Identification of Wnt/GSK3 regulated proteolysis targets in addition to β-catenin

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The Wnt signaling pathway regulates a variety of cellular processes including cell proliferation, differentiation and migration in embryonic development and adult tissue renewal, as well as in diseases such as cancer. In the canonical Wnt signaling pathway, glycogen synthase kinase-3 (GSK3) phosphorylation mediates proteasomal targeting and degradation of β-catenin via the destruction complex. However, it has long been understood that there are branches of Wnt signaling pathways that do not depend on the β-catenin/Tcf mediated transcription activation, and that GSK3 regulates protein stability in a variety of signaling pathways other than Wnt signaling. In this study, we developed and carried out a biochemical screen that discovered multiple additional protein substrates whose stability is regulated by Wnt signaling and/or GSK3 and these have important implications for Wnt/GSK3 regulation of different cellular processes [(1)]. We also designed and executed a bio-informatics based search that identified potential GSK3 and β-Trcp mediated proteolysis targets. Therefore, Wnt/GSK3/destruction complex signaling regulates multiple target proteins to control a broad range of cellular activities in addition to β-catenin mediated transcription activation, and that GSK3 phosphorylation dependent proteolysis is a widespread mechanism that the cell employs to regulate a variety of cell processes in response to signals.

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ABBREVIATIONS AND ACRONYMS

GSK3	glycogen synthase kinase-3
GPCR	G-protein coupled receptor
TGFβ	transforming growth factor beta
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
Fz	Frizzled
SCF	Skp1-Cul1-F-box-protein
PI3K	phosphoinositide-3 kinase
MAPK	mitogen-activated protein kinase
CK1	casein kinase 1
APC	adenomatous polyposis coli
LEF/TCF	lymphoid enhancer factor /T-cell factor
EMT	epithelial-mesechymal transition
BMP	bone morphogenetic protein
Dsh	disheveled
FAP	familial adenomatous polyposis
WT1	Wilms tumor protein
PP1	protein phosphatase 1
Lrp5/6	lipoprotein receptor-related protein 5/6

IVEC	In vitro expression cloning
GST	glutathione S-transferase
IPTG	isopropyl-D-thiogalactoside
LiCl	lithium chloride
GID	GSK3β interaction domain of Axin
dnGSK3β	dominant negative GSK3β (kinase-dead)
AxinΔRGS	Axin mutant that lacks the APC binding domain
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
EST	expressed sequence tag
Lrp6ICD	Cytoplasmic tail of Lrp6
S/T→A mutation	serine/ threonine to alanine mutation
Li-Only	Proteins that are degraded in Xenopus egg extract and rescued by LiCl but not GID
Li/GID	Proteins that are degraded in Xenopus egg extract and rescued by both LiCl and GID
Shifted	Proteins that showed LiCl sensitive mobility shift on SDS-PAGE after incubation in Xenopus egg extract
MRLC	myosin regulatory light chain
TMEM4	transmembrane protein 4
MASP	MIR-interacting saposin-like protein
TIAR	T-cell restricted intracellular antigen-related protein
TACSTD1	tumor-associated calcium signal transducer 1
ATDC	ataxia-telangiectasia group D complementing gene

STAR	signal transduction and activation of RNA
FUS/TLS	*fused in sarcoma/translated in liposarcoma*
Fus	*FUS/TLS* gene product
FusIP1	Fus interacting protein 1
ALS	amyotrophic lateral sclerosis
FGD3	faciogenital dysplasia 3
GEF	guanine nucleotide exchange factor
AKAP220	A-kinase anchor protein 220
EPO-R	erythropoietin receptor precursor
ARD1	ADP-ribosylation factor domain protein 1
LZTS2	leucine zipper putative tumor suppressor 2
Ci	*cubitus interruptus*
Hh	Hedgehog
MafA	musculoaponeurotic fibrosarcoma oncogene homolog A
bZip	basic leucine zipper
SRC-3	steroid receptor coactivator protein 3
BCL-3	B-cell CLL/lymphoma 3
CDK	cyclin-dependent protein kinase
MOMP	mitochondrial outer membrane permeabilization
PCNA	proliferating cell nuclear antigen
NFAT	nuclear factor of activated T-cells
PCP	planar cell polarity

PKC	protein kinase C
CamKII	calmodulin-dependent protein kinase II

1.0 INTRODUCTION

In multicellular organisms, every cell is constantly communicating with its environment and neighboring cells using hundreds of various signal molecules. Depending on their intrinsic characteristics and the states they are in, each cell may proliferate, contract, migrate, differentiate or sometimes even commit suicide (apoptosis) in response to specific signals. This is best exemplified by the orchestration of the behaviors of numerous cells during embryonic development through complicated signaling networks. After decades of research, it is well understood that several major signaling pathways play central roles in regulating cellular behaviors, including the G-protein coupled receptor (GPCR), transforming growth factor/ bone morphogenetic protein (TGFβ/BMP), nuclear factor kappa light chain enhancer of activated B cells (NF-κB), phosphatidylinositol-3-kinase/Akt (PI3K/Akt), JAK-STAT, Notch, Hedgehog, Ras, and Wnt signaling, which is the focus of this study. As a testament to their critical roles in regulating cell behavior and ensuring the survival of the organisms, genes encoding components of these signaling pathways are often conserved during evolution. Mutations of the components of these signaling pathways commonly lead to birth defects and numerous diseases, including various types of cancer.

1.1 THE WNT SIGNALING PATHWAY

The Wnt signaling pathway is one of the major signaling pathways and is conserved across the animal kingdom. It plays central roles in the regulation of a number of cellular processes, including proliferation, migration and differentiation during embryonic development and adult tissue renewal [2-5]. Mutations that affect Wnt signaling components are implicated in developmental and birth defects, and a number of diseases, including various types of cancers. Aberrant Wnt signaling accounts for approximately 90% of colorectal cancer and although less frequently, a wide variety of other cancers such as melanoma, pancreatic cancer and liver cancers [6,7]. In fact, the first Wnt gene identified, *wnt1*, was originally found to be a preferential genomic integration site for viruses in virally induced breast tumors and was thus named *int-1* for integration [8]. The name *wnt* was created after the discovery that *int-1* was homologous to a *Drosophila* gene *Wingless*, which controls segmentation during larval development [9,10]. Ventral injection of *Wnt1* RNA was later found to be able to induce an ectopic body axis in *Xenopus* embryos, indicating the central role of Wnt signaling in axis determination during embryonic development [11]. Many additional proteins involved in the Wnt signaling pathway were later identified through genetic studies in *Drosophila* and axis duplication assays in *Xenopus*, including the Wnt receptor Frizzled (Fz) [12,13], Dishevelled (Dsh) [14-17], the central kinase Glycogen Synthase Kinase 3 (GSK3) [18], a scaffold protein Axin [19], and the main transcription factor β-catenin which was originally identified as a major component of the cadherin mediated adherens junctions [20-22]. Another major cytoplasmic scaffold protein, *adenomatous polyposis coli* (APC), the mutation of whose gene was originally discovered in a hereditary cancer syndrome

called familial adenomatous polyposis (FAP), was found to interact with β-catenin and play important roles in the Wnt signaling pathway, further confirming the critical roles of the Wnt signaling pathway in cancer development [23-25].

After two decades of research, it is now clear that Wnt signaling is among one of the several most indispensable signaling pathways for embryonic development, adult stem cell maintenance, and tissue renewal. The basic scheme of the biochemical mechanisms of the Wnt signaling pathway has been revealed. Wnt is a family of secreted glycolipoproteins (19 Wnts have been identified in human so far). Wnt binding to its receptor Fz, a family of seven-transmembrane proteins, can trigger a variety of cellular responses in different cellular contexts. A few distinct signaling pathways downstream of Wnt have been outlined, and they are separated into the so-called canonical and non-canonical pathways [2-5].

1.2 THE CANONICAL WNT SIGNALING PATHWAY REGULATES β-CATENIN STABILITY

The canonical Wnt signaling pathway refers to a series of signaling events that regulate the β-catenin protein level and consequently β-catenin dependent developmental gene expression programs. Signaling is mediated through the regulation of a "destruction complex" composed of multiple proteins, including the serine/threonine kinases GSK3β, Casein Kinase 1 (CK1) [26] and scaffolding proteins APC and Axin (Figure 1). Several other proteins, including the tumor suppressor protein Wilms tumor protein (WT1) and protein phosphatase 1 (PP1), are also involved in this regulation, but their exact roles are

less well understood [27] [28]. Working together with the tumor suppressor gene APC and Axin in the multi-protein destruction complex, the kinases CK1α (Casein Kinase 1α) and GSK3β serve as the central kinases of the complex. In the absence of upstream Wnt signaling, CK1α constitutively phosphorylates β-catenin at its N-terminal serine 45 (S45). Once phosphorylated, S45 serves as a "primed" recognition site for GSK3β, which then phosphorylates β-catenin at a series of tandem GSK3 recognition sites Threonine 41, serine 37 and serine 33. The serine 33 phosphorylated β-catenin is recognized by the F-box E3 ubiquitin ligase, β-Trcp, and rapidly goes through proteosome mediated degradation. The level of the β-catenin that mediates Wnt signaling is thus kept low in resting cells, resulting in the silencing of downstream target genes [5].

In the presence of Wnt signaling, the Wnt ligands bind to Frizzled (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). The formation of Fz/LRP complex leads to phosphorylation of LRP by GSK3 and CK1 and after the phosphorylation, the Fz/LRP complex recruits Dishevelled (Dsh) and Axin to the plasma membrane. Although the exact biochemical mechanism is still unknown, the destruction complex is inactivated at this step, allowing β-catenin level to increase in the cytosol. The accumulated β-catenin can then translocate to the nucleus and there bind to LEF/TCF transcription factors, where it activates a wide range of downstream target genes that control cell behaviors including proliferation, differentiation and migration. Many of the Wnt target genes are major players in cancer such as c-Myc, c-Jun and Cyclin D1, or important signaling molecules involved in embryonic development such as BMP4 and *Siamois*. In addition, the transcription of many Wnt signaling pathway components are regulated by β-catenin, providing feedback regulations [2,3,5].

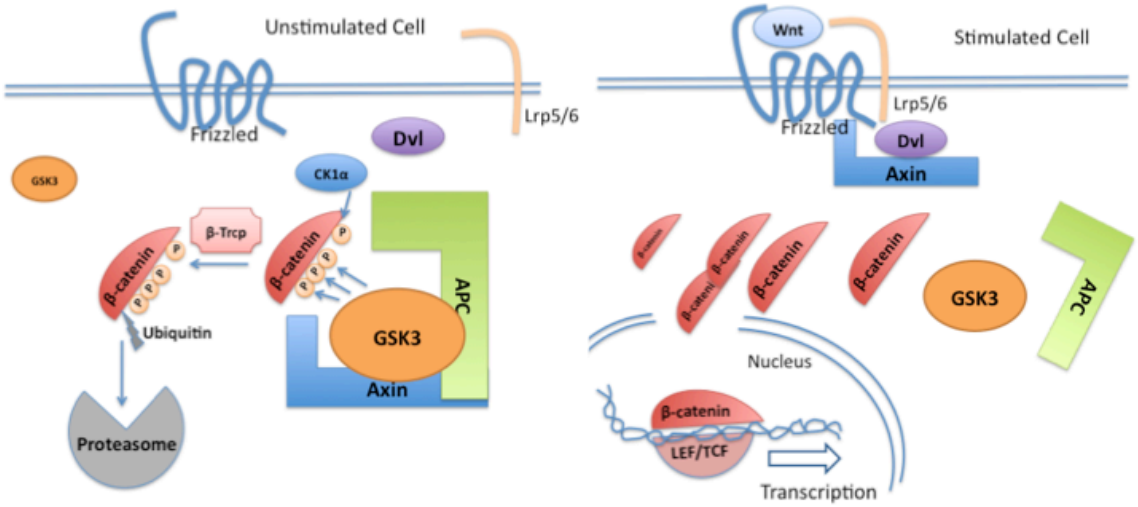

Figure 1. The Canonical Wnt Signaling Pathway. In the absence of Wnt signaling, the destruction complex actively labels β-catenin for subsequent ubiquination and degradation, thus keeping the β-catenin level low in the cell. Upon Wnt signaling the destruction complex is dissociated and inactivated, resulting in an accumulation of β-catenin proteins in the cell and subsequent transcription activation of downstream genes.

1.3 THE NON-CANONICAL WNT SIGNALING PATHWAYS

The non-canonical Wnt signaling pathways are not well-defined and are composed of a few independent signaling pathways [29]. The most well studied among them are the planar cell polarity (PCP) and the Wnt/Ca^{2+} pathways [30]. The PCP signaling pathway is involved in the regulation of cell polarity and migration during embryonic development, especially the convergence-extension movements during gastrulation [31]. The exact biochemical mechanism for PCP signaling pathway is still

elusive but it has been shown to affect cytoskeletal dynamics and cell migration through the regulation of small GTPases such as Rho and Rac [32,33]. A group of cytoplasmic and membrane proteins, include *Diego, Prickle, Flamingo* and *Strabismus*, play important roles in regulating the PCP pathway [30]. Dishevelled is also involved in the PCP pathway and is considered to regulate the bifurcation of canonical and non-canonical Wnt signaling pathways at least in some cell contexts [34,35]. In vertebrates, some Wnt proteins such as Wnt5 [36,37] and Wnt11 [38] can specifically trigger the PCP signaling but not the canonical Wnt signaling cascade. The Wnt/Ca^{2+} pathway refers to the Wnt induced transient spikes of intracellular calcium level and consequent activation of calcium dependent protein kinases such as the calmodulin-dependent protein kinases II (CamKII) and protein kinase C (PKC) [30,39]. In some cellular contexts, the Wnt/Ca^{2+} pathway activates the calcium-responsive transcription factor nuclear factor of activated T-cells (NFAT) and its downstream target gene expression [40].

Different Wnts may trigger different responses in different cellular contexts. How the specificity between the canonical and non-canonical Wnt signaling pathways are regulated is still unknown, but it seems they do not share any of the cytoplasmic components other than Dishevelled. The components of the "destruction complex", e.g. GSK3β, APC and Axin, are not found to be involved in the PCP and Wnt/Ca^{2+} signaling pathways. However, Axin [41], APC [42] and especially GSK3 (described in detail in the following sections) are found to be involved in many other signaling pathways in the cell, providing potential links of Wnt signaling pathway to those cellular processes.

1.4 GSK3: A GENERAL INTRODUCTION

Glycogen Synthase Kinase-3 (GSK3) is involved in a wide variety of signaling pathways in addition to the canonical Wnt signaling pathway. GSK3 was originally identified in the 1980's as a regulator of glycogen metabolism that phosphorylates and inactivates glycogen synthase [43]. When blood glucose level is low, GSK3 constitutively phosphorylates glycogen synthase and inhibits its activity. This inhibition is released by increased insulin signaling resulting from elevated blood glucose levels, in which case GSK3 activity is inhibited through the insulin induced PI3K-Akt signaling and glycogen synthase is dephosophorylated and activated, resulting in synthesis of glycogen from glucose (reviewed in ref [44]). Since its discovery, GSK3 has been found to play important roles in a variety of cellular processes such as cell proliferation, differentiation, microtubule dynamics, cell cycle and apoptosis [45]. In fact, a consensus motif based computational analysis of identified *in vivo* protein phosphorylation sites indicates that GSK3 is one of the most prolific kinases in the cell [46]. Many growth factors and cellular signaling pathways in turn regulate GSK3 activity, including the PI3K-Akt, the MAPK and the Wnt pathways [45,47]. There are two closely related isoforms of GSK3, GSK3α and GSK3β, both of which are widely expressed in mammalian tissues [48]. They share similar over-all structures and a high homology within their kinase domains (98% identity), yet vary in their N-terminal and C-terminal regions [49]. GSK3α cannot rescue the GSK3β null mice from embryonic lethality [50], indicating differences in their function that need to be further clarified. It is worth noting, though, that there seem to be a very high functional redundancy of GSK3α and GSK3β in the canonical Wnt/β-catenin signaling pathway [51].

1.5 GSK3 AND PROTEIN STABILITY REGULATION

An interesting characteristic of GSK3 is that it is usually constitutively active in the cell without inhibitory signals, and in most cases plays a negative regulation role for its downstream substrates. One great example is the classic GSK3 function in regulation of glycogen synthase, as mentioned in the previous section. In some cases, GSK3 phosphorylation negatively regulates downstream targets by earmarking them for ubiquitination and proteolysis. In the absence of upstream inhibitory signals, GSK3 phosphorylation of conserved serine or threonine residues on the substrates creates a binding site for phosphorylation-dependent E3 ubiquitin ligases, such as β-Trcp, allowing rapid ubiquitination and 26S proteasome dependent degradation of the substrates. This is best exemplified by β-catenin stability control in the canonical Wnt signaling pathway described earlier. With more and more such substrates discovered, protein stability control seems to emerge as one of the major mechanisms that GSK3 employs to regulate various cellular functions including cell proliferation, apoptosis and cell cycle (Table 1)

Table 1. Known GSK3 regulated proteolysis substrates

	GSK phosphorylation site		Upstream signal	E3 Ubiquitin ligase	References
	Site	Sequence			
β-catenin	S33, S37, S41	LD**S**GIH**S**GAT**S**TAP**S**LS	Wnt	β-Trcp	(52-56)
Snail	S96,S100,S104	D**S**GKS**S**QPP**S**PP	Wnt	β-Trcp	(57-60)
Smad1	S191,S198, T202, S210	NS**S**YPN**S**PG**S**SSS**T**YPH**S**PTS**S** DPG**S**PF	Wnt	Smurf1	(61)
Hath1	S54	EL**S**LLD**S**TD	Wnt	-	(62)
Smad3	T66	CI**T**IPR**S**LD	Axin involved	-	(63)
SRC-3	S505	VH**S**PMA**S**SG	Akt	Fbw7α	(64)
BCL-3	S394, S398	SS**S**PSQ**S**PP	Akt		(65,66)
p21	T57	DL**S**LSCTLV	ATR, Akt	Skp2	(67-69)
	S114	TE**T**PLEGDF			
HIF-1α	S551, T555	PF**S**TQD**T**DL	hypoxia,PI3K-Akt	-	(70,71)
	S589	SA**S**PESASP			
Mcl-1	S155, 159	NT**S**TDG**S**LPS**T**PP	PI3K-Akt	β-Trcp	(72-75)
c-Jun	T239	GE**T**PPL**S**PI	PI3K-Akt	Fbw7	(76)
c-Myc	T58	LP**T**PPL**S**PS	Ras-PI3K-Akt	Fbw7	(77-80)
Cyclin D1	T286	AC**T**PTDVRD	Ras-PI3K-Akt		(81)
Cyclin E	T380	LL**T**PPQ**S**GK	-	Fbw7	(82)
SREBP	T426, S430	TL**T**PPP**S**DA	insulin signaling	Fbw7	(83)
Cdc25A	S76	MG**S**SES**T**DS	-	β-Trcp	(84)
FGD1	S283, S287	RD**S**GID**S**IS	-	β-Trcp	(85)
FGD3	S72, S76	RD**S**GID**S**PS	-	β-Trcp	(86)
c-Myb	T572	LM**T**PVSED	-	Fbw7	(78)
mCRY2	S553	LS**S**GPA**S**PK	-	Fbxl3	(87)
NACα	T159	TQ**T**PTVQEE	-	-	(88)
MafA	S61, T57, T53, S49	PG**S**LSS**T**PLS**T**PCS**S**VPS**S**PS	-	-	(89,90)
IPF1/PDX1	S61, S66?	QG**S**PPDI**S**PY	-	-	(91)
PS1 CTF	S397	KA**S**ATASGD	-	-	(92)
NF-κB1 (cleavage)	S 903, 907	AH**S**LPL**S**PA	TNF-α	-	(93)
Ci (cleavage)	S852	MQ**S**RRS**S**QS,	Hedgehog	Slimb/β-Trcp	(94-97)
	S888	GC**S**RRS**S**QM		(cleavage)	

-: not reported or not clear

In some cases GSK3 phosphorylation dependent ubiquitination does not lead to full degradation but rather proteolytic cleavage processing of the substrate, as in the case of transcription activator Ci (Cubitus interruptus) in the Hedgehog (Hh) signaling pathway [94-97]. Although less well-studied, GSK3 phosphorylation and subsequent ubiquitination may also regulate p105 to p50 protein cleavage processing in the NF-κB signaling pathway [93].

In some signaling events, phosphorylation by GSK3 plays both positive and negative roles on the same substrates, as in the case of MafA and SRC-3. MafA is a basic leucine zipper (bZip) family transcription factor that promotes oncogenic transformation in embryonic fibroblasts and insulin expression in β cells [98]. SRC-3 is a steroid receptor co-activator that regulates cell growth [99]. Phosphorylation by GSK3 induces both activation and degradation of MafA and SRC-3. Another GSK3 regulated proteolysis substrate, BCL-3, may also be regulated through a similar mechanism [65,66]. This tightly coupled activation and degradation creates a 'rapid spike' of downstream substrate activity by limiting the temporal length of activation that may be necessary for strict regulation [64,89,90].

1.5.1 GSK3 regulated proteolysis in major signaling pathways during embryonic patterning

GSK3 regulation of β-catenin in the canonical Wnt signaling pathway has long been known to play important roles in embryonic development and tumorigenesis [100,101]. GSK3 has also been found to function similarly in the Hedgehog (Hh) signaling pathway, which plays important roles in many developmental processes and diseases (reviewed in

ref[102]). In the absence of the Hh signal, GSK3 phosphorylation results in the Slimb/β-Trcp dependent ubiquitination of transcription activator Ci. As mentioned earlier, ubiquitination does not lead to full degradation but rather cleavage of the full-length Ci protein (Ci155) into a truncated form (Ci75), which acts as a repressor of Hh downstream genes. Hh signaling blocks this process through an unknown mechanism, resulting in the activation of downstream gene expression. GSK3 is also shown to regulate the protein degradation of Smad1 and Smad3 proteins in the TGF-β/BMP signaling pathway [61,63]. The similar roles of GSK3 in Wnt signaling, TGF-β/BMP signaling, and Hh signaling pathways suggest that GSK3 regulated protein proteolysis plays and important role in the regulation of cell proliferation and differentiation during embryonic development.

1.5.2 GSK3 regulated proteolysis in cell cycle and proliferation

GSK3 has been known to play an inhibitory role in cell cycle progression and cell proliferation, at least partly through its regulation of the stability of cyclin E, cyclin D1, cdc25A and c-Myc [81,82,84]. Cyclin D1 and cyclin E protein levels dictate the G1 to S transition and are known to be tightly regulated by mitogens and cell cycle signals (reviewed in ref[103]). GSK3 phosphorylation mediates rapid degradation of both cyclin D1 and cyclin E. Ras signaling inactivates GSK3 through the PI3K-Akt pathway and results in accumulation of stabilized cyclins, triggering cell cycle progression [81,82]. It may also lead to the accumulation of Cdc25A phosphatase, another GSK3 regulated protein degradation substrate, and activate cyclin-dependent protein kinases (Cdks) [84]. At the same time, mitogen signaling also inhibits the GSK3 mediated degradation of c-Myc, resulting in the activation of its target genes, including cyclin D1, cyclin E and

other cell cycle mediators [77,79]. GSK3 thus has both direct and indirect roles in regulation of cell cycle progression. Interestingly, GSK3 activity is high both in quiescent and G1 phase cells, but decreases as cells progress into S phase, reflecting upstream regulation of its activity in cell cycle [84].

1.5.3 GSK3 regulated proteolysis in stress response and apoptosis

In line with its role in cell cycle regulation, GSK3 regulated proteolysis also plays important roles in the cellular response to stress stimuli such as nutrient deprivation or DNA damage. One example is the regulation of $p21^{cip1}$ (p21) turnover in response to UV irradiation. Following low dose UV irradiation p21 is rapidly degraded through ubiquitination, which releases its inhibition of PCNA (proliferating cell nuclear antigen, a cofactor of DNA polymerase δ) and allows DNA repair [68]. The increase in GSK3 activity after UV irradiation is responsible for triggering p21 degradation.[67] GSK3 mediated degradation of cdc25A may also play a role in inducing cell cycle arrest after UV irradiation, thus permitting more time for DNA repair [84].

GSK3 plays pro-apoptotic roles in the cell, as its over-expression induces apoptosis while its inhibition protects cells against apoptotic stimuli [104,105]. GSK3 has recently been found to negatively regulate the protein stability of Mc1-1, a Bcl-2 like anti-apoptotic protein that antagonizes the effects of pro-apoptotic Bcl-2 family proteins on mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release [106]. Some cell stimuli, such as growth factors or increased glucose level, inhibits GSK3 activity through PI3K-Akt and promotes cell survival, while cellular stress, such as UV irradiation increases GSK3 activity and triggers apoptosis [73,75] [72]. It is conceivable that

GSK3 serves as a central kinase that integrates multiple positive and negative stimuli for cell survival, and triggers different cellular response through regulation of the turnover of various signaling protein substrates.

1.6 VARIOUS UPSTREAM SIGNALS REGULATE GSK3 ACTIVITY IN ITS REGULATION OF PROTEIN STABILITY

The upstream signals that control GSK3 activity in the regulation of many of these established or potential GSK3 regulated proteolysis protein substrates are still unknown (Table 1). Several signaling pathways inhibit GSK3 activity, including the PI3K-Akt pathway, the MAPK pathway, the mTOR pathway, and the Wnt pathways. One common mechanism of GSK3 activity inhibition is the inhibitory phosphorylation of its N-terminal serines (Serine 9 in GSK3β and Serine 21 in GSK3α). This phosphorylation creates a primed pseudosubstrate that binds to the kinase activity center of GSK3, thus precluding the binding and phosphorylation of substrates. A number of kinases can phosphorylate GSK3 at these inhibitory serine sites, including Akt, MAPKAP-K1, S6K1, PKA, and PKC, while many growth factors can regulate GSK3 activity through regulation of one or multiple of these kinases [45,47,107]. On the other hand, Wnt signaling does not induce serine 9 phosphorylation in GSK3. Instead, it may inhibit the GSK3 activity by dissociating the destruction complex, thus prohibiting GSK3 from reaching its target. Alternatively, the Wnt triggered phosphorylation of Lrp5/6 cytoplasmic domain may directly bind to GSK3 and inhibit its activity [5].

1.7 WNT REGULATED PROTEOLYTIC SUBSTRATES IN ADDITION TO B-CATENIN

Recently, the levels of a few proteins in addition to β-catenin were reported to be regulated by the canonical Wnt signaling pathway and GSK3, including the E-cadherin transcription repressor Snail and the BMP signaling mediator Smad1. Very similar to β-catenin, Snail protein stability is controlled by GSK3 dependent phosphorylation followed by β-Trcp mediated ubiquitination and proteosome degradation in resting cells. Wnt signaling inhibits the GSK3 mediated Snail phosphorylation and consequently increases Snail protein levels, which in turn triggers epithelial-mesechymal transition (EMT) in certain cell types [58-60]. The BMP signal mediator Smad1 is regulated by Wnt signal in a similar fashion. However, instead of total protein level, only the BMP/MAPK activated form of Smad1 (C-terminal phosphorylated Smad1) is recognized and regulated by Wnt/GSK3, resulting in a fine control of BMP signaling strength and longevity by Wnt signal [61]. Another recent study revealed that the protein stability of another Smad, Smad3, is regulated by Axin and GSK3β, but possibly not via Wnt signaling [63]. Further studies may help to confirm whether Wnt signaling can regulate Smad3 stability in certain contexts. However, these results clearly indicate that the canonical Wnt signaling pathway and the destruction complex regulate the stability of a large and diverse population of signaling proteins in the cell, in addition to the well known target, β-catenin.

1.8 RESEARCH AIM: IDENTIFICATION OF NOVAL WNT AND/OR GSK3 REGULATED PROTEOLYSIS SUBSTRATES

The hypothesis tested in this study is that there are many other signaling proteins in the cell whose turnover is regulated by Wnt signaling. If proven true, this would imply that the canonical Wnt signaling pathway is, by nature, a signaling event that regulates cellular processes through regulation of the stability of various signaling proteins in the cell, other than the traditional linear view of β-catenin level control and the regulation of β-catenin dependent transcription of downstream genes. Identification of these Wnt regulated protein degradation substrates will enable a better understanding of the Wnt signaling dependent regulation of various cellular processes and their implication in both embryonic development and various diseases.

However, these protein degradation substrates are difficult to identify using conventional biochemical purification methods as they are, by definition, labile and tightly controlled in the cell. In order to identify the potential Wnt and the destruction complex regulated protein degradation substrates, a unique biochemical screen was developed and used to examine Wnt and/or GSK3 regulated protein degradation in *Xenopus* egg cytoplasmic extract [(1)]. This screen method is adapted and modified from a method using the in vitro expression cloning (IVEC) technique and *Xenopus* egg cytoplasmic extract system adapted from Marc Kirschner and colleagues [(108,109)].

2.0 MATERIALS AND METHODS

2.1 THE *XENOPUS* EGG CYTOPLASMIC EXTRACT SYSTEM

Xenopus egg extracts were prepared as described in Murray AW (1991) with minor modifications. hCG primed female *Xenopus* frogs were maintained in 1x MMR (Marc's Modified Ringers: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA, pH 7.7) buffer and allowed to lay eggs overnight at 16°C. Collected eggs were dejellied in 2% cysteine in 1x XB (Extraction Buffer: 10 mM KCl, 1 mM $MgCl_2$, 100 nM $CaCl_2$, 10 mM Hepes, 50 mM sucrose, pH 7.7) buffer and washed 5 times in 1x XB buffer, followed by wash in 1x XB buffer containing protease inhibitors (10 µg/ml each of leupeptin, pepstatin and chymostatin) and transferred to pre-chilled centrifuge tubes (Beckman #326819) containing 500 µL of 1x XB buffer with protease inhibitors and 100 µg/ml cytochalasin B. Eggs were packed for 30 seconds at low speed (<100 x g) and excess buffer was removed before the eggs were crushed at 21,000 x g for 5 mins. The resulting clear cytoplasmic layer was transferred to fresh tubes. Protease inhibitors and cytochalasin B were added to the extract. Four more rounds of centrifugation were then performed to obtain clear egg extracts. 10 ng/ml cycloheximide and energy mix (7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, 1 mM $MgCl_2$) were added into the final *Xenopus* egg extract right before the degradation assay.

2.2 DEGRADATION ASSAY

The degradation assay was performed as described [109] with minor modifications. The degradation reactions contained 6.5 μL of *Xenopus* egg extract, 0.2 μL of 14 mg/ml bovine ubiquitin in 1x XB buffer and 0.1-0.3 μL of ^{35}S-Met labeled *in vitro* translated proteins. Reactions were carried out at 22°C for 3 hours and then analyzed by to SDS-PAGE and autoradiography. The quality of the *Xenopus* egg extracts were evaluated by degradation of β-catenin as a positive control and cleavage of procaspase-3 as an indication of apoptotic activity, as described in the text. 25 mM LiCl and 0.8 μM recombinant GID protein were used as GSK3 and the destruction complex inhibitors during the screen.

2.3 GEL ELECTROPHORESIS AND AUTORADIOGRAPHY

Protein samples were boiled in SDS sample buffer (10% (by volume) glycerol, 62.5 mM Tris-HCL pH 6.8, 2% SDS, 0.01 mg/ml bromophenol blue, 5% β-mercaptoethanol (BME)) and loaded on 12-15% gradient SDS acrylamide gels. Gel electrophoresis were carried out at 20mA/gel for ~8 hours. The acrylamide gels containing samples were then incubated in fixation buffer (5% glycerol, 40% methanol, 10% glacial acetic acid) for one hour before vacuumed dry under 65°C in a gel dryer (Bio-rad Model 583). After drying, gels were subjected to autoradiography using Kodak BioMax TransScreen Intensifying screen and Kodak BioMax MS films in -80°C.

2.4 THE IVEC SCREENING METHOD

A gastrula stage Xenopus embryo cDNA library (a courtesy from Dr. Todd Stukenberg, [110] was transformed into E. coli and titrated to grow 50~100 colonies per plate. The colonies were pooled together and plasmid DNA was isolated from each pool. 35S labeled in vitro translated proteins were made from the resulted plasmid pools using the TNT Quick Coupled Transcription/Translation System (Promega catalog #L2080) and used in the degradation assays. Once a positive band has been identified in a pool, the bacteria containing positive pools were grown again and plasmids were isolated from 96 individual colonies picked from each pool. The plasmids of each row of the 96 well plate were pooled together as a 'sub-pool', from which in vitro translated proteins were made and subjected to degradation assay to identify the positive sub-pool. Positive single clones were then identified from the positive sub-pools by protein band size, verified again by the degradation assay and sequenced to identify the genes encoded (Figure 2).

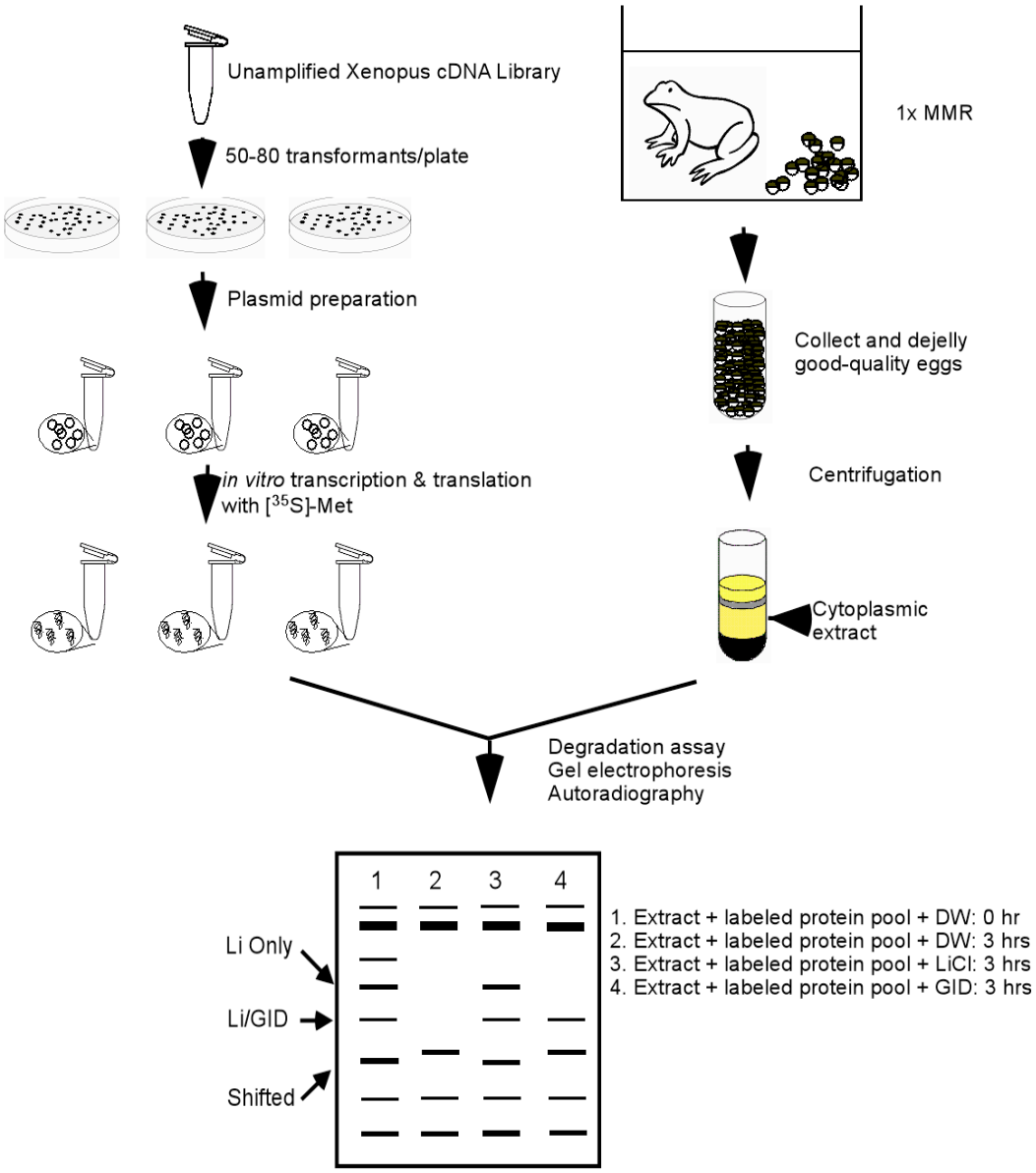

Figure 2. Schematic of the screening procedure. The in vitro expression cloning (IVEC) technique was used with the Xenopus egg extract degradation assay to identify novel phosphorylation and proteolytic target genes of the Wnt/GSK3 signaling pathway. DW: distilled water; LiCl: 25mM lithium chloride; GID: 0.8 μM recombinant GID protein; Li Only: protein bands that were degraded after three hour incubation with the *Xenopus* egg extract and rescued by LiCl but not by GID; Li/GID: protein bands that were degraded in the *Xenopus* egg extract and rescued by both LiCl and GID; Shifted: protein bands that showed mobility shift after three hour incubation in the *Xenopus* egg extract and the shift was abolished by LiCl.

2.5 PREPARATION OF RECOMBINANT PROTEINS

GSK3 interacting domain of human Axin (GID, a.a. 321-495, Genbank accession No.: NM_003502) was cloned into pET21a vector. Dominant negative *Xenopus* GSK3β (K85R, Genbank accession No.: L38492) was cloned into pGEX4T3 vector. BL21 bacterial cells containing pET21a-GID and pGEX-dnGSK3β were grown to OD_{600} of 0.6 at 37°C and induced with isopropyl-D-thiogalactoside (IPTG) (0.5 mM) for overnight at room temperature. Recombinant His-tagged GID, and glutathione S-transferase (GST) fusion dnGSK3β, Lrp6ICD proteins were purified by Ni-NTA agarose (Qiagen) or Glutathione Sepharose 4B (GE Healthcare), respectively, according to manufacturer's instructions. Purified recombinant proteins were loaded onto PD-10 desalting column (GE Healthcare), and finally eluted with elution buffer [10 mM Hepes (pH 7.7), 100 mM KCl and 10% Glycerol]. Mouse Axin ΔRGS (Δ123-220, Genbank accession No.: NM_009733) and *Xenopus* Dsh (Genbank accession No.: BC090218) were cloned into pFast-Bac-HT vectors (Invitrogen) for insect cell expression. Baculoviruses were prepared according to standard procedures. Sf9 cells ($1.8x10^6$ cells/ml) were infected with MOI of 2 for 36 hours. Recombinant dnGSK3β, AxinΔRGS, Dsh proteins were purified by Ni-NTA agarose and PD-10 column, and finally eluted with elution buffer [10 mM Hepes (pH 7.7), 100 mM KCl and 10% Glycerol].

2.6 MOLECULAR CLONING

Coding region of candidate genes were amplified by PCR and cloned into the pCS2+ vector that contains C-terminal Flag and HA tag. *Xenopus* c-myc (Genbank accession No.: X53717) was amplified by RT-PCR from stage 10 Xenopus embryo cDNA and cloned into the same vector. Point mutations were carried out using QuikChange Site-Directed Mutagenesis Kit (Stratagene Catalog #200518) using standard methods described in the product manual. Tau expression plasmid was a generous gift from Dr. George S. Bloom (University of Virginia, Charlottesville, VA).

2.7 XENOPUS EMBRYO RNA INJECTION

10 nl of β-catenin (10pg/nl) or candidate RNAs (20 pg/nl) synthesized with Riboprobe system (Promega) were co-injected with 30pg of GFP or Wnt8 mRNA into one of the ventral blastomeres of the *Xenopus* embryos at 4-cell stage. In all cases, 10 pg Myc-EGFP RNA was co-injected as an internal control for injection and loading. The embryos were then allowed to develop into stage 12~12.5 before the animal caps were dissected and lysed in 2x SDS sample buffer. Expressed protein levels were detected by Western Blot using anti-Myc antibody (Upstate; 4A6, 1:10,000) and anti-Flag antibody (Sigma; M2, 1:5,000).

3.0 RESULTS

3.1 VALIDATION OF THE *XENOPUS* EGG EXTRACT AS AN *IN VITRO* SYSTEM FOR THE IDENTIFICATION OF WNT/GSK3/DESTRUCTION COMPLEX TARGET PROTEINS

The *Xenopus* egg extract provides a powerful *in vitro* system to recapitulate many intracellular processes and signaling pathways, including the cell cycle and apoptotic pathways [110,111]. Moreover, the *Xenopus* egg extract system was reported to be able to accurately recapitulate the cytosolic part of the canonical Wnt signaling pathway: the destruction complex mediated β-catenin degradation and the inhibition of this degradation by various Wnt pathway activators [108]. When added into the reaction, the GSK3 inhibitor, lithium chloride (LiCl), and other Wnt pathway activators can inhibit the degradation of β-catenin [108]. This provides an *in vitro* system that is ideal to screen for proteolytic target proteins of the Wnt/GSK3 signaling pathway.

Before screening, the robustness of the *Xenopus* egg extract system was verified using β-catenin as a positive control. *In vitro* translated *Xenopus* β-catenin protein was degraded in the *Xenopus* egg extract within three hours, and the GSK3 inhibitor LiCl blocked this degradation in a concentration dependent manner (Figure 3A). β-catenin degradation was mediated specifically by GSK3-dependent phosphorylation, because a

GSK3 phosphorylation deficient mutant β-catenin (S33D) was stable in the extract (Figure 3*A*).

The specificity of the reaction was tested using additional Wnt signaling pathway activating proteins. Among them, GID (GSK3β interaction domain of Axin), kinase-dead GSK3β (dnGSK3β), and an Axin mutant that lacks the APC binding domain (AxinΔRGS) all work as dominant negative proteins that disrupt the destruction complex, whereas Dsh is a potent upstream Wnt pathway mediator that inhibits the destruction complex [19,101,112,113]. Purified recombinant proteins GID, dnGSK3β, AxinΔRGS and Dsh efficiently blocked the degradation of β-catenin by the *Xenopus* egg extract (Figure 3*B*), indicating that degradation of β-catenin is specifically mediated by the destruction complex, as previously reported [108].

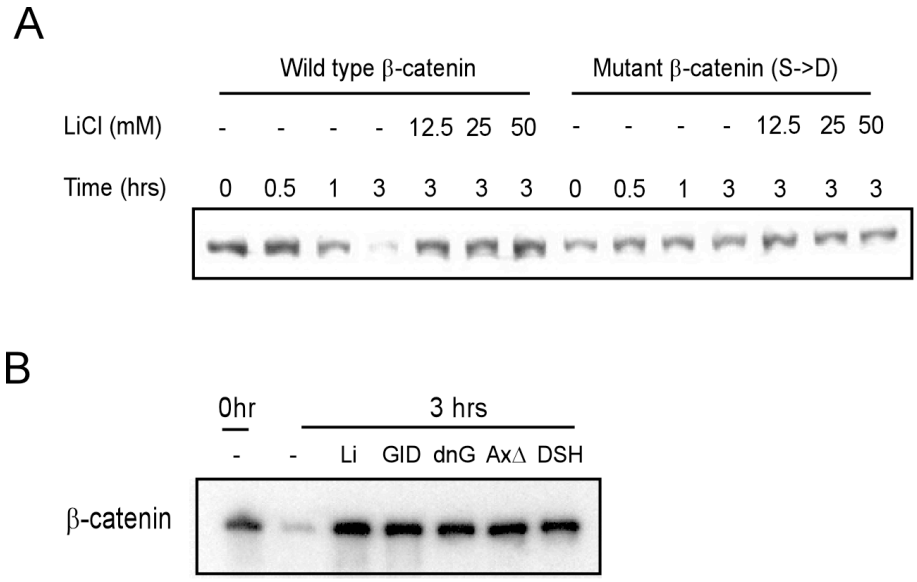

Figure 3. The *Xenopus* egg extract recapitulates the cytosolic part of the canonical Wnt signaling pathway. (*A*) Wildtype and mutant (S33D) *Xenopus* β-catenin proteins were incubated in the *Xenopus* egg extract with different concentrations of LiCl for different incubation time. (*B*) Wnt activators that inhibit the destruction complex stabilize *Xenopus* β-catenin in the *Xenopus* egg extract. (–)**:** 1x XB buffer added in the reactive as a negative control; **Li:** 25 mM Lithium Chloride; **GID:** GSK3β-interaction domain of Axin; **dnG:** dominant negative GSK3β (kinase dead); **AxΔ:** AxinΔRGS, Axin protein missing the RGS domain that binds to APC; **DSH:** recombinant Dishevelled protein.

3.2 SCREENING OF PROTEOLYTIC TARGETS OF WNT/GSK3 SIGNALING IN *XENOPUS* EGG EXTRACTS BY *IN VITRO* EXPRESSION CLONING (IVEC)

IVEC screening using the *Xenopus* egg extract has been used to identify numerous proteins that are mitotically phosphorylated or degraded and participate in the regulation of mitotic events [110,114,115]. In order to identify novel proteolytic target proteins of the Wnt/GSK3 pathway, this method was employed with some modification (Figure 2). Small pools containing 50-100 clones of cDNAs from a gastrula stage *Xenopus* cDNA library were *in vitro* translated to generate [^{35}S-Met]-labeled proteins. These radio-labeled protein pools were incubated with freshly-prepared *Xenopus* egg extracts at room temperature for three hours. The three hour limit favors the identification of proteolytic target proteins with relatively short half lives. Incubation times greater than three hours were avoided because the apoptotic activities of the *Xenopus* egg extract become significant after three hours. In the initial screens, LiCl and the recombinant GID protein were used to inhibit GSK3 activity and the Wnt regulated destruction complex, respectively. *In vitro* translated β-catenin protein was used as a positive control and pro-caspase 3 protein was used to monitor the apoptotic activity of *Xenopus* egg extracts in every assay. *Xenopus* extracts that cleaved pro-caspase 3 or did not degrade β-catenin were discarded. Protein bands that exhibited either a mobility shift or degradation after the reaction and were rescued by LiCl or GID were regarded as putative phosphorylation and/or proteolytic targets of the Wnt/GSK3 pathway. For the identification of proteolytic targets, only bands with more than 50% signal reduction after 3 hour incubation compared to the starting material in the autoradiograph were included. Positive protein

bands were validated in at least three independent experiments using different *Xenopus* egg extracts. Representative examples of pools having at least one positive band were shown in Figure 4.

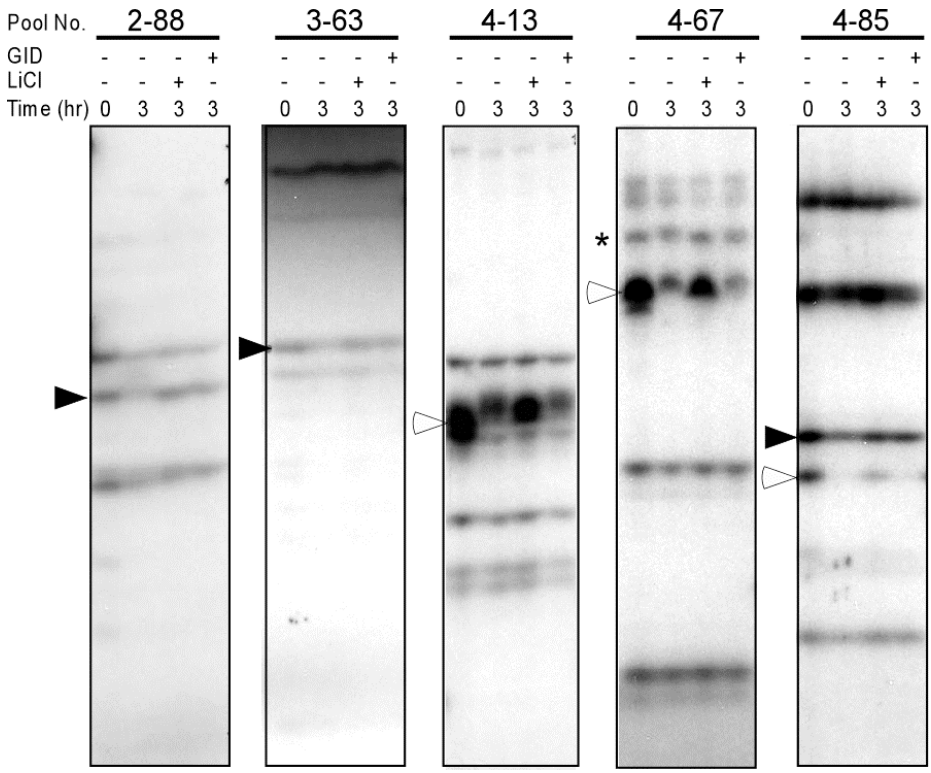

Figure 4. Examples of the degradation assay of *in vitro* translated *Xenopus* cDNA pools used in the screen. Small pools containing 50-100 clones of gastrula stage *Xenopus* cDNA library were *in vitro* translated with [^{35}S]-methionine. Radio-labeled protein pools were incubated with *Xenopus* egg extract for three hours at room temperature and then analyzed by SDS-PAGE and autoradiography. 25mM LiCl or 0.8 μM of recombinant GID protein were used to inhibit GSK3 activity and the destruction complex, respectively. Closed arrowhead indicates the protein bands that are degraded in the *Xenopus* egg extract and rescued by LiCl and GID (Li/GID). Open arrowheads indicate protein bands that are degraded in the *Xenopus* egg extract and rescued by LiCl but not GID (Li-Only). Asterisk indicate protein bands that showed mobility shifts in SDS-PAGE after incubation in the *Xenopus* egg extract and LiCl blocked the mobility shift.

In total, 549 *Xenopus* cDNA pools encoding close to 10,000 distinguishable protein bands were screened in this study. Most protein bands exhibited no change in mobility or stability after incubation in the extract and thus served as internal controls. There were also a small number of protein bands that were shifted or degraded by the *Xenopus* egg extract but not rescued by LiCl or GID, indicating Wnt/GSK3 independent phosphorylation and degradation. These were not pursued further. Curiously, there were also three proteins whose degradation was stimulated by LiCl, and they turned out to be ribosomal proteins (Figure 5). Two other ribosomal proteins whose degradation was rescued by LiCl in the *Xenopus* egg extract were also identified in the screen (Figure 6, Figure 7), suggesting that GSK3 may have both positive and negative roles in ribosomal protein turnover.

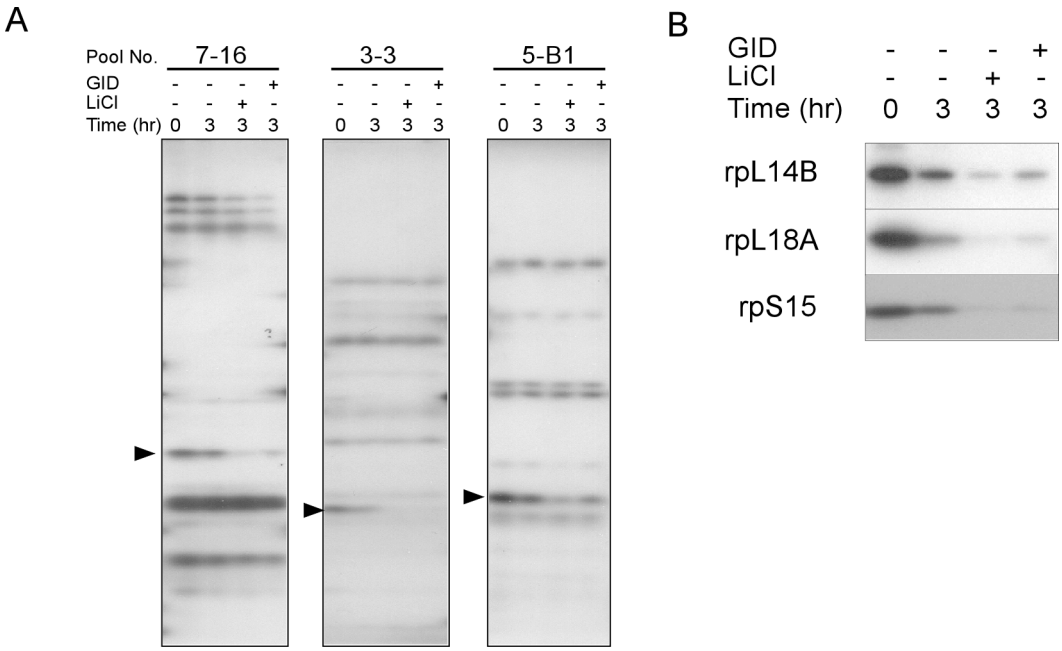

Figure 5. Ribosomal proteins whose degradation in the Xenopus egg extract was stimulated by LiCl. (A) Examples of pools containing the proteins whose degradation was stimulated by LiCl (closed arrowheads). (B) Ribosomal proteins whose degradation in the Xenopus egg extract was stimulated by LiCl. (–): 1x XB buffer added in the reactive as a negative control; LiCl: 25mM Lithium Chloride; GID: GSK3β-interaction domain of Axin.

LiCl and/or GID blocked the degradation or mobility shift of 57 protein bands from 45 different cDNA pools. This represents ~0.6% of all bands detected in the screen. Of these 57 protein bands, 9 exhibited mobility shifts that were inhibited by LiCl. LiCl inhibited the degradation of 32 protein bands and 16 protein bands were degraded and rescued by both LiCl and GID. There were no protein bands rescued by GID alone, indicating that the regulation of protein phosphorylation and degradation by GID depends on GSK3 activity.

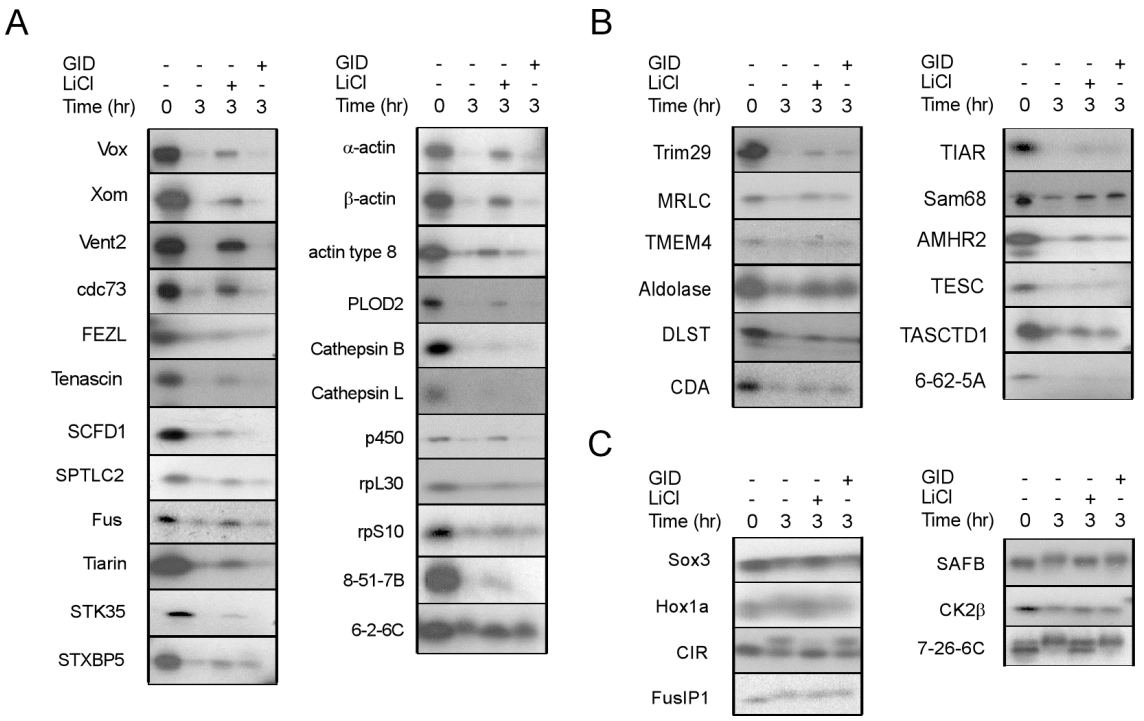

Figure 6. Degradation assay of isolated putative Wnt/GSK3 target proteins identified in the screen. The genes encoding positive protein bands were identified by progressive sub-selection (see methods). Positive individual clones were sequenced and verified with the degradation assay. Out of 42 identified proteins, the degradation of 23 proteins by the *Xenopus* egg extract were inhibited by LiCl but not GID (*A*), 12 proteins were positive for both LiCl and GID (*B*). 7 proteins showed mobility shift after incubation in the *Xenopus* egg extract that was inhibited by LiCl (C). (**–**)**:** 1x XB buffer added in the reactive as a negative control; **Li:** 25 mM Lithium Chloride; **GID:** 0.8 μM of GID (GSK3β-interaction domain of Axin).

3.3 CLONING AND IDENTIFICATION OF SPECIFIC TARGET PROTEINS DETECTED IN THE SCREEN

The genes encoding the positive protein bands were identified by subsequent sub-selection (see methods), cloning and sequencing. Once identified, the LiCl or GID-dependent degradation or mobility shift of proteins from positive cDNA single clones was confirmed again in the degradation assay with *Xenopus* egg extract. Out of the 57 positive protein bands, 42 unique genes were identified. 29 of these are known genes in *Xenopus,* while 9 could not be found in *Xenopus* EST database (Table 2) but exhibited high sequence similarity to human genes. Four genes were completely unknown and could not be found in any database. Eight genes were identified in more than two independent pools (Table 2).

Table 2. Novel phosphorylation and/or proteolysis targets of Wnt/GSK3

Name	Gene bank	Function	coding region	No. of clones
Trim29	NM_001086106	Ataxia-telangiectasia group D-associated protein	366-497	1
Cdc73	NM_001086043	Parafibromin, Tumor suppressor	374-531	1
Vox1	U53528	ventralizing homeodomain gene	1-336	5
Xom	X98454	ventralizing homeodomain gene	1-327	2
Vent2	NM_001088138	ventralizing homeodomain gene	1-328	3
FEZF2	BC110761	Transcription repressor, fore-brain embryonic zinc-finger like	36-434	1
sox3	BC072222	control of nervous system development	1-309	2
CIR	BC081191	CBF1 binding co-repressor, Notch pathway	1-240	1
HOX1a	M26884	Regulator of cell fate and pattern formation	93-232	1
MRLC	NM_001087310	Myosin Regulatory Light chain	1-172	3
Tmem4	NM_001092222	Positive regulator of neurite outgrowth, MIR-interacting saposin-like protein	1-184	1
Tenascin	N/A	Extracellular matrix protein	N/A	1
SCFD1	NM_001092557	Syntaxin-binding protein	444-632	1
STXBP5	N/A	Syntaxin binding protein	N/A	1
α-actin	X03470	cytoskeleton	134-377	1
Actin type 8	M24770	cytoskeleton	119-376	1
β-actin	AF079161	cytoskeleton	82-375	2
Adolase	AB002267	Ubiquitous glycolytic enzyme	233-364	1
CDA	BC118900	Cytidine deaminase	1-151	1
Dlst	NM_001087234	Dihydrolipoamide S-succinyltransferase	226-452	1
PLOD2	BC043893	Lysyl hydroxylase	N/A	1
CathepsinB	NM_001086941	lysosomal cysteine proteinase	1-333	1
CathepsinL	NM_001094020	lysosomal cysteine proteinase	1-335	1
P450	AB284118	cholesterol monooxygenase	N/A	1
SPTLC2	NM_001092294	Serine palmitoyltransferase	N/A	1
TIAR	AJ416631	Cytotoxic granule-associated RNA-binding protein	237-389	1
Sam68	NM_001017045	mRNA stability, signal transduction	124-360	1
SAFB	N/A	Scaffold attachment factor of nuclear matrix	N/A	1
rpL30	NM_001087152	Ribosomal protein	1-116	1
rpS10	BC073601	Ribosomal protein	12-174	1
Fus	NM_001086914	Nuclear RNA-binding protein	214-536	1
Fus ip1	BC084231	FUS interacting protein	1-251	1
AMHR2	NM_001011098	TGF-β-related type II receptor protein	N/A	1
Tescalcin	BC084830	Ca2+- and Mg2+-binding protein	N/A	1
TACSTD1	BC044698	Homotypic calcium-independent cell adhesion molecule	1-314	1
Tiarin	NM_001088746	Secreted glycoprotein implicated in development of the nervous system	294-467	1
STK35	NM_001091120	serine/threonine kinase 35	N/A	1
CK2β	NM_001090657	Casein Kinase 2 beta subunit	1-215	2
Unknown1	N/A	unknown	N/A	1
Unknown2	N/A	unknown	N/A	1
Unknown3	N/A	unknown	N/A	4
Unknown4	N/A	unknown	N/A	1

N/A: Data not available for Xenopus

Of these 42 unique genes, 7 encoded proteins that exhibited a mobility shift after incubation in the *Xenopus* egg extract and LiCl blocked the mobility shift, indicating a potential GSK3 mediated phosphorylation. GID did not block their mobility shifts. The degradation of 23 proteins by the *Xenopus* egg extract was inhibited by LiCl alone (Li-Only), and 12 proteins were positive for both LiCl and GID (Li/GID) (Figure 6). The latter 12 proteins are strong candidates for regulation by the Wnt signaling pathway and the destruction complex.

Based on their cellular functions, positive proteins can be categorized in seven different groups: transcription factors, cytoskeleton and membrane proteins, metabolic enzymes, RNA associated proteins, signaling molecules, and unknown function (Figure 7), indicating that Wnt and/or GSK3 can specifically regulate a broad range of intracellular mediators, in addition to the well-studied β-catenin in the canonical Wnt signaling pathway.

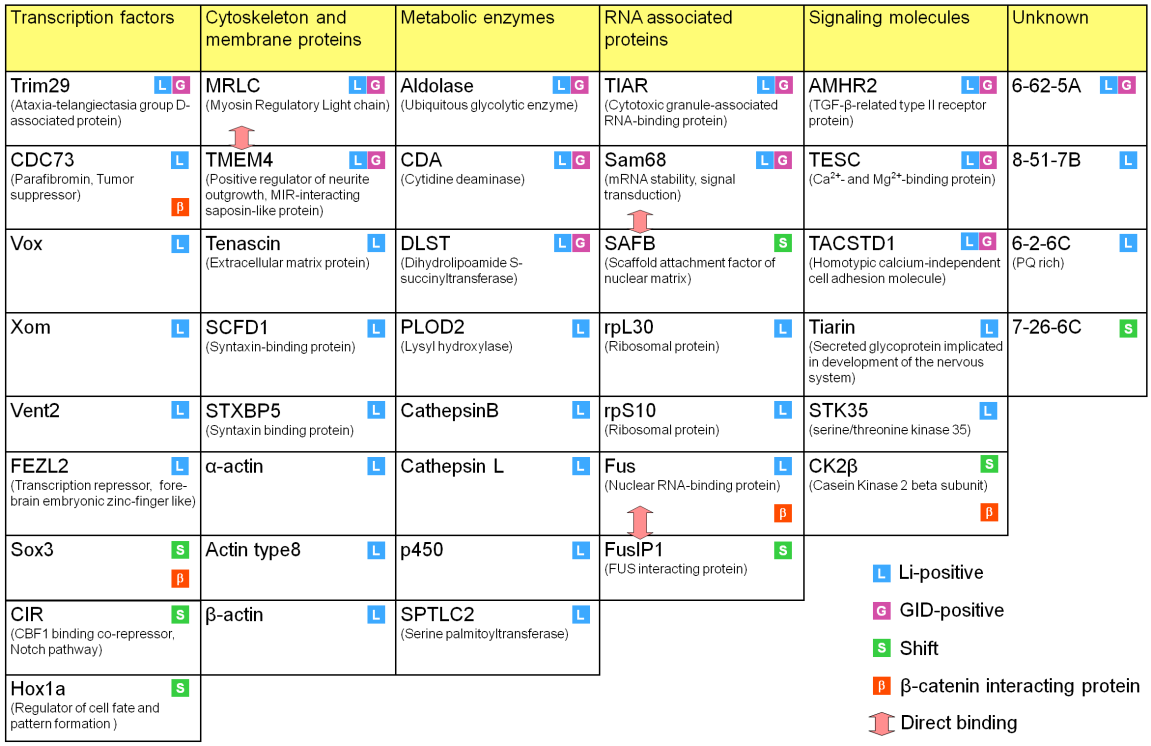

Figure 7. Proteins identified in the screen arranged in functional groups. 42 novel phosphorylation or proteolytic targets of Wnt and/or GSK3 are identified by whether their degradation is inhibited by LiCl or GID, or their mobility shift is inhibited by LiCl. Also indicated are proteins reported to interact with b-catenin. Known interactions between candidate proteins are indicated by double-headed arrows.

3.4 CHARACTERIZATION OF THE LI/GID POSITIVE PROTEINS IDENTIFIED IN THE SCREEN

GID was used to screen for destruction complex dependent targets, since it should interfere with the binding of GSK3β to Axin in the complex, thereby inhibiting the activity of the complex. Indeed, we were able to clearly distinguish the Li/GID from LiCl-Only candidates. However, GID has been claimed to act as a more general GSK3 inhibitor [112]. Therefore the specificity of GID in the *Xenopus* egg extract system was further tested using two known GSK3 targets not thought to be regulated by the Wnt signaling pathway. The microtubule-associated protein Tau is phosphorylated by GSK3 in the *Xenopus* extract [116], as detected by a mobility shift (Figure 8). Phosphorylation of Tau was inhibited by LiCl, but not at all by GID (Figure 8). c-Myc is also known to be a GSK3β substrate, which targets it for degradation [77,80]. Although the degradation of c-Myc by the extract is inhibited by LiCl, it is unaffected by GID (Figure 8). Therefore, GID does not block all GSK3β mediated activities in the extract and is probably more specific for destruction complex targets.

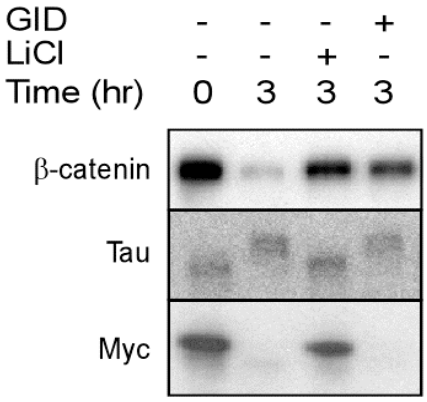

Figure 8. GID does not inhibit the activity of GSK3β towards all protein targets in *Xenopus* egg extracts. GID blocked the degradation of β-catenin but not the mobility shift of Tau or the degradation of Myc protein. **(–):** 1x XB buffer added in the reactive as a negative control; **Li:** 25 mM Lithium Chloride; **GID:** 0.8 μM of GID

Candidate proteins were further characterized using the destruction complex inhibitors dnGSK, AxinΔRGS, Dsh and the cytoplasmic tail of Wnt co-receptor Lrp6 (Lrp6ICD). The Li/GID positive candidates were stabilized by all the inhibitors tested, whereas the LiCl-Only positive candidates were not (Figure 9), further confirming the specificity of GID as a Wnt destruction complex inhibitor. The stability of the Li/GID positive proteins thus seems to be specifically regulated by the destruction complex and therefore by the Wnt signaling pathway.

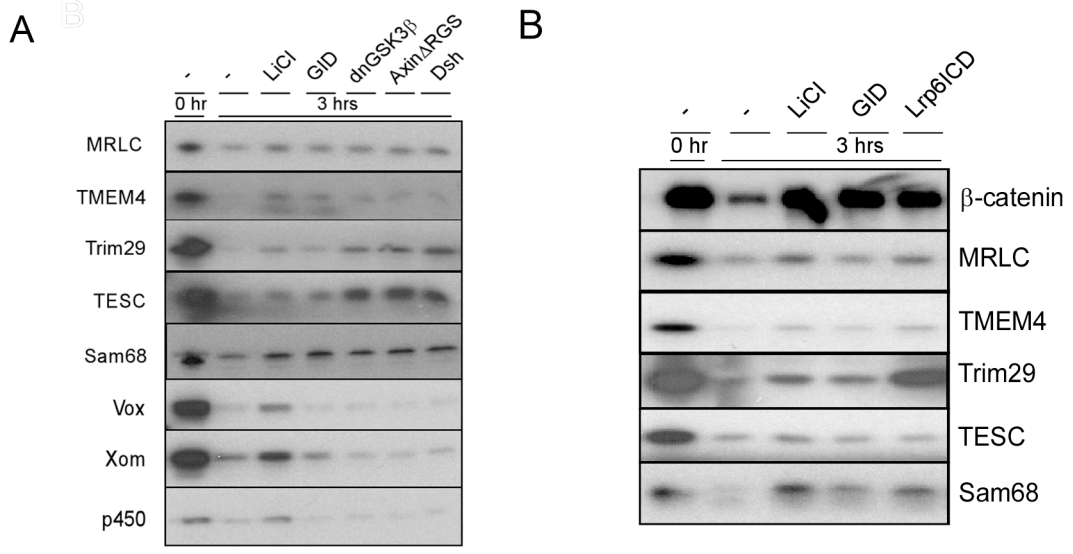

Figure 9. Selected proteolytic candidates of the Wnt/GSK3 signaling pathway were incubated in the *Xenopus* egg extract with various inhibitors of the destruction complex. (*A*) The degradation of Li/GID positive proteins were rescued by all the Wnt pathway activators, whereas Li-Only positive genes were not. (**–**)**:** 1x XB buffer added in the reactive as a negative control, **LiCl:** 25mM Lithium Chloride, **GID:** 0.8 μM of GID; **dnGSK3b:** 0.3 μM of dominant negative GSK3β (kinase dead); **AxΔRGS:** 0.2 μM of Axin protein missing the RGS domain (which binds to APC); **Dsh:** 0.2 μM of recombinant Dishevelled protein. (*B*) Selected proteolytic candidates of the Wnt/GSK3 signaling pathway were incubated in the *Xenopus* egg extract with or without low concentration of recombinant Lrp6ICD. (**–**)**:** 1x XB buffer added in the reactive as a negative control; **Li:** 25mM Lithium Chloride; **GID:** GSK3β-interaction domain of Axin; **Lrp6ICD:** 0.6 μM recombinant *Xenopus* Lrp6 intracellular domain.

The *Xenopus* embryo was used as an *in vivo* system to test whether the stability of some of these Li/GID positive proteins is regulated by Wnt signaling *in vivo*. Li/GID positive candidates were selected from each functional group (Figure 7) and *in vitro* transcribed mRNAs were injected along with myc-EGFP as a control into one of the ventral blastomeres of 4-cell stage *Xenopus* embryos. Either Wnt8 mRNA or a control mRNA (GFP) was co-injected, and at stage 12-12.5, levels of the expressed target

proteins in dissected animal caps (which have little endogenous Wnt activity [117]) were analyzed by Western blot (Figure 10). As expected, Wnt8 mRNA co-injection promoted the stabilization of β-catenin in animal caps without affecting the level of myc-EGFP protein (Figure 10*A*, Figure 10*B*). The levels of selected candidate target proteins, MRLC, TMEM4 and TESC, were also increased by Wnt8 co-injection (Figure 10*B*), indicating that their stabilities are similarly regulated by Wnt signaling. On the other hand, the protein expression levels of some candidate proteins, Sam68, Trim29, aldolase and DLST, were not increased (and for Sam68 and Trim 29, actually decreased) by Wnt8 co-injection, indicating that they may not be regulated by exogenous Wnt8 in early stage *Xenopus* embryo (Figure 10*C*). Instead, they may be regulated by the Wnt signaling pathway in other cellular contexts.

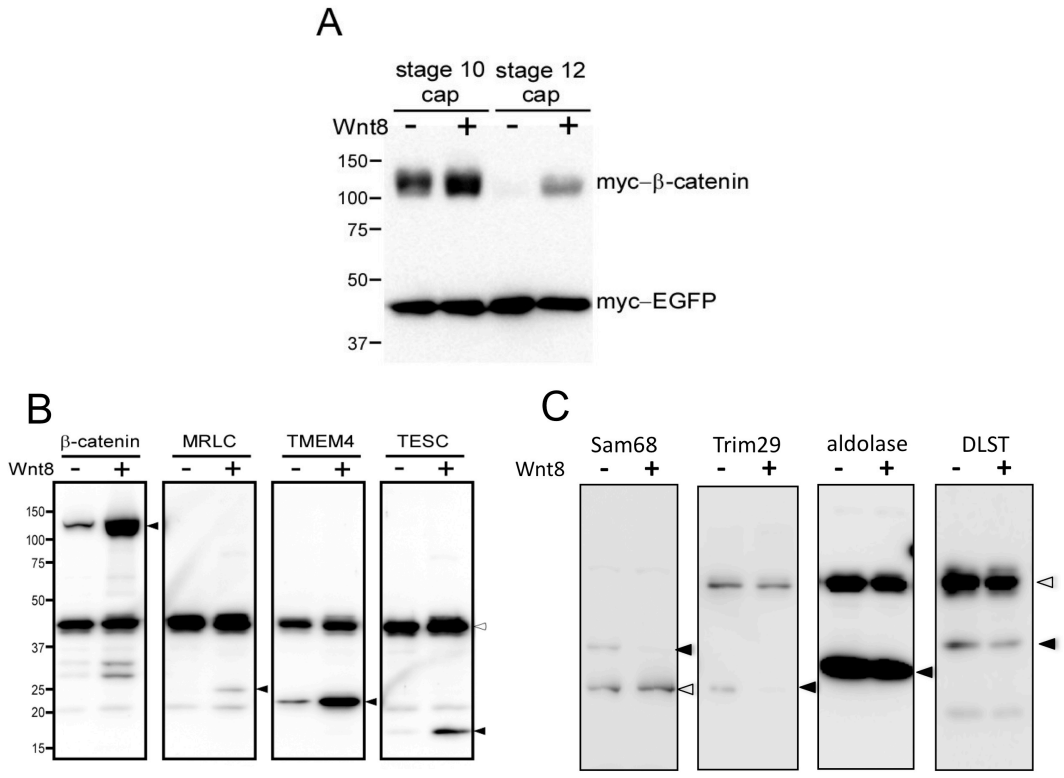

Figure 10. Wnt dependent protein stabilization in *Xenopus* embryos. (*A*) β-catenin level is regulated by Wnt8 co-injection *in vivo*. mRNA encoding β-catenin was injected into one of the ventral blastomeres of the 4 cell embryo along with Myc-EGFP mRNA as a non-target control. Cells were also co-injected with either Wnt8 mRNA or a control mRNA. Animal caps were dissected from either stage 10 or stage 12 injected embryos and lysed in SDS sample buffer. (*B*) Protein level of selected candidates were regulated by Wnt8 co-injection *in vivo*. mRNA encoding one of the target proteins indicated was injected into one of the ventral blastomeres of the 4 cell embryo along with Myc-EGFP mRNA as a non-target control. Cells were also co-injected with either Wnt8 mRNA or a control mRNA. Animal caps were harvested at stage 12-12.5. Closed arrowheads: β-catenin and target proteins; Open arrowhead: Myc-EGFP. (*C*) Candidates whose expression levels were not increased by Wnt8 co-injection *in vivo*. Experiments were performed as in (*B*). Closed arrowheads: candidate proteins; Open arrowhead: Myc-EGFP.

3.5 MUTATION OF PUTATIVE GSK3 SITES IN SELECTED CANDIDATE SUBSTRATES

It is important to find out whether our candidate Wnt/GSK3 regulated protein degradation substrates identified in the screen are direct phosphorylation substrates of GSK3. S/TxxxS/T^{p} is the GSK3 consensus phosphorylation site, in which the second (c-terminal) serine or threonine is usually primed by phosphorylation by another kinase (CK1α in the case of β-catenin) before GSK3 phosphorylates the N-terminal serine/threonine. Mutation of the GSK3 sites of β-catenin render it unable to be phosphorylated by GSK3, and in turn stablize it both in cells and in *Xenopus* egg extract, as described earlier (Figure 3).

Selected candidate protein sequences were scanned for putative GSK3 phosphorylation sites. The serine/threonines (S/T) in the identified sites were mutated into alanines (A) using standard site-directed mutagenesis procedure (S/T→A mutations). The stability of resulting mutants were then tested in the *Xenopus* egg extract degradation assay (Figure 11). Only one putative GSK3 consensus site was found in MRLC, two in TMEM4, and three in Sam68. Surprisingly, in none of the tested candidates (MRLC, TMEM4, Sam68 (Figure 11), and Tescalcin and Trim 29 (data not shown)), did the mutation of the putative GSK3 consensus phosphorylation sites block degradation (Figure 11). These candidate proteins therefore may not be direct GSK3 phosphorylation targets. Alternatively, there may be other potential GSK3 phosphorylation sites that we did not mutate.

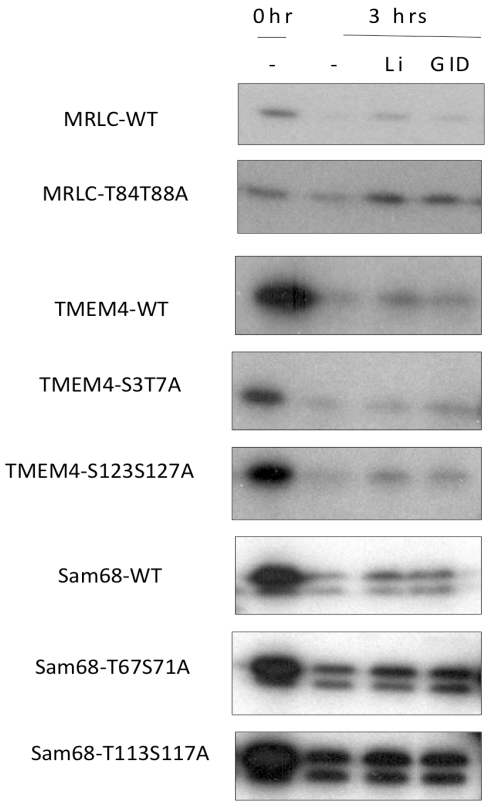

Figure 11. Mutation of putative GSK3 phosphorylation sites in selected Li/GID positive candidates did not block their degradation in the *Xenopus* egg extract. *In vitro* translated proteins of indicated constructs were added into freshly prepared *Xenopus* egg extract and incubated for three hours with or without the indicated inhibitors. **(–): 1x** XB buffer added in the reactive as a negative control, **LiCl:** 25mM Lithium Chloride, **GID:** 0.8 µM of GID, WT: Wildtype, S3T7A etc.: serine 3 and threonine 7 both mutated to alanine, and so on.

3.6 PLAKOGLOBIN AS A POTENTIAL CARRIER PROTEIN FOR THE DEGRADATION OF SELECTED CANDIDATES

The inability of S/T→A mutations on GSK3 consensus phosphorylation sites in the selected candidates to block their protein degradation in *Xenopus* extract (Figure 11) suggests that some of the proteolytic target proteins may not be direct GSK3 phosphorylation targets. Instead, there may be carrier proteins that bridge between GSK3 and the destruction complex and the candidate substrates identified and that some of the target proteins may be directed to degradation via their binding to unidentified carrier proteins.

Plakoglobin (γ-catenin) is a potential candidate for such a carrier protein. Plakoglobin is a member of the armadillo repeat family of proteins that plays an essential role in desmosomes and adherens junctions [53,118]. It is highly homologous to β-catenin, especially among the armadillo repeat regions where most of the interactions between β-catenin and the components of the destruction complex happen, and it also has conserved GSK3 phosphorylation sites at the N-terminus. Indeed, the stability of plakoglobin is regulated by Wnt and GSK3 dependent phosphorylation and subsequent degradation in a similar manner as β-catenin, although plakoglobin appears to be less susceptible to this proteolytic regulation [119]. Plakoglobin has also been show to bind LEF transcription factors, and to trigger axis duplication in Xenopus embryos when its mRNA is injected into the ventral blastomeres at early stages [120]. However, in contrast to β-catenin, the exact role of plakoglobin in the Wnt signaling pathway is not clear. Plakoglobin's structural similarity to β-catenin and its elusive role in the Wnt signaling pathway leads to

the hypothesis that plakoglobin may be a potential carrier protein for some of the candidate target proteins identified in the screen.

In order to test this hypothesis, plakoglobin was examined in the degradation assay to see if it is regulated by Wnt and by the destruction complex regulated protein degradation in our experimental systems. Although to a significantly less amount than β-catenin, *in vitro* translated plakoglobin proteins were degraded in freshly prepared *Xenopus* egg extract, and LiCl and GID protein inhibited their degradation, indicating that plakoglobin protein stability is indeed regulated by the destruction complex in the *Xenopus* egg extract in a similar manner to β-catenin (Figure 12A). When plakoglobin mRNA is injected into the *Xenopus* embryos, its expression level in the animal caps is regulated by Wnt8 RNA co-injection. Moreover, mutation of the conserved GSK3 phosphorylation site at the N-terminus of plakoglobin (serine 24 in *Xenopus* plakoglobin) increased the plakoglobin level and abolished the Wnt regulation (Figure 12B). These results indicate that plakoglobin protein stability is regulated by both Wnt signaling and the destruction complex in both systems, *in vitro* and *in vivo*.

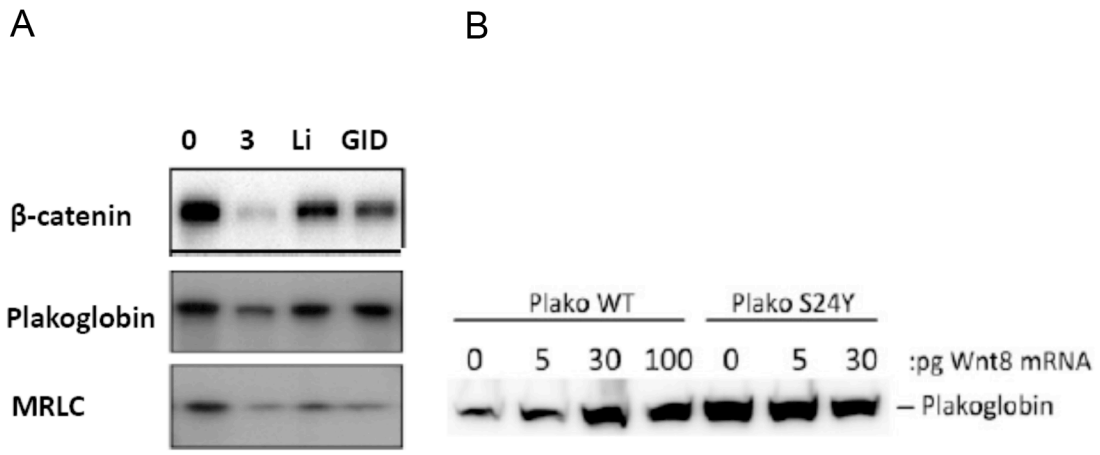

Figure 12. Plakoglobin protein stability is regulated by the Wnt signaling *in vitro* and *in vivo.* (A) *In vitro* translated proteins of indicated genes were added into freshly prepared *Xenopus* egg extract and incubated for three hours with or without the indicated inhibitors. (–)**:** 1x XB buffer added in the reactive as a negative control, **LiCl:** 25mM Lithium Chloride, **GID:** 0.8 μM of GID. (B) mRNA encoding either wildtype or mutant plakoglobin was injected into one of the ventral blastomeres of the 4 cell embryo. Cells were also co-injected with either Wnt8 mRNA or a control mRNA. Protein express levels in animal caps were observed at stage 12.

Next we sought to look at whether plakoglobin can regulate the protein stability of selected candidate target proteins from the screen. Knockdown of plakoglobin expression in early *Xenopus* embryos induces a severe reduction of cortical actin network in the embryo, providing a hint that plakoglobin may be involved in the regulation of proteins that play roles in actin cytoskeleton assembly and dynamics [(118)]. Therefore as a preliminary experiment, we checked whether excessive amount of wild-type and/or stabilized mutant form of plakoglobin or β-catenin could affect the protein degradation of

MRLC (myosin regulatory light chain) and β-catenin in the *Xenopus* egg extract, as MRLC has been know to play central roles in the regulation of actin filament dynamics [121,122]. β-catenin was used as a control for potential inhibitory saturation effects on the destruction complex. Addition of both wild and mutant type plakoglobin in the *Xenopus* egg extract inhibited the degradation of MRLC but not β-catenin, while addition of wildtype or mutant β-catenins inhibited the degradation of β-catenin but not of MRLC. These results indicate that plakoglobin specifically inhibits the degradation of MRLC through a mechanism that is not simply explained by saturation of the destruction complex machinery. Although less specifically, plakoglobin also inhibits the degradation of Sam68, another candidate target proteins identified in the screen, indicating a widespread role of plakoglobin in the regulation of Wnt controlled protein degradation (Figure 13). The stabilized mutant plakoglobin blocks the degradation of target proteins as effectively as wildtype plakoglobin, probably due to the fact that plakoglobin degradation in the *Xenopus* egg extract is inefficient (Figure 12), and the mutation of the GSK3 site only slightly increases the stability of plakoglobin in the *Xenopus* egg extract (data not shown).

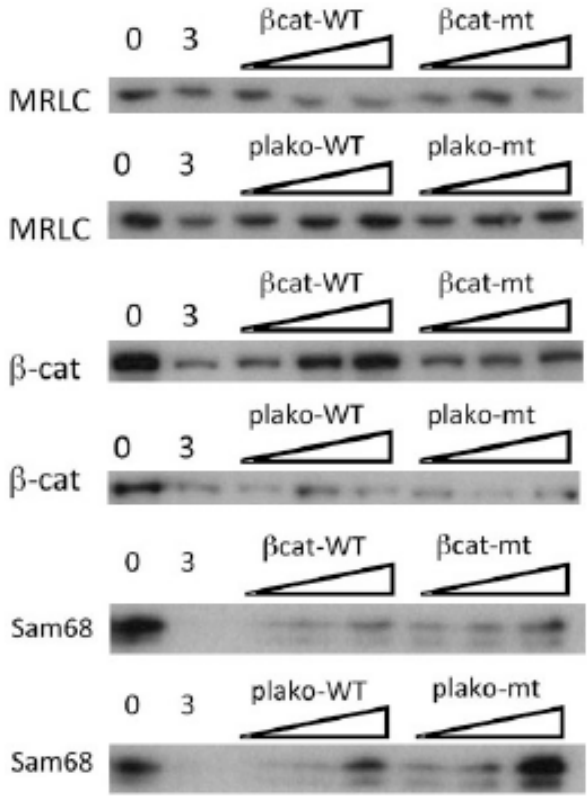

Figure 13. Excessive amount of plakoglobin specifically inhibits the degradation of selected protein targets in the *Xenopus* egg extract. Various amount of *in vitro* translated proteins of wildtype or mutant forms of plakoglobin or β-catenin are incubated with indicated target proteins in the *Xenopus* egg extract for three hours.

3.7 LICL-ONLY POSITIVE CANDIDATES ARE LIKELY GSK3 REGULATED PROTEOLYSIS SUBSTRATES

The LiCl-Only positive candidates identified in the screen may be substrates whose degradation is regulated by GSK3 independently of the destruction complex. It is also possible that LiCl can affect some other unknown kinases/enzymes in the *Xenopus* egg

extract that could potentially control the stability of these proteins. Interestingly, at a high concentration (1.3 μM), dnGSK3β blocked the GSK3 mediated phosphorylation of Tau protein in the *Xenopus* egg extract, indicating that it acts as a general GSK3β inhibitor at this concentration (Figure 9). Therefore high concentrations of dnGSK3β were tested for its effects on the stability of some representative Li-Only candidates in the *Xenopus* egg extract. Although low concentrations of dnGSK3β selectively stabilized β-catenin and Li/GID positive targets (Figure 9, Figure 14*A*), high concentrations of dnGSK3β effectively blocked the degradation of the Li-Only targets (Figure 14*A*). The cytoplasmic domain of the Wnt co-receptor protein Lrp6 (Lrp6ICD) has also been shown to regulate β-catenin stability in *Xenopus* egg extracts, due to its interaction with GSK3β [(123)]. Lrp6ICD selectively stabilized β-catenin and Li/GID positive target proteins in the extract when used at low concentrations (Figure 9*B*). However, similar to dnGSK3β, at higher concentrations Lrp6ICD also stabilized several Li-Only targets (Figure 14*B*). The stabilization of these Li-Only targets by the two GSK3 selective inhibitors, dnGSK3β and Lrp6ICD, as well as LiCl, suggests that they are regulated by GSK3 dependent phosphorylation.

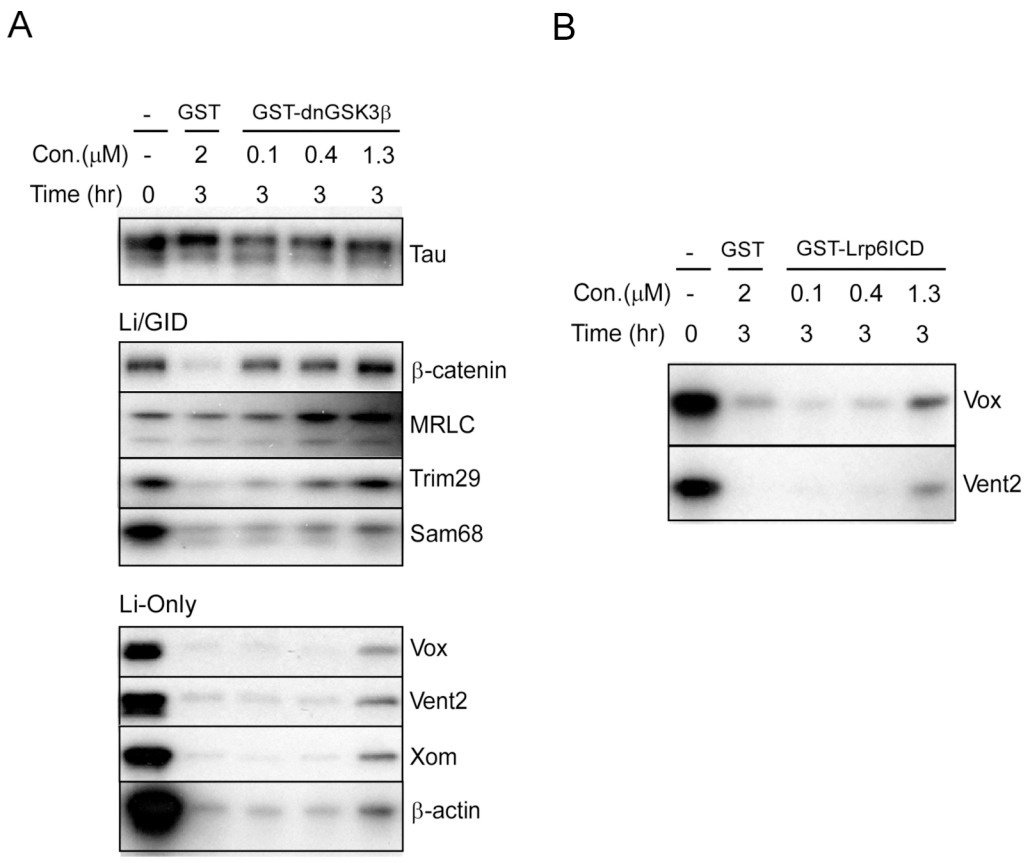

Figure 14. dnGSK3β and Lrp6ICD inhibit general GSK3β activity at high concentration. (*A*) Selected proteins were incubated with recombinant dominant GSK3β (dnGSK3β, kinase dead) at different concentrations in the *Xenopus* egg extract. Recombinant GST protein was used as a negative control. At high concentration (>=1.3μM), dnGSK3β generally inhibit GSK3 activity in the *Xenopus* egg extract. At low concentration (0.1μM ~ 0.4μM), dnGSK3β specifically blocked degradation of β-catenin and Li/GID positive candidate MRLC, but not general GSK3 phosphorylation of Tau protein or degradation of Li-Only candidate proteins. (**–**)**:** 1x XB buffer; (*B*) Higher concentration of Lrp6ICD inhibited the degradation of Li-Only target proteins. Selected proteins were incubated with recombinant Lrp6ICD at different concentrations in the *Xenopus* egg extract. Recombinant GST protein was used as a negative control. At high concentration (>=1.3μM), Lrp6ICD inhibited the degradation of Li-Only candidate proteins. (**–**)**:** 1x XB buffer.

Interestingly, three members of the Vent2B/Vox family (Vox1, Vent2 and Xom), a homeobox containing family of ventralizing transcriptional repressors, were identified as putative proteolytic target proteins regulated by GSK3 (Li-Only) from different cDNA pools. Xom is already known to be degraded in early gastrula stage *Xenopus* embryo extract and rescued by treatment with LiCl [(124)]. The putative GSK3 phosphorylation sites that are important for the proteolytic regulation of Xom in *Xenopus* embryos are well conserved among the Vent2B/Vox sub-family members, but not the closely related Vent-1B sub-family that has a different response to upstream BMP signaling [(124)] (Figure 15). To determine whether this proteolytic event is specific and applicable to the whole Vent/Vox sub-family, another Vent2B/Vox sub-family member Vent-2B, as well as Vent-1 of the distinct Vent-1B sub-family was cloned from a *Xenopus* cDNA library and tested in the degradation assay. The degradation assay showed that Vent-2B protein is degraded by the *Xenopus* egg extract and rescued by LiCl, similar to the other Vent2B/Vox sub-family members, whereas Vent-1 is stable in the *Xenopus* egg extract (Figure 15*B*). Mutation of the conserved GSK3- consensus phosphorylation sites completely blocked the degradation of Vox1, Vent2 and Xom (Figure 15*C*), and dnGSK3β and Lrp6ICD effectively inhibited their degradation (Figure 14). This suggests that protein half lives of all the Vent2B/Vox sub-family members are specifically regulated by GSK3β, and that the conserved GSK3 consensus sites are important for this regulation.

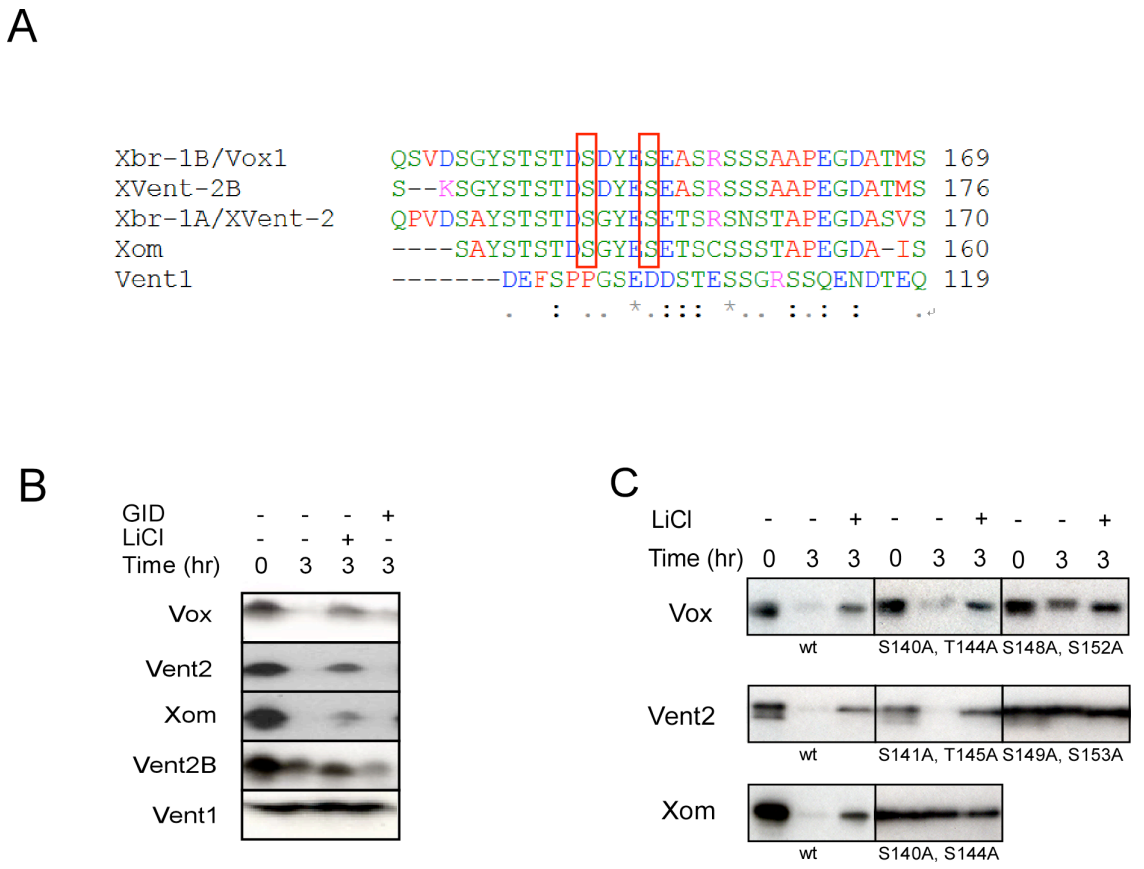

Figure 15. Degradation assay of the Vent/Vox family members. (*A*) Protein sequence alignment of Vox1, Vent2B, Vent2, Xom (Vent2B/Vox sub-family) showing the highly conserved GSK3-like phosphorylation sites (red box) that are absent in Vent1 (Vent-1B sub-family). (*B*) Vent2B/Vox sub-family member proteins were degraded in the *Xenopus* egg extract and rescued by LiCl, whereas Vent1 (Vent-1B sub-family) was not degraded. (–)**:** 1x XB buffer added in the reaction as a negative control; **Li:** 25 mM Lithium Chloride; **GID:** GSK3β-interaction domain of Axin. (*C*) When the serines at the conserved GSK3-like consensus phosphorylation sites (S148, S152 for Vox; S149, S153 for Vent2 and S140, S144 for Xom) were mutated to alanine, the degradation of Vent2B/Vox sub-family member proteins in the *Xenopus* egg extract was blocked. On the contrary, mutation of another GSK3 like consensus site (S140, T144 for Vox; S141, T145 for Vent2) did not stabilize the proteins. (–)**:** 1x XB buffer added in the reaction as a negative control; **Li:** 25 mM Lithium Chloride;

3.8 A BIOINFORMATICS BASED SEARCH FOR POTENTIAL GSK3/B-TRCP REGULATED PROTEINS

Both β-catenin and Snail contain a "DSGxxS/TxxxS/T" motif that is necessary for their regulation by Wnt signaling [59]. D/ESG$(X)_{2+n}$S is the recognition site for the E3 ubiquitin ligase β-Trcp: when the serine in the D/ESG motif is phosphoyrlated, β-Trcp binds to this motif and adds ubiquitin onto a lysine usually located upstream [125]. On the other hand, "S/TxxxS/T^{p}" is the GSK3 consensus phosphorylation site, in which the second (c-terminal) serine or threonine is usually primed by phosphorylation by another kinase (e.g. CK1α in the case of β-catenin) before GSK3 phosphorylates the N-terminal serine/threonine. In many cases there are tandem repeats of this consensus motif, resulting in processive phosphorylation as in the case of β-catenin [107] (Figure 16).

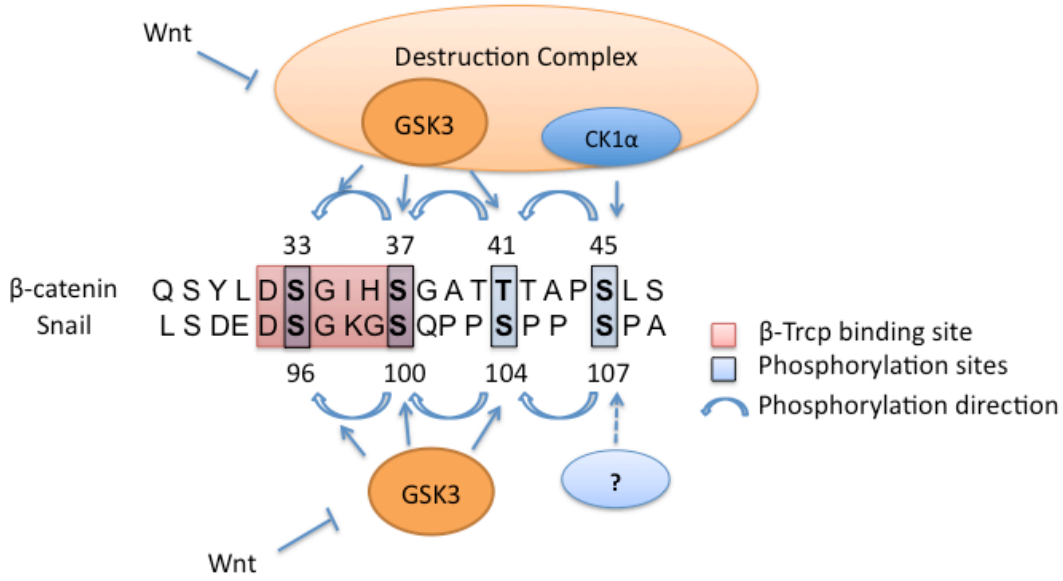

Figure 16. Tandem phosphorylation of β-catenin and Snail N-terminal residues by GSK3 and recognition by β-Trcp. Once prime phosphorylated at the downstream conserved serines (S45 by CK1α for β-catenin and potentially S107 for Snail), GSK3 progressively phosphorylates the tandem S/TxxxS/T sites and ultimately creates a binding site for β-Trcp E3 ubquitin ligase, resulting the degradation of the substrate.

It is therefore hypothesized that proteins containing the DSGxxS β-Trcp binding motif followed by tandem GSK3 consensus sites are likely regulated by GSK3 and β-Trcp in a similar fashion as β-catenin and Snail, and thus possibly by Wnt signaling. With this hypothesis, a search for proteins containing a "D/ESGxxS/TxxxS/TxxxS/T" motif was performed in the curated UniProtKB/Swiss-Prot protein database. The search identified 605 hits in 579 sequences in the database that contains ~ 500,000 sequence entries (release 57.4, eukaryota). As important regulatory amino acid motifs are usually evolutionarily conserved, each hit sequence was blasted against its homologs and those contained motifs that were evolutionarily conserved among numerous species were

identified. This narrowed the list down to 38 potential protein substrates regulated by GSK3 and β-Trcp. They can be included in functional groups (Table 3), indicating potential GSK3/β-Trcp regulation of the respective cellular processes.

Table 3. Potential GSK3/β-Trcp regulated proteolysis substrates

Cell Motility and Cytoskeleton	Transcription Factors	Cell Signaling	Protein modification	Adaptor / scaffold	GTPase/GEF	Unknown
ABLIM2	NACα	BNIP3	WNK2	Gephyrin	FGD3	BAZ2B
Cappuccino	DMRT	EPOR	ARD1	DOK7	RAP GEF1	C1orf172
KIF1B	β-catenin	I10R1	BRD3	JIP3	TBC15	FAM135A
KIF1C	Snail	LZTS2	KDIS	AKAP 220		PRIC3
Ninein	WWTR1	NP1L3	PLCH1			TRI67
Plakoglobin	ZBT20	PRP16				YQ013
Unc-84	ZN395					

Both β-catenin and Snail were, by design, identified in the search. Interestingly, the search identified FGD3 (faciogenital dysplasia 3), a putative Cdc42 guanine nucleotide exchange factor (GEF) whose stability was reported to be regulated by GSK3 and β-Trcp [86] and plakoglobin, which was known to be regulated by Wnt signaling in a similar manner as β-catenin as mentioned before [53,55,56]. These two positive hits serve as validation for the screen.

The search also identified many interesting potential GSK3/ β-Trcp targets. NACα (nascent-polypeptide-associated complex alpha polypeptide), which plays a role in nascent polypeptide localization, has been reported to be regulated by GSK3 phosphorylation dependent ubiquitination, but not the DSG motif and β-Trcp (Table 2). Further studies may help clarify this apparent discrepancy. Additionally, AKAP220 (A-kinase anchor protein 220), which regulates the subcellular localization of protein kinase

A (PKA) and Ninein, a centrosomal protein, have been found to bind GSK3β in yeast two-hybrid experiments. [126,127] EPO-R (erythropoietin receptor precursor) is known to be a β-Trcp substrate [128]. These protein are therefore strong candidates for GSK3 and β-Trcp regulated proteolysis. There are also two hits, ARD1 (N-terminal acetyltransferase complex subunit) and LZTS2 (Leucine zipper putative tumor suppressor 2), that have been shown to interact with β-catenin and play roles in the canonical Wnt signaling pathway: ARD1 acetylates β-catenin and increases its transcriptional activity, thus promoting cell proliferation; while LZTS2 induces nuclear export of β-catenin and thus reducing β-catenin dependent transcription activity [129,130]. Therefore, this bioinformatics based search provided some interesting hypotheses for further testing.

A few candidate proteins from this bioinformatics based search were tested in the *Xenopus* egg extract degradation assay. Primary results showed BNIP3, a pro-apoptic bcl2 family protein, to be Li-Only positive in the assay, in line with the roles of GSK3 in regulation of apoptosis; and ARD1 was stable in the *Xenopus* egg extract (data not shown). Further experiments are needed to fully test the potential GSK3 dependent proteolysis regulation of these candidate proteins. Confirmation of these potential GSK3/β-Trcp regulated proteolysis targets will provide exciting opportunities to explore the roles of GSK3 controlled protein degradation in the cell. As β-catenin and Snail are both regulated by GSK3/β-Trcp and they are regulated by Wnt signaling, it is also conceivable that some of these proteins may be regulated by the Wnt signaling pathway in a similar fashion, thus providing more evidence in support of the finding that Wnt signaling pathway regulates the ubiquitination and degradation of multiple proteins in the cell.

4.0 DISCUSSION

4.1 IDENTIFICATION OF NOVEL WNT/GSK3 REGULATED PROTEIN SUBSTRATES

Wnt signaling has been found to play significant roles in a broad range of cellular behaviors and it has long been understood that not all the functions of Wnt signals can be attributed to the canonical β-catenin mediated transcription regulation. In this study, the working hypothesis is that there are multiple mediators of the Wnt signaling pathway that are regulated by Wnt/GSK3 in addition to β-catenin,. Indeed, there were several reports that Wnt/GSK3 regulates the stability of the E-cadherin repressor Snail in the epithelial-mesenchymal transition (EMT) [58-60]. More recently during the study, another report showed that the activated form of the TGFβ signaling pathway molecule Smad1 is regulated by Wnt/GSK3 in a similar fashion, providing another piece of evidence for the hypothesis [61].

In order to provide additional evidence in support for the hypothesis and draw a more complete picture of the β-catenin independent branches of the Wnt/GSK3 signaling

pathway, this study aimed at identification of novel phosphorylation and proteolytic targets for the Wnt/GSK3 signaling pathway in a systematic fashion. Such proteolytic target proteins are difficult to identify using conventional biochemical methods, as they are by nature labile and may present only in very small quantities in the cell. Also, the details of regulation could be cell-context specific, as exemplified in the case of β-catenin in many epithelial cell lines, where the majority of β-catenin actually binds to cadherins and is not subject to GSK3 phosphorylation and degradation under normal conditions. A biochemical screen was designed to screen for these Wnt/GSK3 regulated proteolysis substrates, taking advantage of the IVEC technique to circumvent the strict requirement for protein purification and the *Xenopus* egg extract system to recapitulate cytoplasmic events in an *in vitro* setting.

42 potential novel target proteins of the Wnt signaling pathway and/or GSK3 regulation were successfully identified in a screen using IVEC and the *Xenopus* egg extract degradation assay, including 35 proteolytic and 7 phosphorylation targets. They fall into several categories, including transcriptional regulators, RNA associated proteins, cytoskeletal proteins and regulators, signaling proteins, and metabolic enzymes. Identification of this very selective but diverse number of novel Wnt/GSK3 regulated target proteins provided direct evidence in support of the hypothesis that there are multiple signaling mediators besides β-catenin whose degradation is specifically regulated by the Wnt/GSK3 pathway. Instead of the traditional linear view of the canonical Wnt signaling pathway through regulation of β-catenin dependent transcription activation, we propose that the Wnt signaling pathway is better understood as a broad signaling network in which Wnt signaling can regulate the activity and/or stability of a

diversity of proteins, including transcription factors, RNA binding proteins and cytoskeletal proteins, that in turn control gene expression, cell physiology, and cell behavior (Figure 17).

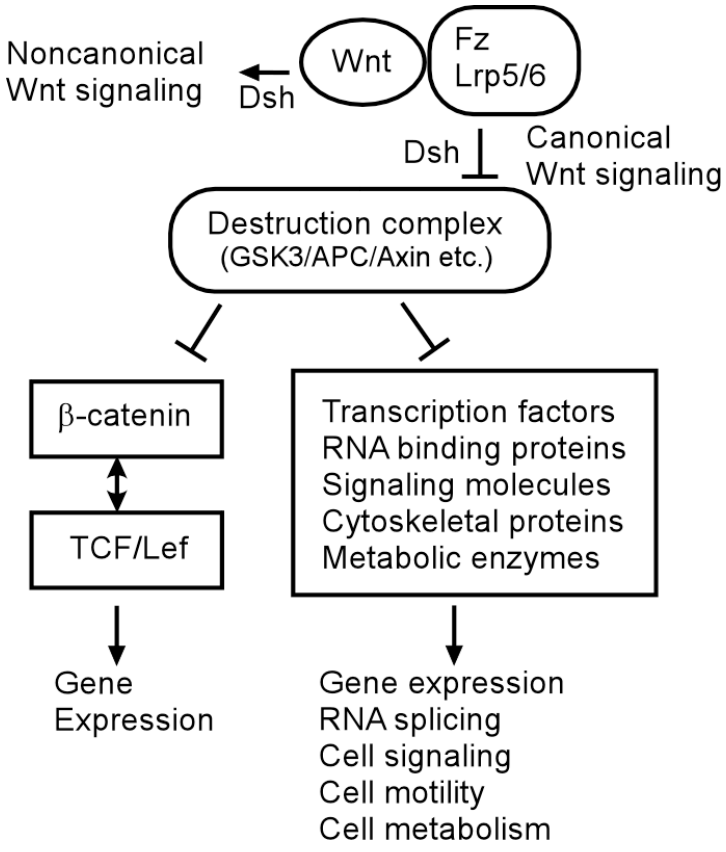

Figure 17. A model for the expanded canonical Wnt/GSK3 signaling pathway. Binding of Wnt proteins to receptors triggers canonical (β-catenin and the destruction complex dependent) or non-canonical (β-catenin and the destruction complex independent) Wnt signaling. In the canonical Wnt signaling pathway, the stability of β-catenin is controlled by destruction complex composed by GSK3, APC and Axin etc. In addition to β-catenin, the degradation of many different proteins in various functional classes is regulated by the destruction complex and Wnt receptor signaling.

With modification, the biochemical screen method designed in this study can also potentially be used to identify other signaling proteins, such as upstream regulators of the Wnt signaling pathway. On the other hand, this screen method has its limitations. The screening conditions favor protein substrates that have relatively short half-life (less than 3 hours) and small size (<50KD). Also, many of the cDNAs in the cDNA library are not full-length clones and lack N-terminal regions due to the construction procedure of cDNA library (cDNA synthesis using Poly-T as primer and restriction enzyme digestion during clone procedures), therefore many proteins whose N-terminal region contain critical motifs that are responsible for Wnt and/or GSK3 mediated regulation were potentially not identified in the screen. Moreover, the *Xenopus* egg extract system, as powerful as it is in recapitulating cellular events in cytoplasm, only represents one of the many possible physiological states of the cell and may miss some important cell signaling regulators. All these limitations of the screen design and methodology determined that this screen is far from comprehensive and probably explains why we did not identify β-catenin, our positive control, from the screen itself.

4.2 POTENTIAL CANDIDATE TARGETS OF THE WNT SIGNALING PATHWAY AND THE DESTRUCTION COMPLEX REGULATED PROTEOLYSIS

Among the 42 potential novel Wnt and/or GSK3 regulated proteolysis target proteins, of particular interest are 12 proteins whose degradation is inhibited by the GID

protein, a potent inhibitor of Axin-dependent GSK3 phosphorylation, making them strong candidates for targets of the Wnt signaling pathway and the destruction complex. The GID sensitive candidates were a small subset of all the LiCl sensitive targets. Also, GID was not able to inhibit the phosphorylation or degradation of known GSK3β targets in the *Xenopus* egg extract, indicating that GID works specifically on the destruction complex, whereas LiCl directly inhibits GSK3 kinase activity.

Further analysis of the specificity of the degradation of the 12 Li/GID candidate proteins using additional Wnt pathway activators confirmed that their stability was regulated, similar to β-catenin, by Axin, APC and Dsh, and therefore the destruction complex. Furthermore, we verified that Wnt signaling regulates the levels of at least three of these proteins (MRLC, TMEM4, TESC) *in vivo*, as a result of Wnt8 expression in *Xenopus* embryos.

4.3 LI-ONLY CANDIDATES ARE LIKELY TO BE TARGETS OF GSK3 BUT NOT WNT REGULATED PROTEOLYSIS TARGETS

At least some of the Li-Only candidates are likely to be targets of different non-Wnt, but GSK3-dependent signaling pathways, because dnGSK3β and Lrp6ICD inhibit their degradation. GSK3 phosphorylates a great number of proteins and resides in the middle of many important signaling pathways that respond to cellular stimuli, such as growth factors and stress, and GSK3 has been found to regulate the ubiquitination and proteolysis of a number of important signaling proteins. The results from our recent

screens indicate that there are many more proteins whose stability may be regulated by GSK3 phosphorylation. Protein stability control is thus emerging as a major mechanism that GSK3 employs to modulate cellular processes. Moreover, many different signaling pathways utilize this GSK3 dependent mechanism to target proteins for turnover. We speculate that GSK3 is a conserved kinase that regulates protein turnover in response to cellular signals, and in turn regulates a variety of cell processes such as cell proliferation, differentiation, apoptosis, embryonic patterning, and tumorigenesis.

4.4 WNT SIGNALING PATHWAY PLAYERS IDENTIFIED IN THE SCREEN

Some proteins identified in the screen are known to interact with β-catenin and play roles in the canonical Wnt signaling pathway. Two of the LiCl and GID positive targets, Trim29 and TACSTD1, have recently been reported to be positive regulators of β-catenin signaling. TACSTD1, also called EpCAM, is a membrane protein that is up regulated in cancer cells and was one of the first identified cancer antigens.[131-133] It undergoes proteolytic cleavage upon extracellular domain homophilic binding and releases the intracellular domain (EpICD). EpICD then enters the nucleus and binds to transcription factors including β-catenin and Lef-1, and activates downstream gene expression.[134] Trim29, also known as ATDC (ataxia-telangiectasia group D complementing gene), was found to positively regulate Dishevelled levels and inhibit the destruction complex, thus stabilizing β-catenin.[135] Potential Wnt regulation of the stability of EpCAM and ATDC therefore suggests that there exists feedback control of the canonical Wnt/β-catenin

signaling pathway, probably resulting in fine control of the signaling strength and length in various cellular contexts.

Some Li-Only positive and Shifted candidates also play roles in the regulation of Wnt signaling pathway. Parafibromin, the mammalian homolog of yeast cdc73, is a tumor suppressor gene that was originally identified as the gene associated with the hyperparathyroidism-jaw tumor (HPT-JT) syndrome [136] and was later found to be part of the RNA polymerase II/Paf1 complex important for histone modification and transcriptional regulation [137,138]. Interestingly, it was recently reported to directly associate with β-catenin in the nucleus and together with Pyogopus, another transcription co-activator, mediate the transcriptional activity of β-catenin/TCF [139]. Casein kinase 2b (CK2b) is also a positive regulator of the canonical Wnt signaling pathway [140,141]. CK2b is the regulatory subunit of the ubiquitous serine/threonine kinase CK2, and it was shown to be able to induce ectopic dorsal axis when the RNA was injected into the ventral side of the Xenopus embryo [140]. It can phosphorylate β-catenin at the central armadillo repeat domain and stabilize the β-catenin protein [142,143]. It also phosphorylates Lef-1, enhances its binding to β-catenin and subsequent transcriptional activation of downstream genes[141]. On the other hand, Sox3 and Fus negatively regulate β-catenin/Tcf transcription activity when they bind to β-catenin [144-146]. Sox3 belongs to the Sox family of HMG box transcription factors that play important roles in embryonic development. Sox3 and Sox17 have been found to bind to β-catenin and inhibit its TCF-mediated signaling activity[144], and recently, it was reported that different Sox family members can either positively or negatively regulate the β-catenin mediated transcription[145]. However, the detailed mechanism and regulation of this signaling pathway is not yet known. Fus

was also found to bind to β-catenin and negatively regulate its transcriptional activity via unknown mechanisms [146]. GSK3 regulation of the stability and/or phosphorylation of these proteins could suggest the existence of novel layers of regulation in the canonical Wnt signaling pathway, or provide possible crosstalk between other signaling pathways and the Wnt pathway.

4.5 POTENTIAL ROLE OF WNT/GSK3 REGULATED PROTEOLYSIS IN REGULATION OF CYTOSKELETAL DYNAMICS

Many of the proteins identified in the screen are known to interact with each other or belong to specific functional groups (Figure 7), raising the possibility of coordinated regulation of corresponding cellular processes. One example includes MRLC (myosin regulatory light chain) and TMEM4 (transmembrane protein 4, also known as MASP for MIR-interacting saposin-like protein). MRLC is a main regulator of myosin contractility and cell motility, and its activation has been known to be regulated by multiple signaling pathways.[121,122,147] On the other hand, there have been several studies suggesting that Wnt signals may regulate the dynamics of the actin cytoskeleton and increase cell spreading and migration [148-152]. Particularly interesting is a recent report that in *C.elegans*, Wnt/Frizzled signaling regulates embryo gastrulation through regulation of actomyosin [153]. Little is known about TMEM4, but it has been shown to interact with MRLC and positively regulate its activity and stability [154,155]. This finding indicates that canonical Wnt signaling may also control cell motility in some cell contexts through the

destruction complex mediated regulation of MRLC and TMEM4 stability. Interestingly, components of the destruction complex- GSK3 and APC- have been implicated in regulation of cell polarity and cell migration through their regulation of microtubule polarity and dynamics [156,157]. It is also intriguing that some types of actins were identified in the screen as Li-Only candidates, indicating a role of GSK3 in regulation of actin protein stability (Figure 7).

4.6 POTENTIAL ROLE OF WNT/GSK3 REGULATED PROTEOLYSIS IN REGULATION OF RNA PROCESSING

Another interesting example of functional groups identified in the screen is a group of RNA binding proteins, including the RNA binding proteins TIAR (T-cell restricted intracellular antigen-related protein), the STAR (signal transduction and activation of RNA) family protein Sam68, Fus (*fused in sarcoma/translated in liposarcoma* (*FUS/TLS*) gene product) and FusIP1 (Fus interacting protein 1). TIAR and Sam68 are both Li/GID positive in the screen and therefore potential Wnt signaling regulated destruction complex substrates. TIAR has been shown to bind to U-rich sequences near 5' splice sites of pre-mRNAs and modulates alternative splicing.[158,159] Indeed, one bioinformatics study estimates that ~15% of alternative cassette exons in the genome are regulated by TIA1/TIAR, suggesting a widespread role of TIAR in the regulation of alternative splicing.[160] Sam68 has been reported to regulate the splicing of genes, such as CD44 and Bcl-x, and its activity is regulated by cellular signaling and phosphorylation

[161,162]. Fus is a RNA binding protein and the product of *fused in sarcoma/translated in liposarcoma* (*FUS/TLS*) protooncogene, and mutations of the gene cause amyotrophic lateral sclerosis (ALS) [163,164]. Fus degradation was regulated by Li-only in the screen, and it has been suggested to work together with β-catenin to regulate pre-mRNA splicing [146]. Fus Interacting Protein 1 (FusIP1), a splicing factor that binds to Fus [165] and regulates RNA splicing in response to cellular signals [166], was identified in the screen by GSK3 dependent mobility shift. Taken together, these results raise the exciting possibility that Wnt/GSK3 signaling may affect gene expression via regulation of multiple mRNA splicing and processing proteins. The majority of eukaryotic genes go through pre-mRNA splicing during the expression to remove introns or generate alternatively spliced isoforms. However, the regulation of mRNA splicing, especially by the various cell signaling pathways, is poorly understood despite recent progresses[167]. Therefore, potential Wnt signaling dependent regulation of mRNA splicing in the cell may have important implications in our understanding of the regulation of RNA splicing.

4.7 GSK3 MAY REGULATE THE STABILITY OF VENT/VOX FAMILY PROTEINS DURING EMBRYONIC DEVELOPMENT

Another interesting family of proteins identified in the screen as candidates for GSK3 regulated degradation is the Vent/Vox protein family. The Vent/Vox homeobox containing transcriptional repressor is known to play critical roles in early patterning of *Xenopus* and zebrafish embryos by antagonizing the dorsalization effect of the Spemann

organizer, and the transcription of the Vent/Vox family genes are regulated by the BMP and Wnt signaling pathways [124,168-170]. Consistent with the findings from this screen, one of the family members, Xom, was reported to be degraded during early gastrulation and regulated by a LiCl sensitive activity via a GSK3-like consensus sequence [124]. The GSK3 consensus sequence of Xom protein is well conserved in the other protein family members, which in *Xenopus* includes Vent2B, Vent2 and Vox. We found that all Vent2B/Vox family proteins were similarly degraded in the *Xenopus* egg extract and rescued by LiCl, dnGSK3β and Lrp6ICD. Mutation of the conserved GSK3-consensus phosphorylation sites stabilized the proteins in the *Xenopus* egg extract. Thus, our findings indicate that rapid proteolytic regulation of Vent2B/Vox family proteins during embryonic development is likely to be universal, and this regulation might be dependent on the GSK3 activity but not Wnt signaling.

Although degradation of Xom in the previous study was found to be mediated by a GSK3 consensus sequence, immune-depletion of GSK3 from the *Xenopus* egg extract did not prevent degradation [124]. However, our findings that two additional GSK3 inhibitors, dnGSK3β and Lrp6ICD, blocked the degradation of these family members, suggests that GSK3 might be involved in their regulation. We cannot completely exclude that Xom degradation is regulated by another kinase that is highly similar to GSK3β.

4.8 PLAKOGLOBIN AS A POTENTIAL CARRIER PROTEIN FOR THE WNT SIGNALING REGULATED PROTEOLYSIS FOR SOME CANDIDATE PROTEINS

Some of these degradation substrates of the destruction complex may not be direct phosphorylation targets of GSK3, as mutation of the putative GSK3 phosphorylation sites in selected candidate substrates failed to stabilize the proteins in the *Xenopus* egg extract (Figure 11). Instead, there may be carrier proteins that bind to these protein substrates and components of the destruction complex, and at the end mediate the degradation of these substrates. The armadillo repeat protein family member plakoglobin may be one such carrier protein, due to its structural similarity to β-catenin, its capability to interact with the destruction complex, and its elusive role in the canonical Wnt signaling pathway. Indeed, plakoglobin was found to be regulated by the destruction complex in a similar manner to β-catenin in our experimental systems (Figure 12), consistent with previous report [53]. Plakoglobin degradation in the *Xenopus* egg extract is significantly less compared to β-catenin (Figure 12), which may be due to its interaction with membrane/cytoskeleton proteins [171], or possible O-Glycosylation close to the N-terminus where the GSK3 and β-Trcp binding sites and ubiquitination sites are located [172]. Addition of excessive amounts of plakoglobin specifically inhibited the degradation of MRLC, and to a less extent Sam68, two of the target proteins identified in the screen, but not the degradation of β-catenin, supporting the hypothesis that plakoglobin may act as a carrier protein for the degradation of some of the substrates in our screen. On the other hand, plakoglobin may regulate the destruction complex via some other unknown mechanisms.

4.9 CONCLUSION

The identification of the novel Wnt regulated proteolysis targets in this study, in addition to the recent reports of Wnt regulated stability control of Snail and Smad proteins, indicates that protein stability control by the GSK3/destruction complex is the essence of the Wnt signaling pathway, rather than the β-catenin dependent gene transcription. A lot of important questions arise with the identification of these novel target proteins. Are they ubiquitously regulated by Wnt signaling in all cell types, or are there different proteins mediating Wnt signaling in different cell contexts and processes? In the latter case, how does the temporal and spatial regulation of the different mediators happen in the cells and how do other major signaling pathways in the cell contribute to the regulation of these proteins? Further study of these novel target proteins should lead to answers to these questions and provide valuable insights into the regulation and mechanism of Wnt and GSK3 signaling pathways during embryonic development and tumorigenesis, and open up exciting new possibilities for further explorations.

On the other hand, GSK3 has been found to regulate the ubiquitination and proteolysis of a number of important signaling proteins or transcription factors. The variety of potential novel GSK3 regulated proteolysis target proteins identified in this screen provide strong evidence that GSK3 phosphorylates a great number of proteins and resides in the middle of many important signaling pathways that respond to cellular stimuli, such as growth factors and stress. Protein stability control is thus emerging as a major mechanism that GSK3 employs to modulate cellular processes. GSK3 is therefore emerging as a conserved kinase that regulates protein turnover in response to cellular

signals, and in turn regulates a variety of cell processes such as cell proliferation, differentiation, apoptosis, embryonic patterning, and tumorigenesis.

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