	142	-0.60467	0.029813
Zinc finger protein ubi-d4	Dpf2	176	-0.98766	0.040282
Eukaryotic translation initiation factor 3 subunit B	Eif3b	75	-2.8659	0.01838
Cytosolic endo-beta-N-acetylglucosaminidase	Engase	16	-0.7836	0.025554
Pre-mRNA 3'-end-processing factor FIP1	Fip1l1	280	-1.1743	0.007888
UAP56-interacting factor	Fyttd1	23	-0.72178	0.0452
Golgin subfamily A member 4	Golga4	93	-0.8199	0.033139
Host cell factor 1	Hcfc1	2029	-0.60065	0.00569
High mobility group protein HMG-I/HMG-Y	Hmga1	102	-0.88865	0.040156
Transcription factor AP-1	Jun	73	-0.61912	0.041908
Beta-lactamase-like protein 2	Lactb2	279	-0.74984	0.009452
Microtubule-associated protein 1A	Map1a	1580	-1.13349	0.040758
Microtubule-associated protein 1A	Map1a	526	-0.75336	0.026593
Microtubule-associated protein 1A	Map1a	1789	-0.91477	0.008007
Microtubule-associated protein 1A	Map1a	527	-0.75336	0.026593
Microtubule-associated protein 4	Map4	914	-0.65291	0.030172
7SK snRNA methylphosphate capping enzyme	Mepce	188	-0.99668	0.047541
Matrix-remodeling-associated protein 7	Mxra7	79	-1.84082	0.014214
Nucleophosmin	Npm1	225	-0.72118	0.014094
Palladin	Palld	1129	-0.69284	5.42E-05
Astrocytic phosphoprotein PEA-15	Pea15	116	-1.32164	0.035662
Phosphoglycerate mutase 1	Pgam1	14	-0.76149	0.017139
PHD finger protein 6	Phf6	155	-2.27063	0.002128

Protein names	Gene names	Phos-Site	Average all	t-test
Liprin-beta-1	Ppfibp1	753	-1.339634	0.044058
Serine/threonine-protein phosphatase 4 regulatory subunit 2	Ppp4r2	226	-1.664075	0.014451
Prothymosin alpha	Ptma	107	-0.609891	0.033828
RNA-binding protein 39	Rbm39	125	-0.583935	0.040376
RNA-binding protein 8A	Rbm8a	166	-0.935241	0.018583
RNA-binding protein 8A	Rbm8a	168	-0.935241	0.018583
REST corepressor 2	Rcor2	202	-0.626135	0.00041
Telomere-associated protein RIF1	Rif1	1683	-0.684252	0.030218
60S ribosomal protein L18	Rpl18	130	-1.32524	0.034024
40S ribosomal protein S28	Rps28	23	-1.833228	0.00899
Ribosomal RNA processing protein 1 homolog B	Rrp1b	405	-0.745959	0.028383
Ribosomal L1 domain-containing protein 1	Rsl1d1	360	-0.77868	0.000419
Sodium-coupled neutral amino acid transporter 1	Slc38a1	52	-0.779791	0.007526
SWI/SNF complex subunit SMARCC2	Smarcc2	302	-0.908945	0.035088
SWI/SNF complex subunit SMARCC2	Smarcc2	304	-0.908945	0.035088
Serine/arginine repetitive matrix protein 1	Srrm1	450	-0.691792	0.00358
Serine/arginine repetitive matrix protein 2	Srrm2	1864	-0.974095	0.041468
Serine/arginine repetitive matrix protein 2	Srrm2	1535	-0.602159	0.025127
Serine/arginine-rich splicing factor 9	Srsf9	212	-0.627465	0.049767
Serine/arginine-rich splicing factor 9	Srsf9	205	-0.79851	0.02929
Serine/arginine-rich splicing factor 9	Srsf9	190	-0.908317	0.02975
Supervillin	Svil	960	-0.975017	0.037178
Treacle protein	Tcof1	1128	-0.630423	0.011945
Tight junction protein ZO-2	Tjp2	265	-2.822597	0.002248
Tight junction protein ZO-2	Tjp2	267	-2.822597	0.002248
Tight junction protein ZO-2	Tjp2	263	-2.822597	0.002248
Transformer-2 protein homolog beta	Tra2b	85	-0.756033	0.024763
Transformer-2 protein homolog beta	Tra2b	201	-0.800516	0.017183
Transformer-2 protein homolog beta	Tra2b	87	-0.811499	0.016927
Transformer-2 protein homolog beta	Tra2b	95	-0.642136	0.04846
Transformer-2 protein homolog beta	Tra2b	266	-0.656968	0.024048
Transformer-2 protein homolog beta	Tra2b	39	-0.665452	0.022632
Transformer-2 protein homolog beta	Tra2b	83	-0.756033	0.024763
Transformer-2 protein homolog beta	Tra2b	99	-0.651262	0.021001
Transformer-2 protein homolog beta	Tra2b	29	-0.699293	0.035037
Transformer-2 protein homolog beta	Tra2b	264	-0.680797	0.024555
Transformer-2 protein homolog beta	Tra2b	270	-0.744461	0.026006
Transcription intermediary factor 1-beta	Trim28	752	-0.775232	0.045878
Tumor protein p53-inducible protein 11	Trp53i11	14	-1.415493	0.015656
Ubiquitin-associated protein 2-like	Ubap2l	625	-0.620222	0.002221
WW domain-binding protein 11	Wbp11	237	-0.591035	0.01703
WD repeat-containing protein 46	Wdr46	41	-0.621319	0.035833
5'-3' exoribonuclease 2	Xrn2	448	-1.069088	0.049817
Zinc finger protein 423	Znf423	1168	-1.276373	0.011944
Zinc finger protein 638	Znf638	606	-0.93211	0.021051
Zinc finger protein 638	Znf638	881	-0.913098	0.034597
Uncharacterized protein C17orf85 homolog		382	-0.617977	0.000425
Uncharacterized protein C18orf25 homolog		69	-0.673958	0.042789

CHAPTER 5

General Discussion

Overview

The studies outlined in this work have provided insight into the broad range of targets and functions potentially regulated by glycogen synthase kinase-3 (GSK-3). GSK-3 was first identified for its role in metabolism, specifically for inhibition of its eponymous substrate, glycogen synthase (Embi et al., 1980; Rylatt et al., 1980). Insulin activates receptor tyrosine kinase signaling and the PI3K/Akt pathway, which leads to inhibition of GSK-3 by Akt-mediated phosphorylation (Cross et al., 1995). GSK-3 is also well characterized in the Wnt/β-catenin signaling pathway where GSK-3, bound to axin in a cytoplasmic complex, can phosphorylate and target β-catenin for ubiquitin-mediated proteosomal degradation. Wnt activation leads to inhibition of GSK-3 and activation of Wnt target genes (MacDonald et al., 2009). It is now recognized that GSK-3 has a much broader role and can influence many signaling pathways and cellular processes using its diverse network of effectors. Thus, the bulk of the thesis work focused on characterizing the GSK-3 phosphoproteome. To explore this network of downstream effectors, we used *Gsk3a/b* double-knockout (DKO) murine embryonic stem cells (ESCs).

GSK-3's role as a global cellular regulator has to this date been attributed to its direct inactivation of protein function, modulation of transcription factors that control the production of new proteins, or targeting of proteins for degradation. We observed that many of these known functions of GSK-3 can be recapitulated by looking at the spectrum of GSK-3 substrates in one cell type. Furthermore we discovered that by modulating a network of splicing factors, GSK-3 appears to exert an additional level of cellular control by regulating the splice variants transcribed from mRNA. Together, our data underlines the importance of GSK-3 as a critical node that directs many effectors in response to multiple upstream signals.

Findings from our analysis also provided several insights into the general characteristics of GSK-3. First, we identified many substrates of GSK-3 that do not have the consensus sequence, including two that were validated by direct kinase assays – RBM8A and NPM1. Second, our results demonstrate that the proteins with the consensus sequence require another level of specification, such as a phosphorylation by the priming kinase that determines phosphorylation by GSK-3. Our results provide compelling evidence that the consensus sequence alone may not be a reliable predictor of phosphorylation by GSK-3. An interesting follow-up study would be to assess how many substrates with a S/TxxxS/T motif that are phosphorylated at the C-terminal S/T residue can be phosphorylated by GSK-3. Our data is limited by the fact that we cannot determine the phosphorylation status of the priming residue.

Finally, our analysis also revealed that *Gsk3* deletion leads to a similar number of proteins with increased and decreased abundance. β-catenin has set the precedent for the discovery of many other substrates of GSK-3 that are degraded in response to phosphorylation, which has led to the general concept that GSK-3 inhibition will lead to protein stabilization (Acebron and Niehrs, 2016; Kim et al., 2009; Taelman et al., 2010; Xu et al., 2009). Our study is the first to provide an unbiased view of proteomic changes in response to *Gsk3* deletion. In support of our findings, there are examples of GSK-3 substrates that are stabilized by GSK-3 phosphorylation, such as the nuclear receptor Rev-erbα (Yin et al., 2006). Direct validation of targets identified and functional consequences will be the subject of future studies.

In an early study, outlined in chapter 3, we tested the role of GSK-3 and mechanistic target of rapamycin (mTOR) signaling pathways in ESCs. We found that a combination of GSK-3 and mTOR inhibitors was not sufficient to maintain ESCs in a pluripotent state. Our hypothesis remains that GSK-3 inhibition results in activation of multiple pathways that may have opposing affects on pluripotency. From our phosphoproteomic analysis, it is clear that GSK-3 has many

effectors in ESCs that still remain unaddressed. Implications of GSK-3 as a regulator of splicing in ESCs will be discussed below.

Overall, our analysis revealed many candidate substrates of GSK-3 that could be the subject of future studies. An important consideration is that we cannot distinguish between direct and indirect substrates in our analysis, as we are assessing cells with a long-term deletion of *Gsk3*. The first next step would be to repeat this analysis using inhibitors of GSK-3 to account for long-term effects of *Gsk3* deletion. Although pharmacological inhibitors have off target effects, a comparison of both analyses could be used to validate some of the findings and select more direct substrates of GSK-3. The most intriguing finding from our work was discovery of GSK-3 as a regulator of alternative splicing, which will be the subject of the rest of the discussion.

GSK-3 as a regulator of alternative splicing

The existence of alternative splicing has been known for decades, and there are estimates that over half the genome can be alternatively spliced (Berget et al., 1977; Chow et al., 1977). Alternative splicing, the process of variable inclusion of exons, has several effects including broadening the complexity of the genome and proteome (Maniatis and Tasic, 2002; Modrek and Lee, 2002). For example, alternative splicing of TCFs can alter their DNA binding domain and as a result, affect its DNA binding (Cadigan and Waterman, 2012). Alternative splicing events often alter the stability of the transcript or resulting protein (Lareau et al., 2007). Splicing events can produce mRNA splice forms that have a premature termination codon (PTC), which targets this product for non-sense mediated mRNA decay (NMD) (Lareau et al., 2007). NMD was first thought of as a mechanism to clean up splicing errors, but recent studies have pointed to the cooperation of NMD and alternative splicing to regulate overall mRNA transcript abundance (Lareau et al., 2007). The splicing factor SRSF2 autoregulates its own expression by splicing a PTC into its precursor transcripts, resulting in mRNA degradation by NMD (Sureau et al., 2001). Despite the observations describe that exemplify the importance of alternative splicing,

the cellular signals that control these events are not well understood. Our studies point to GSK-3 regulating alternative splicing events and call for future experimentation to elucidate the mechanism of how GSK-3 influences this process.

Alternative splicing events are catalyzed by the assembly of the spliceosome complex. The spliceosome complex is a multiprotein complex that contains 5 small nuclear ribonucleoproteins (snRNPs) and a number of associated proteins (Maniatis and Tasic, 2002). Splicing is catalyzed by a series of sequential steps that bring the 5' and 3' ends of splice sites together to from an intron lariat. This is followed by ligation of the exons and release of the intron lariat (Maniatis and Tasic, 2002). The serine/arginine (SR) family of splicing factors is involved in various steps of this process, including splice-site selection, and can activate or repress splicing events (Bradley et al., 2014). We identified three SR splicing factors in our screen (TRA2B, SRSF9, and RBM39) that were phosphorylated by GSK-3 on or next to the RS domain. This C-terminal domain is enriched in arginine and serine (RS) dipeptides and is required for specificity of splicing events and for protein-protein interactions (Bradley et al., 2014; Long and Caceres, 2009). Phosphorylation of SR proteins can modulate cellular localization of SR proteins as well as influence spliceosomal complex assembly (Graveley, 2000; Zhou and Fu, 2013). As GSK-3 phosphorylation is already shown to influence nuclear localization of transcription factors, GSK-3 phosphorylation could have a similar effect on splicing proteins. Alternatively, phosphorylation could also modulate protein-protein interactions. GSK-3 has been proposed to phosphorylate of PTB-associated splicing factor, PSF, and promoting binding partner TRAP150. This interaction prevents binding to CD45 pre-RNA and inhibits exon skipping (Heyd and Lynch, 2010). A follow-up study has also demonstrated that GSK-3 can influence other splicing events in T-cells as well (Martinez et al., 2015).

Our studies have also implicated GSK-3 in regulation of splicing by direct phosphorylation of RBM8A. RBM8A is one part the exon junction complex (EJC), which is assembled during

splicing reaction (Le Hir et al., 2015). The EJC is involved in regulation of pre-mRNA splicing events, mRNA maturation following splicing and NMD. The EJC is formed at the splice site and stays bound to the splice site as the mRNA progresses through further maturation steps, including during translation initiation (Le Hir et al., 2015). Regulation of translation initiation by GSK-3 phosphorylation of RBM8A could be another avenue of exploration, as GSK-3 is already known to inhibit translation through mTOR (Inoki et al., 2006). Interestingly, RBM8A also has a RS dipeptide, which was identified as the site of GSK-3 phosphorylation. This particular RS peptide is located in a region involved in protein-protein interactions, raising the possibility that GSK-3 phosphorylation inhibits EJC formation (Hsu et al., 2005).

Another validated target, NPM1, has also been linked to negative regulation of alternative splicing. Phosphorylation of NPM1 by Cdk1 causes it to localize to nuclear speckles and inhibit mRNA splicing (Tarapore et al., 2005).

Overall, there are several possible mechanisms by which GSK-3 phosphorylation affects splicing factor function that could influence splicing events. Our RNA-sequencing results indicate that GSK-3 most likely inhibits splicing events and several interesting contexts for GSK-3 regulation of splicing will be discussed below. Further analysis, using site-directed mutagenesis, would be required to test if the effect of splicing is mediated through GSK-3 phosphorylation of these substrates.

Alternative splicing and Wnt signaling

The finding that GSK-3 could potentially regulate alternative splicing has brought to our attention that the transcriptional effectors of Wnt signaling, TCF/LEFs, can be alternatively splice and lead to differential DNA binding capacity (Cadigan and Waterman, 2012). One study looking at TCF4 splice in neonatal tissues, ESCs and neural progenitors from mice identified at least 13 different variants (Weise et al., 2010). These splice variants have differential DNA binding capacities, therefore diversifying target gene activation and functional outcomes (Weise et al.,

2010). Signal dependent alternative splicing of LEF1 is also important during T cell activation, where GSK-3 has been propose to influence many splicing events (Mallory et al., 2015; Martinez et al., 2015). Therefore, it is plausible that GSK-3 regulation of splicing factors results in alternative splicing of TCF/LEFs. These alternative splicing events could alter target genes, and add another level of complexity to Wnt signaling regulation by GSK-3.

Another intriguing idea is that Wnt signaling acts as the upstream signal to control alternative splicing through GSK-3. This would be relevant not only for alternative splicing of TCF/LEFs, but also impact the diverse array of developmental processes and diseases Wnt signaling is known to influence (Anastas and Moon, 2013; Logan and Nusse, 2004). One study has reported that Wnt activation can indirectly alter splicing events in colorectal cancer cells by increasing expression of SFSR3 gene that encodes the splicing factor SRp20 (Gonçalves et al., 2008). Activating mutations in the Wnt signaling pathway occur in many cancers (Anastas and Moon, 2013). Specifically, truncating mutations in APC, which are highly linked to colorectal cancer, could be a result of alternative splicing (Sumithra et al., 2016). Taken together, these suggest a role Wnt signaling in regulation of splicing, likely mediated through GSK-3, is highly conceivable.

Interestingly, there has also been a report looking at global changes in alternative splicing events in response to insulin and Wnt signaling pathways in *Drosophila* (Hartmann et al., 2009). This study finds that 40% of the genes can be alternatively spliced in response to activation of these pathways (Hartmann et al., 2009). As both insulin and Wnt pathways can inhibit GSK-3, these data support our finding that GSK-3 is a regulator of alternative splicing. It also highlights the likely complexity of upstream mechanisms that act on GSK-3 to regulate downstream splicing events.

Alternative splicing in ESCs

One of the earlier hypotheses discussed in this thesis was the possibility that GSK-3 regulated pathways, in addition to Wnt signaling, may play a role in ESC pluripotency. Our finding that GSK-3 is a potential regulator splicing provides an intriguing avenue for further exploration as an additional role for GSK-3 in ESCs. As the upstream signals that influence GSK-3 regulation of splicing are not yet known, this could ultimately be a Wnt-dependent effect.

Alternative splicing has been shown to be a critical determinant of pluripotent state in ESCs (Graveley, 2011). Genome-wide studies demonstrated that alternative splicing signatures in pluripotent ESCs (both human and mouse) are distinct from more differentiated cell types (Cloonan et al., 2008; Salomonis et al., 2010; Wu et al., 2010; Yeo et al., 2007). During reprogramming, somatic cells revert their alternative splicing profile to resemble pluripotent cells further supporting a role for splicing in influencing pluripotency (Ohta et al., 2013). One study has proposed that an alternative splicing event in the forkhead family transcription factor, FOXP1, can act as a switch in regulation of ESC pluripotency (Gabut et al., 2011). They identified ES specific form of FOXP1, FOXP1-ES, which has a different DNA binding specificity than FOXP1 and could increase expression of pluripotency genes (e.g. Oct4 and Nanog) (Gabut et al., 2011). Another study in ESCs also detected two alternatively spliced forms for Tcf3 that had distinct transcriptional targets (Salomonis et al., 2010). Although these alternative splicing events have been described, the upstream signals directing these splicing events have not been studied. A role for GSK-3 in the regulation of pluripotency by modulation of alternative splicing events would be therefore a novel and compelling finding.

Implications in Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is characterized by defective differentiation of white blood cells lead to accumulation of myeloid blasts. Myelodysplastic syndrome (MDS) is a deregulated production of immature blood cells that often develops into AML. Mutations in spliceosomal genes

are prevalent in patients with MDS (Graubert et al., 2011; Lindsley et al., 2015; Papaemmanuil et al., 2011; Yoshida et al., 2011). Not only are these genes frequently mutated, but a genome-wide screen determined that 29% of the genome was abnormally spliced in AML patients compared to healthy donors (Adamia et al., 2014). A recent study demonstrated that a spliceosomal inhibitor could reduce tumor burden in mice and patient-derived xenograft AMLs with mutations in Srsf2 (Lee et al., 2016). These studies indicate dysregulation in alternative splicing is an integral part of AML pathogenesis.

As a regulator of hematopoietic stem cell homeostasis, GSK-3 is likely involved in leukemia pathogenesis, where maturation of blood cells is compromised (Huang et al., 2009b; 2012a; Trowbridge et al., 2006). An elegant study from the Bhatia lab has recently proposed that a series of allelic deletions of GSK-3 isoforms specifically in the hematopoietic compartment can model AML progression (Guezguez et al., 2016). Knockout of GSK-3β caused an MDS-like phenotype that progressed to an AML-like phenotype with additional deletion of GSK-3α in these mice (Guezguez et al., 2016). These data point to the involvement of GSK-3 in AML disease pathogenesis, however the downstream effectors of GSK-3 that lead to AML are not known.

Our finding that GSK-3 regulates alternative slicing provides a potential mechanism for GSK-3 deletion leading to AML-like state. An interesting follow-up to link GSK-3 regulation of splicing to leukemogenesis could be to assess the effect of the allelic deletions of GSK-3 isoforms described above on alternative splicing events.

We also discovered another direct target of GSK-3, NMP1 that could further link GSK-3 to AML pathogenesis. Npm1 is mutated in 27% of AML patients and has been proposed to be a potential driver mutation for AML (Cancer Genome Atlas Research Network, 2013). Npm1 is also mutated in a variety of other solid tumors, including colon cancer (Di Matteo et al., 2016). Thus, functional consequences of GSK-3 phosphorylation of NPM1 could provide insight into transformation caused by NPM1 mutation for many cancers.

Implications of our findings for therapeutic mechanism of lithium

Lithium, a known inhibitor of GSK-3, has been used to treat bipolar disorder for decades, but the mechanism of action is not known (Klein and Melton, 1996; Stambolic et al., 1996). There is compelling evidence that some of its therapeutic functions are mediated through GSK-3 as lithium sensitive behaviors can be mimicked by *Gsk3* loss of function or pharmacological inhibition (Beaulieu et al., 2004; 2008; O'Brien, 2004; O'Brien et al., 2011)

Neurogenesis has been proposed to play a role in response to antidepressants, which stimulate neurogenesis (Balu and Lucki, 2009; Petrik et al., 2012; Santarelli, 2003). Although the requirement for neurogenesis in lithium response has not been tested, lithium activates Wnt signaling and stimulates hippocampal neurogenesis in mice and rats (Chen et al., 2010; Kitamura et al., 2011; O'Leary et al., 2012; Wexler et al., 2008). These data reveal an apparent connection between lithium and neurogenesis, thus it is probable that neurogenesis could be involved in lithium therapeutic response. Moreover, GSK-3 is a regulator of neural stem and progenitor cell homeostasis, and thus could be involved in mediating lithium's neurogenic effects (Ka et al., 2014; Kim et al., 2009). However, our findings have identified several novel effectors of GSK-3 that could be important in a neural setting and mediate the response to lithium.

Alternative splicing has been proposed to have a critical role in neurodevelopment and mature neurons (Vuong et al., 2016; Zheng, 2016; Zheng and Black, 2013). A recent study has implicated changes in alternative splicing events as a response to antidepressants (Piechota et al., 2015). Another study demonstrated that increased expression of the splicing factor SRp20, in blood samples from BD patients, could cause defects in alternative splicing (Watanuki et al., 2008). Therefore, alternative splicing mediated by GSK-3 inhibition could be important for lithium response and provides an intriguing avenue for further exploration. Notably, preliminary analysis of our RNA-sequencing results showed that ankyrin-G was differentially spliced in DKO ESCs. If validated, this would be a very relevant alternative splicing event to explore, as several,

independent, genome wide studies in different ethnic groups have detected significant association between polymorphisms in the ANK3 gene with BD (Baum et al., 2007; Dedman et al., 2012; Lee et al., 2010; Schulze et al., 2008; Scott et al., 2009; Takata et al., 2011).

RBM8A is an interesting candidate as an effector of GSK-3 in the brain. RBM8A overexpression in the mouse dentate gyrus (DG) has been proposed to affect anxiety behaviors (Alachkar et al., 2013). The behavioral effects of RBM8A overexpression were linked to increased double cortin staining, a marker of immature neurons, in the DG and the binding of RBM8A to neuronal mRNAs (Alachkar et al., 2013). Another study has implicated RBM8A as a regulator of embryonic neural progenitors. This study demonstrates that RBM8A expression is highest in neural progenitor cells (NPCs) and decreases as these cells differentiate (Zou et al., 2015). RBM8A knockdown decreases NPC proliferation and promotes exit from the cell cycle (Zou et al., 2015). Moreover, RNA-sequencing analysis also indicated that RBM8A regulates many risk genes implicated in neuropsychiatric diseases and genes important for early neurodevelopment (Zou et al., 2015). Taken together, these studies make RBM8A an intriguing potential mediator of GSK-3-dependent effects of lithium. Studies demonstrating GSK-3 phosphorylation of RBM8A affects mouse behaviors and NPC proliferation would further support GSK-3 as a molecular target of lithium.

NPM1 is not as well studied in the neural setting but has been linked to regulation of neuronal survival and NPC proliferation (Pfister and D'Mello, 2016; Qing et al., 2008). A recent study showed that Npm1 cytoplasmic localization, increased oligomerization and increased expression could lead to cytotoxicity in otherwise health neurons. Thus, NPM1 could be another effector of lithium, which is known to have neuroprotective effects in the brain (Dell'Osso et al., 2016).

Conclusion

We have presented here an unbiased analysis of the GSK-3 phosphoproteome. Our data has contributed several findings, outline in this thesis, to the field. Overall, our results highlight the breadth of the GSK-3 phosphoproteome, and the importance of understanding the regulatory signals that inhibit GSK-3 activity and determine specificity of its diverse effectors. The discovery of GSK-3 as a regulator of alternative splicing presents many opportunities for further experimentation. Our findings have implications in several diseases, including hematologic malignancies and bipolar disorder.

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