NOVEL AND DIVERSE ROLES OF STRAP IN MAINTENANCE OF MESENCHYMAL MORPHOLOGY AND GSK3β SIGNALING

By

Nilesh Kashikar

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

August, 2010

Nashville, Tennessee

Approved:

Professor Pran K. Datta

Professor Robert J. Matusik

Professor Sarki A. Abdulkadir

Professor Anna L. Means

Professor Utpal P. Davé

To my parents and my dear wife for their love and support …

ACKNOWLEDGEMENTS

Throughout my time as a graduate student at Vanderbilt, I have received great help from my mentors, friends, and co-workers. Without their help, it would be impossible for me to complete my research and my study at Vanderbilt. I would like to thank my PI, Dr. Pran Datta, for mentoring me and providing his knowledge and experience for my research. I would also like to thank my committee members, Dr. Robert Matusik, Dr. Sarki Abdulkadir, Dr. Utpal Dave and Dr. Anna Means, who gave me helpful suggestions for my research.

The electron microscopic analysis was done under guidance of Dr. Jay Jerome (Electron Microscopy Core Center, Vanderbilt University.

Dr. Sunil Halder provided helpful suggestions during a lot of experimental techniques and Arunima Datta helped during many experimental procedures. I want to thank Jennifer Reiner for providing the STRAP re-expressing MEFs and also for many helpful suggestions. I want to thank all the members in my lab for helping me with my experiments and giving me suggestions.

I would really like to thank my family and friends for putting up with me all these years, for their love, and for their support. I would like to thank my parents, Digvijay Bhavsar and Charushila Bhavsar, and my brother Sunil Kashikar who encouraged me to go further in my academic career and for their tender care throughout my life. Finally, I would like to thank my beloved wife,

Smita Kashikar, for her constant understanding, support and unconditional love. I might never have achieved this goal if she has not been by my side. Thank you very much Smita.

Table of contents

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

- ASK1 Apoptosis signal-regulating kinase 1
- Cdk Cyclin-dependent kinase
- Cdk2 Cyclin dependent kinase 2
- CK1 Casein Kinase 1
- PDGF Platelet Derived Growth Factor
- ERK Extracellular signal-Related Kinase
- EWS Ewing sarcoma
- GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase
- GFP Green Fluorescence Protein
- GS Glycogen Synthase
- GSK-3 Glycogen Synthase Kinase-3
- GSK-3α Glycogen Synthase Kinase-3alpha
- GSK-3β Glycogen Synthase Kinase-3beta
- GST Glutathione-S-Transferase
- ICN Intracellular Notch
- IgG Immunoglobulin G
- IHC Immunohistochemistry
- IRES internal ribosomal entry site
- JNK c-Jun N-terminal kinase
- LEF Lymphoid-Enhancer Factor
- LiCl Lithium Chloride

- MAP Microtubule Associated Protein
- MAP1B Microtubule Associated Protein 1 Binding Protein
- MAPKAP-K1 MAPK-Activated Protein Kinase 1 or p90Rsk
- MAPK Mitogen-Activated Protein Kinase
- Mdm2 Double minute 2 protein
- MEFs Mouse embryonic fibroblasts
- MEK MAPK/ERK kinase
- NXF nuclear export factor
- NLS Nuclear Localization Signal
- NM23 non-metastatic cells 1, protein
- PAK1 p21-activated kinase 1
- PCR Polymerase Chain Reaction
- PDK1 3-Phosphoinositide-Dependent Protein Kinase-1
- PKA Protein Kinase A
- PKB Protein Kinase B or Akt
- PKC Protein Kinase C
- PI3K Phosphatidylinositol 3-Kinase
- PMSF Phenylmethylsulphonyl Fluoride
- p38 MAPK Mitogen Activated Protein Kinase p38
- RPMI Roswell Park Memorial Institute cell culture medium
- RT-PCR Reverse Transcriptase- Polymerase Chain Reaction
- SDS Sodium Dodecyl Sulfate
- shRNA short hairpin RNA

- siRNA small interfering RNA

- SMN survival of motor neurons

- SMA spinal muscular atrophy

- STRAP Serine Threonine Receptor-Associated Protein

- SUMO Small ubiquitin-related modifier

- TCF T-cell factor family of transcription factors or LEF

- TGFβ Transforming Growth Factor β

- Thr (T) Threonine, amino acid

- TNFα Tumor Necrosis Factor alpha

- TβRI and TβRII TGFβ Receptor I and II

- U snRNPs Uridine-rich small nuclear ribonucleoproteins

- unrip unr-interacting protein

- VEGF Vascular Epithelial Growth Factor

- Wnt vertabrates secreted glycoprotein equivalent to the Wingless in drosophila

- WT1 Wilm's Tumor Homolog 1

CHAPTER I

INTRODUCTION

STRAP is a 39 kDa protein of WD-40 family involved in probable scaffolding function during the formation of multiprotein complexes, first shown to be active in the Transforming growth factor-β (TGF-β) receptor signaling network. Oncogenic STRAP is up-regulated in human cancers and may be involved in tumor progression.

Characteristics

STRAP was first cloned from mouse embryonic cDNA library while searching for novel proteins that bind the cytoplasmic region of TGF-ß type I receptor (TβRI) using the yeast two-hybrid screening (Datta el al., 1998). Later it was found that STRAP could associate with both type I and Type II (TßRII) serine-threonine kinase receptors (Datta et al., 2000). Southern blot analyses demonstrate that STRAP is evolutionarily conserved from yeast to mammals. The importance of this conservation was revealed when STRAP knockout mice were generated using the gene-trap mutagenesis technology coupled with microarray in the process of identifying probable targets of platelet-derived growth factor (PDGF) signaling (Chen et al., 2004). STRAP was found to be a PDGF-BB-inducible gene. The gene trap insertion results in embryonic lethality between embryonic day (E) 10.5 and E12.5. Later STRAP was also identified in humans as an interacting protein with upstream of N-ras (unr) that is involved in the internal initiation of translation of

human rhinovirus RNA and is implicated in cap-independent translation (Hunt et al., 1999).

The human STRAP gene is located in chromosome 12p12.3 near the marker D12S1593 (Matsuda et al., 2000). Northern blot analysis, using different tissues in mice, indicated a major transcript of 1.8 kb, though in some tissues a larger transcript was detectable. This may suggest alternative splicing of the STRAP RNA at least in some tissues. It is ubiquitously expressed in all mouse tissues with highest levels in liver and testis and less abundantly in spleen. In humans, STRAP expression has also been shown to be ubiquitous and it forms a 2 kb transcript. Both human and mouse STRAP gene contains 10 exons which finally forms a 350 amino acid protein migrating with an apparent mass of 39 kD on SDS-PAGE. Murine STRAP has more than 97% amino acid identity over the entire sequence with its human version. Sequence analysis indicates that STRAP contains seven WD40 repeats. STRAP shows a 55 % similarity in base pairs and a 19 % similarity in amino acid sequence to another known WD40 protein TRIP-1. Some of these similarities are among the conserved amino acid residues within the WD repeats. STRAP is localized predominantly in the cytoplasm but a good level is also present in the nucleus. It forms homo-oligomers probably through the WD repeats and this may be important for the multi-protein complex assembly. The physical interaction of STRAP with the TGF-ß receptor complex raises the possibility that STRAP is a substrate of the receptors. Our findings showed that an increase in the phosphorylation of STRAP requires the kinase activity of receptors in vivo, but STRAP does not appear to be a direct

substrate of the receptors during in vitro kinase assays. The C-terminal 57 amino acids are important for this phosphorylation.

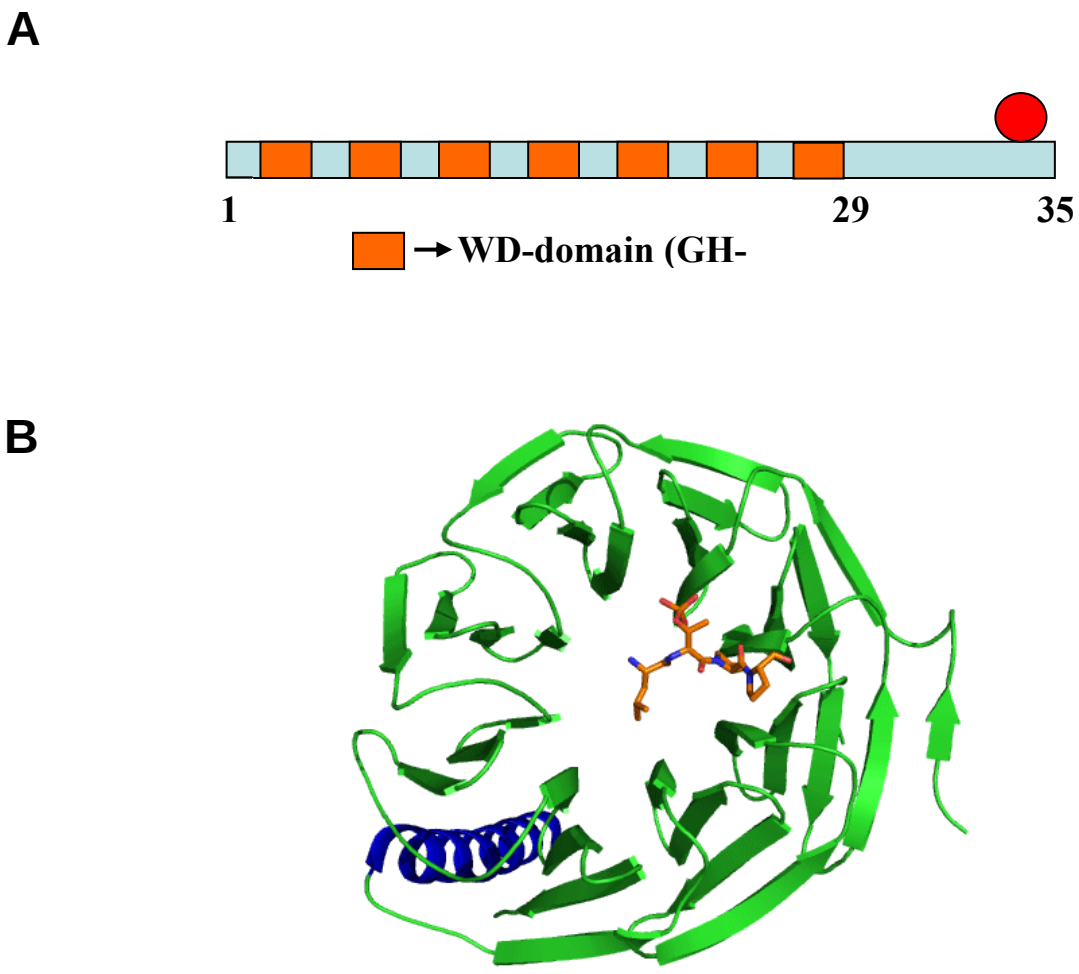


Figure 1. Structural considerations for STRAP (A) STRAP has 7 WD-40 repeats in the N-terminal region and a low complexity short C-terminal chain that is known to be phosphorylated. **(B)** WD-40 domains together form a β-propeller structure. Here is a sample structure of the yeast Cdc4 protein having 8 WD40 repeats forming an eight-bladed β-propeller structure. (Adopted from Orlicky S. et al. (2003) Cell. 112(2): 243-256)

As stated earlier, STRAP belongs to the family of WD repeat proteins that are known to have four or more repeating units containing a conserved core of approximately

40 amino acids that mostly end with tryptophan-aspartic acid (WD). Most of them are thought to form a circularized β propeller structure. Though the underlying common function is coordinating multiprotein complex assemblies, these proteins are also involved in various cellular processes like signal transduction, transcriptional regulation, programmed cell death, RNA synthesis/processing, chromatin assembly, cell cycle progression and vesicular trafficking. Other common examples of WD repeat proteins are the β subunit of the G proteins, TATA box binding protein associated factor II (TAFII), apoptotic protease-activating factor 1 (APAF-1), retinoblastoma-binding protein p48 (RbAp-48), receptor of activated protein kinase C 1 (RACK1), phospholipase A2-activating protein (PLAP) or TGF-beta receptor-interacting protein 1 (TRIP-1), which is also known to interact with TβRII (Chen et al., 1995). Structural analysis of the WD repeat proteins in general shows that they act as a very rigid platform or scaffold irrespective of the proteins they interact with.

This is evident from the fact that apart from TβRI and TβRII (Datta et al., 1998), STRAP also binds with Smad2, Smad3, Smad6, Smad7, 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Seong et al ., 2005), Ewing's sarcoma protein (EWS) (anumanthan et al., 2006), hMAWD binding protein (MAWBP) (Matsuda et al., 2000), unr (Hunt et al., 1999), microtubule associated protein 1B (MAP1B) (Iriyama et al., 2001), nuclear export factor (NXF) proteins (Tretyakova et al., 2005), Gemin6, Gemin7, and 3 small nuclear ribonucleoproteins (SmB), SmD2, SmD3 (Meister et al., 2001 and Carissimi et al., 2006), ASK1 (Jung et al., 2010), NM23-H1 (Jung et al ., 2007) and also GSK3β and Notch3 as found in our current study. Figure 1B shows an example of the yeast WD40-

repeat protein Cdc4 with the substrate being able to interact with the WD40-repeates only after appropriate phosphorylation.

STRAP in TGF-β Signaling Pathway

TGF-beta binds with the TGF-β receptor to induce a hetero-dimeric complex of TBR-I and TBR-II and signals though both Smad-dependent and the Smad-independent pathways (Derynck et al., 2003; Bierie et al., 2006 and Feng et al., 2005). TGF-β signaling is known to regulate diverse biological functions such as growth, differentiation, EMT, invasion, and apoptosis (Zavadil et al., 2005; Schuster and Kriglstein, 2002). As far as cancer progression is concerned, Smad-dependent pathway seems to suppress tumor formation by inhibiting cell cycle progression and inducing apoptosis. TBRI phosphorylates receptor associated Smads (R-Smads) -2 and -3 that binds to the common Smad-4 and translocate to the nucleus. This complex can activate or suppress transcription from TGF-beta target genes depending on the type of transcriptional regulators it associates with.

In the Smad-independent pathway, TGF-β can activate MAP kinase signaling pathway, TGF-beta activated kinase 1 (TAK1) (Kimura et al., 2000), p38 (Morigouchi et al., 1996), JNK (Shirakabe et al., 1997; Engel et al., 1999), RhoA (Bhowmick et al., 2001), Ras and phosphoinositide 3-kinase (PI3K) pathways (Bakin et al. 2000; Chen et al. 1998). In contrast to the Smad pathway, the Smad-independent pathways are believed to play a role in the pro-oncogenic functions of TGF-β (Wakefield and Roberts 2002). Smad-independent pathways have been reported to promote cell survival, EMT, and migration; and several independent studies suggest that these pathways can induce

malignant characteristics to normal and neoplastic cells (Massague 2008). Whether a Smad-dependent or Smad-independent pathways will prevail in a certain cell depends on the specific cell type and still very unclear. Our recent report does indicate that mechanisms like loss of Smad4 expression can be one such event that can favor the Smad-independent pathways ahead of Smad-dependent pathway (Zhang et al., 2010).

STRAP binds with both TβRI and TβRII in a ligand independent manner. It synergizes specifically with Smad7, but not with another inhibitor Smad6, in the inhibition of TGF-ß signaling (Datta et al., 2000). STRAP stabilizes the association between Smad7 and activated receptor complex, thus assisting Smad7 in preventing phsophorylation and activation of Smad2 and Smad3 by the receptor complex. Though Smad6 is also shown to interact with STRAP, this association does not seem to interfere with bone morphogenic protein (BMP) signaling in which Smad6 acts as an inhibitor. STRAP inhibits TGF-ß-induced nuclear translocation of Smad2/3 and Smad4 and as a result, activation of TGF-ß responsive reporter genes including plasminogen activator inhibitor 1 (PAI-1) and p21Cip1 is abrogated. Downregulation of p21Cip1 by STRAP leads to hyperphosphorylation of retinoblastoma protein (pRb). In vitro kinase assay demonstrated that overexpression of STRAP can induce extracellular signal-regulated kinase (MEK/ERK) activity in a TGF-ß-independent manner. Activation of MEK/ERK pathway by endogenous STRAP was further confirmed by knocking STRAP down using small interfering RNA (siRNA) (Halder et al., 2006). Although STRAP is not a kinase, it may facilitate the activation of MAPK pathway through functioning as a scaffold for upstream kinases. Therefore, STRAP may inhibit activation and nuclear translocation of Smad2 and Smad3 by interacting with receptors and Smad7 and/or by activating

MAPK/ERK pathway. Additional unpublished data from our lab now indicates that STRAP may mediate activation of other Smad-independent pathways. Further work will be needed to confirm these findings and understand the exact role of STRAP in activating these pathways.

Role of STRAP in PDK1-mediated Signaling

3-phosphoinositide-dependent protein kinase-1 (PDK1) has been shown to phosphorylate and activate many members of the protein kinase A, G, and C subfamily that include protein kinase B (PKB), p70 S6 ribosomal kinase, p21-activated kinase 1 (PAK1) and serum/glucocorticoid regulated kinase (SGK) (Mora et al., 2004). PDK1 signaling is implicated in cellular proliferation, survival, migration, and invasion of tumor cells. STRAP interacts with the catalytic domain of PDK1 and modulates PDK1 activity. Seong et al. (2005) showed that STRAP promotes phosphorylation of PDK1 substrates S6K, Akt, and Bad. This interaction of STRAP and PDK1 is inhibited by TGF-β and induced by insulin. The induction in binding by insulin is abrogated by wortmannin, a PI3K inhibitor. The mechanism behind PDK1 activation by STRAP is thought to be displacement of the 14-3-3 protein from PDK1 complex, which negatively regulates it (Fig. 2). The binding of PDK1 with STRAP also potentiates negative regulation of TGF-β mediated transcription by STRAP. This repression occurs through increased association of Smad7 with both STRAP and TβRI. Finally, interaction between STRAP and PDK1 induces cell survival probably through phosphorylation of Bad and attenuation of tumor necrosis factor-alpha (TNF-α) induced apoptosis.

STRAP interacts with NM23-H1

NM23-H1 is currently thought of as a putative metastasis suppressor because of its reduced expression in a few highly metastatic cell lines and tumors. There are eight NM23 genes in humans namely NM23-H1, NM23-H2, NM23-H3, NM23-H4, NM23-H5, NM23-H6, NM23-H7, and NM23-H8 that encode for DNA-binding nucleoside diphosphate kinases (Lacombe et al., 2000). Multiple studies have shown that these kinases affect a broad spectrum of cellular responses, including development, differentiation, proliferation, endocytosis, and apoptosis (Steeg et al., 2008; Fan et al., 2003). The molecular mechanisms underlying the role of NM23-H1 as a metastasis suppressor, however, have so far remained unclear.

There have been contrasting reports about the effect of NM23 on TGFβ signaling. Earlier studies indicate a potentiating role for NM23-H1 during Smad-dependent signaling in HT29 colon cancer cells but a later report indicates that NM23-H1 can associate with STRAP to reduce transactivation of Smad-dependent reporter genes (Hsu et al., 1994; Seong et al., 2007). A more recent study shows that NM23-H1 and STRAP interact with p53 and potentiate p53 activity (Jung et al., 2007). Both NM23-H1 and STRAP were shown to directly interact with the central DNA binding domain of p53 and remove Mdm2 from the p53-Mdm2 complex. Mdm2 is a known negative regulator of p53. The knowledge about the exact role of STRAP-NM23-H1 complex formation in p53 signaling is still in early phase and warrants further investigation.

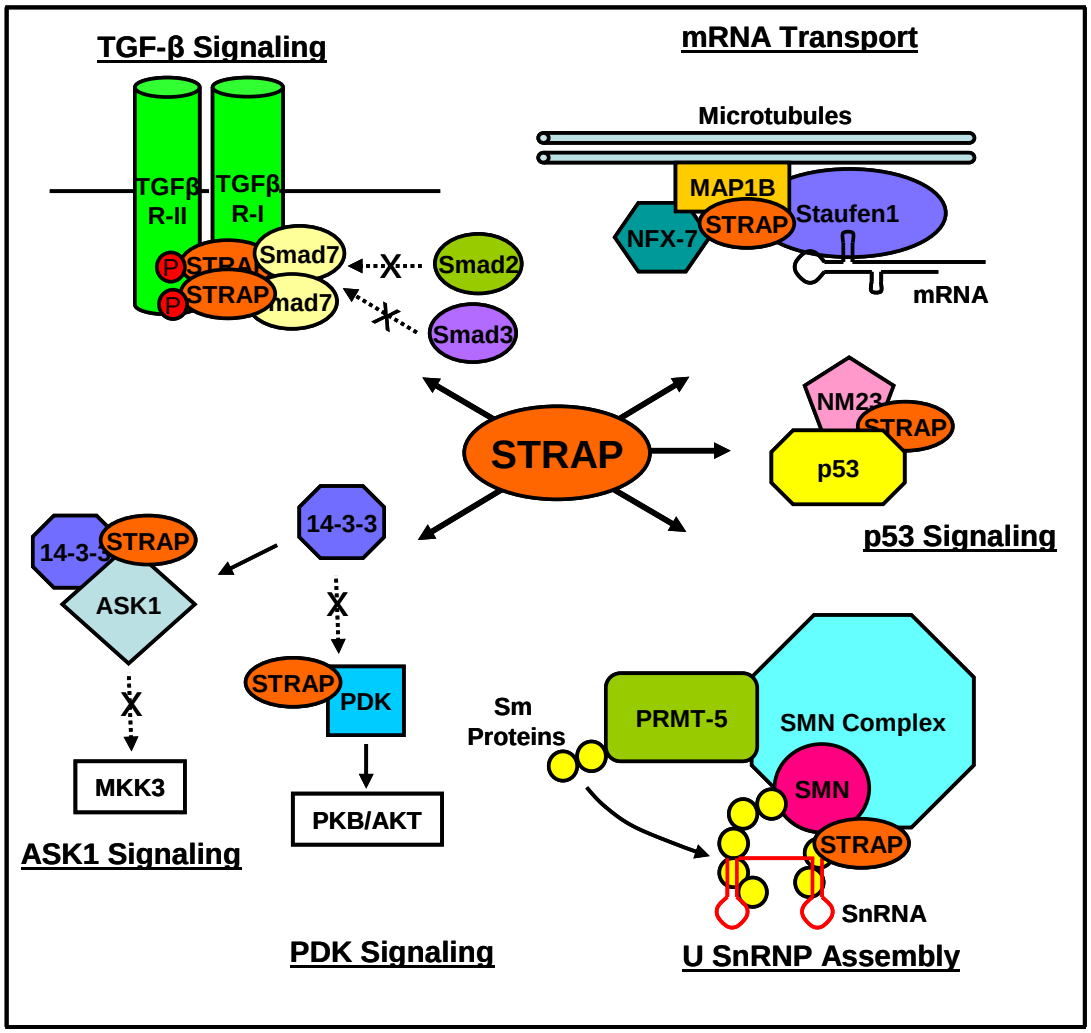


Figure 2. Role of STRAP as a scaffold in diverse signaling pathways. Please see the text for detailed discussion.

STRAP in ASK1-dependent signaling

ASK1 (apoptosis signal-regulating kinase 1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that phosphorylates mitogen-activated protein kinase kinases MKK3, -4, -6 and -7 and in turn activate the c-Jun N-terminal kinase (JNK)3/p38 signaling cascade (Chen et al., 1999). A diverse range of factors including stress-related

stimuli, tumor necrosis factor-α, Fas ligand, endoplasmic reticulum stress and calcium excess can stimulate ASK1 activity and thioredoxin (Trx), glutaredoxin, glutathione S-transferase μ (GSTμ), heat shock protein 72, 14-3-3 etc. can inhibit ASK1 activity (Matsukawa et al., 2004). This regulation has been proposed to be achieved through protein-protein interactions. Consistent with this, ASK1 was shown to interact with STRAP through the C-terminal region of ASK1. Functionally, STRAP inhibited ASK1-mediated signaling to both JNK and p38 kinases (Jung et al., 2010). STRAP has a stabilizing effect on a complex between ASK1 and thioredoxin and 14-3-3 that are known negative regulators of ASK1. This prevents complex formation between ASK1 and its downstream substrate MKK3 thus effectively inhibiting ASK1 signaling. Functionally, this effect on ASK1 activity resulted in STRAP inhibiting H_2O_2-mediated apoptosis in a dose-dependent manner. This is in direct contrast to the role of STRAP in PDK1 signaling where STRAP has an activating effect on PDK1 by displacing protein 14-3-3 and thus enabling PDK1 to phosphorylate its downstream substrates. This suggests that role of STRAP can be dependent on the specific protein it associates with and not dependent on a certain fixed mechanism. Additionally, ASK1 phosphorylates STRAP at Thr175 and Ser179 within a region between the WD4 and WD5 domains that subsequently leads to ASK1 activity inhibition though the exact mechanism of this is not well understood.

Role of STRAP in the SMN complex and cap-independent translation

The survival of motor neurons (SMN) gene is considered to be responsible for the neurodegenerative disorder spinal muscular atrophy (SMA) (Battaglia et al., 1997). In

cells, SMN is part of a stable multiprotein complex in the cytoplasm and in nuclei. Apart from the SMN protein itself, the SMN complex contains six other proteins, called Gemins2–7. The SMN complex plays a vital role in the assembly of the spliceosomal Uridine-rich small nuclear ribonucleoproteins (U snRNPs). STRAP seems to be involved in this assembly through its interaction with STRAP with Gemin6 and Gemin7 as well as SmB, SmD2 and SmD3 components of SMN complex (Grimmler et al., 2005; Carissimi et al., 2005). Interestingly, Gemin5 which is one of the SMN complex components STRAP does not interact with, is also a WD40-repeat protein. Although STRAP is localized in both cytoplasm and nucleus, it is present predominantly in the cytoplasm and may help the nuclear-cytoplasmic distribution of the SMN complex. The presence of STRAP in the SMN complexes was shown to be essential for U snRNP assembly as depletion of STRAP using a specific antibody markedly reduces snRNP assembly (Carissimi et al., 2005). In contrast, STRAP was also shown not to be essential for this assembly by another report from Grimmler et al. (2005). Thus presently, there is no conclusive understanding of the role of STRAP in this macromolecular SMN complex.

unr is a cytoplasmic RNA binding protein that plays an important role in the initiation of HRV-IRES dependent (cap-independent) translation of the animal picornavirus RNA like the rhinoviral RNA. STRAP interacts with unr and was named unr-interacting protein (unrip) for this role. It was found that STRAP did not appear to play any role in the initiation of viral translation. But STRAP could increase c-myc internal ribosomal entry site (IRES) activity though again the significance of STRAP in this process is not well understood.

Miscellaneous functions of STRAP

Ewing's sarcoma is a rare malignant round-cell tumor found in the bone or in soft tissue. Though fusion product of EWS gene and ETS family gene has been implicated in Ewing's Sarcoma, little is know about the wild type EWS protein (Uren et al., 2005). STRAP is localized in both cytoplasm and nucleus. It colocalizes and associates with the oncogenic EWS protein in nucleus through its NH2 and COOH termini (Anumanthan et al., 2006). STRAP inhibits the interaction between EWS with p300, a protein that is a transcriptional co-activator of EWS. This results in downregulation of EWS target genes like ApoCIII and c-fos. Although TGF-β has no effect on the interaction between STRAP and EWS, TGF-β-dependent transcription is inhibited by EWS.

In an interesting finding, STRAP along with EWS was shown to be present in the kinesin driven mRNA transport granules in the dendrites of murine neurons (Tretyakova et al., 2005). In eukaryotes, the nuclear export of mRNA is mediated by nuclear export factor 1 (NXF1) receptors. As shown in mouse neuroblastoma N2a cells, NXF proteins bind to brain-specific microtubule associated proteins (MAP) such as MAP1B and also STRAP. Additionally, MAP1B also binds with STRAP. This assembly helps in nuclear export of mRNA. In an independent setting, NXF-7 binds with MAP1B and STRAP only in the cytoplasm and colocalizes with Staufen1 containing mRNA transport granules in the neurites of these cells (Fig. 2) (Kanai et al., 2004). As in other cases, STRAP seems to play a role in the multiprotein complex assembly required for both nuclear export of mRNA and the cytoplasmic transport of mRNA containing granules along microtubules.

Recently, it was shown that STRAP is a substrate for SUMO4 (a novel member of the SUMO gene family) sumoylation. Although the significance of this is not yet known, SUMO4 sumoylation is predicted to have a role in the regulation of intracellular stress. It will be interesting to determine whether sumoylation of STRAP has any effect on its biological functions.

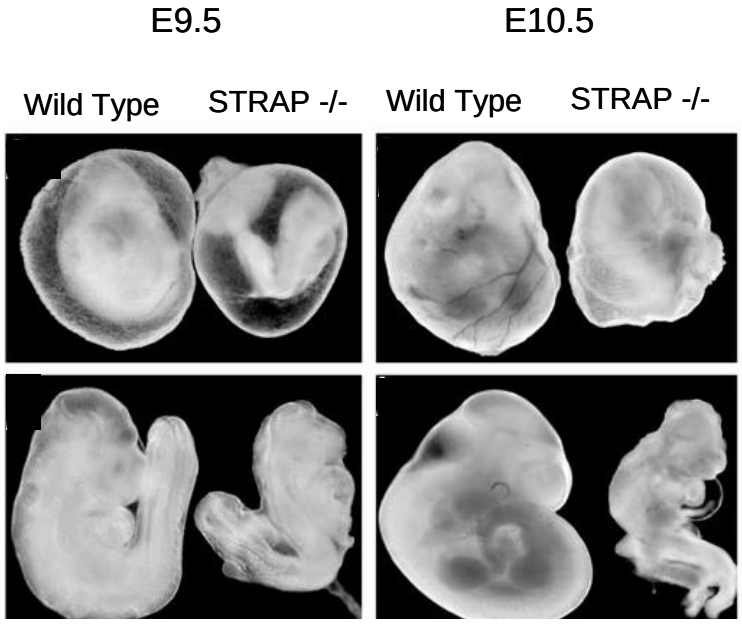


Figure 3. STRAP in development. Gross morphology of wild-type (left) STRAP -/- (right) embryos at E9.5 and E10.5. Mutants had underdeveloped yolk sac vasculature, arrested neural tube closure and embryonic turning, as well as abnormal hearts and somites. (From W. Chen, J. Delrow, P. Corrin, J. Frazier, P. Soriano, Nat. Genet. 2004, 36; 304–312)

STRAP in development

Chen et al. (2004) identified STRAP as a PDGF-BB-inducible gene in a pilot gene-trap array screen. The gene-trap insertion resulted in a null allele and recessive

embryonic lethality between embryonic day (E) 10.5 and E12.5. Homozygous mutant embryos had defects in angiogenesis, cardiogenesis, somitogenesis, neural tube closure and embryonic turning (Figure 3). Coupled with conservation of STRAP from yeast to humans, this highlights an indispensable function for STRAP during early development. Recently, drosophila STRAP homologue, pterodactyl, has been implicated in tubulogenesis and branching morphogenesis defects (Khokhar et al., 2008).

Clinical aspects

Although STRAP seems to be involved in mutually independent biological functions, there is increasing clinical and experimental evidence suggesting that STRAP acts as an oncogene. The level of STRAP is found to be altered in different cancers. The protein level is elevated in 60% of colorectal, 78% of lung and 46 % of the breast carcinomas (Halder et al., 2006; Matsuda et al., 2000 and Kim et al., 2007). Several lines of evidence suggest that carcinoma cells frequently lose the tumor suppressor function of TGF-β (Bierie et al., 2006). Upregulation of TGF-β signaling inhibitors like STRAP and Smad7 and their synergistic function presents a novel intracellular mechanism by which a portion of human tumors become refractory to antitumor effects of TGF-β. STRAP also exerts several other biological functions in a TGF-β independent manner that contributes to cell proliferation and inhibition of apoptosis.

Ectopic expression of STRAP in different cell lines promotes cellular proliferation, induces anchorage-independent growth and increases tumorigenicity during in vitro and in vivo experiments (Halder et al., 2006). Downregulation of $p21^{Cip1}$ that results in hyperphosphorylation of pRb as well as activation of MAPK/ERK pathway

may contribute to tumorigenic effects of STRAP during tumor formation and progression. As noted earlier, STRAP also has an anti-apoptotic role probably through Bad phosphorylation and inhibition of TNF-α induced apoptosis (Seong et al., 2005). It has been recently shown that STRAP interacts with Ewing Sarcoma protein (EWS), an oncoprotein known to be involved in 80% of Ewing tumors after chromosomal translocations (Anumanthan et al., 2006). Normal EWS protein is also upregulated in human cancers, which correlates with the upregulation of STRAP in 71% of colorectal cancers and 54% of lung cancers, suggesting a cooperative role of these two proteins in human cancers. In an attempt to determine whether STRAP is of prognostic value or predictive of chemotherapy benefit, STRAP gene was found to be amplified in 23% of colorectal tumors and amplification of STRAP in patients without adjuvant chemotherapy was found to exhibit better prognosis (Buess et al., 2004). Interestingly, these patients had a worse survival when treated with adjuvant therapy when compared with patients without chemotherapy. In contrast, patients carrying tumors with diploidy or deletion of STRAP benefited from the treatment. These results suggest that STRAP is an unfavorable prognostic marker for 5-FU-based adjuvant chemotherapy. Taken together, STRAP appears to facilitate multiple steps in the process of tumorigenesis and possibly during metastasis, and it could be a potentially important drug target for therapeutic intervention in human cancers.

Aims of Dissertation

The aims of this dissertation were:

1) To evaluate the role of STRAP behind the altered morphology of STRAP null mouse embryonic fibroblasts (MEFs)

During a study to find PDGF target genes using a microarray coupled gene trap mutagenesis, STRAP was functionally deleted. The STRAP null mice showed embryonic lethality between embryonic day E10.5 and E12.5 with multiple organ system defects. The embryonic fibroblasts (MEFs) isolated from STRAP null fibroblasts exhibited striking phenotypical alterations. STRAP null MEFs aggregated and formed epithelial islands compared to the regular elongated spindle shaped fibroblasts isolated form the embryos with same genetic background except for wild type STRAP. This indicated a need to study the possible role in maintaining the fibroblastic phenotype of MEFs to get additional clues about novel functions of STRAP. The differences between wild type and STRAP null MEFS were studied using a variety of techniques and STRAP stable MEFS were generated from STRAP null MEFs. A possible role for STRAP in modulating WT1 expression was identified.

2) To determine the role of STRAP in GSK3β and Notch signaling.

The serine/threonine protein kinase glycogen synthase kinase 3 (GSK-3) is a key regulator of multiple enzymes and many important transcription factors. GSK3β phosphorylates and thereby regulates the functions of many metabolic, signaling and structural proteins.

Ewing et al. first (2008) reported the possibility of an interaction between STRAP and GSK3β in a large scale analysis of protein-protein interactions using mass spectroscopy. We verified this interaction and since STRAP is known to act as a scaffold protein in multiprotein assemblies, we decided to study the possible role of STRAP as a scaffold in GSK3β signaling. We showed for the first time that intracellular fragment of Notch3 is a possible substrate of GSK3β. STRAP also interacted with ICN3 and has a stabilizing effect on ICN3 by aiding its deubiquitination.

CHAPTER II

MATERIALS AND METHODS

Cell culture and plasmids

Wild type and STRAP null mouse embryonic fibroblasts (MEFs), HEK-293, HeLa and NIH3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), antibiotics, and glutamine (GIBCO BRL). STRAP null MEFs were used to generate clones stably re-expressing STRAP using the STRAP pBabe Puro retroviral vector and clones were selected in 0.75 μg/ml puromycin. The E-cadherin luciferase construct (-178/+92) was a gift from Dr. Amparo Cano (Universidad Autónoma de Madrid, Instituto de Investigaciones Biomédicas Alberto Sols, Madrid, Spain). Plasmids expressing A and B isoforms of murine WT1 were obtained from Dr. Jerry Pelletier (McGill University, Montreal, Quebec, Canada). Axin-myc (in pCDNA3.1) was a gift from Dr. Michele Kimple (Duke University). HA-tagged GSK3β (in pCDNA3) and myc-tagged GSK3β (in pJ3M vector) were gifts from Dr. Gordon Mills (MD Anderson Cancer Center) and Dr. Alan Diehl (University of Pennsylvania Cancer Center) respectively. Murine STRAP and CT-1-STRAP constructed using the pCDNA3 vector have been described previously (Datta et al., 1998). HA-tagged mICN3 and myc-tagged mICN1 (both in pCDNA3) were a gift from Dr. Jon Aster (Brigham and Women's Hospital, Harvard University). HA-tagged β-catenin was a gift from Dr. Stephen Byers (Georgetown University School of Medicine, Washington). GSK3β inhibitors SB216763

and SB416286 were purchased from Sigma and AR-A01441 was purchased from Calbiochem and used as directed by manufacturers.

Recombinant adenovirus

A STRAP expressing adenovirus was generated through homologous recombination between a linearized transfer vector pAD-Track and the adenoviral backbone vector pAD-Easy (He et al., 1998). pAD-STRAP contained the full length murine STRAP cDNA with a carboxy-terminal HA or FLAG-tag. In addition to the STRAP transgene the virus encoded the green fluorescent protein (GFP) transcribed from a second independent CMV promoter. GFP expression was used to monitor viral infection efficiency. An adenovirus coding for GFP only (pAD-GFP) was used as a control in all experiments. For both adenoviruses, a titer of 200 MOI was used in all the experiments to infect the cells for 8 hours in a serum free medium. The cells were then kept in a serum containing medium for 60 hours where not specifically mentioned.

Western Blot analysis

For immunoblotting, whole-cell lysates were prepared in a cold lysis buffer with 0.01 M Tris-HCl (pH 7.4), 0.01 M NaCl, 1 mM EDTA, sodium ortho-vanadate, 0.1 % SDS and protease inhibitors (Aprotinin, Leuprptin and PMSF) and sonicated before centrifugation at 14,000 rpm for 15 min. The proteins were separated by 10% SDS/PAGE, transferred to nitrocellulose membrane (Biorad), and probed with primary antibodies from the following sources: Santa Cruz Biotechnologies (vimentin, WT1, fibronectin, HA and Myc), BD Biosciences (E-cadherin, β-catenin and N-cadherin) and

Sigma (FLAG) as described in Halder et al., 2006. Primary antibodies were incubated for 3 hr at room temperature followed by incubation with species-specific secondary antibodies for 1 hr at room temperature. The signal was visualized by enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Pittsburgh, PA).

Co-Immunoprecipitation

HEK-293T cells were plated in 60 mm dish and transfected next day at a 40% confluency with appropriate combination of plasmids using Lipofectamine reagent (Invitrogen) using 1:3 ratio in serum-free media. The serum-free media was changed with serum-containing media 3 hours after transfection. Where needed, cells were treated with proteasomal inhibitor MG132 (4 hr) or GSK3β inhibitors (12 hr) as indicated in respective figures. Cells were solubilized in 1ml of lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.02% NaN3, 50 mM NaF, 1 mM Na3VO4, 0.7% NP-40, 0.5 mM dithiothreitol, 0.02% SDS and protease inhibitors aprotinin, PMSF and leupeptin). An equal amount of each protein lysate was incubated with the appropriate antibodies as indicated in the figures for 3 hours at $4^{o}C$, followed by incubation with 20 µl of protein G-Sepharose beads (dry volume) equilibrated with lysis buffer (Sigma Biochemicals, St. Louis, MO) for 1 hour. The immune complexes were washed with the lysis buffer 5 times. The beads were finally boiled in 50 µl of 2X SDS sample buffer (125mM Tris-HCL pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue) and the samples then were separated on 10% SDS-PAGE and transferred to PVDF membranes (Biorad). Bound proteins were analyzed by Western blot analysis using appropriate antibody. Protein lysates used for immunoprecipitation were also analyzed by

Western blot analysis with other antibodies to check for comparable expression of proteins across all transfections.

Reverse Transcription-PCR Analyses

Total RNA was isolated from each cell line using Trizol method and RT–PCR amplification was carried out using MMLV reverse transcriptase. The RNA samples (2 µg) were retrotranscribed into cDNA using oligo-dT primers in a total volume of 20 µl containing 5 mM MgCl2, 1 mM dNTPs (Boheringer), 1 U RNase inhibitor (Perkin Elmer) and 2.5 U MuLV reverse transcriptase (Perkin Elmer) at 37^{o}C for 1 hour. Amplification by PCR was carried out using 2µl of the cDNA with the Red Taq polymerase according to the manufacturer's protocol. The thermal cycles were: denaturing at 94^{o}C for 1 min, annealing at 54^{o}C for 1 min, and extension at 72^{o}C for 1 min. The cDNA was amplified for 28 cycles. The primer pairs of E-cad, WT1, LEF1, Snail, SIP1, Slug, E2A, Twist, S100A and GAPDH are shown in table 1. GAPDH was amplified in each sample as an internal control. All experiments were repeated at least three times.

Table 1
Primers used for RT-PCR.

Gene	Upstream primer	Downstream primer	Size (bp)
E-cadherin	ggaatccttggagggatcctc	gtcgtcctcgccaccgccgtacat	560
WT1	cagagagcaaggcaccag	taagagcccagtgctagtg	221
LEF1	ccaactttccggaggaggc	gtaggagggtcccttgttgtac	313
Snail	cagctggccaggctctcggt	gcgagggcctccggagca	370
Slug	ccaaggatcacagtggttca	cagtgcagctgcttgtgttt	843
ZEB1	cgccaacaagcagactattc	tgaggcctcttacctgtgtg	295
SIP1	agtccaatgcagcacttaggt	ttcatgctgatgcaggggaat	490
S100A	aggagctactgaccagggagct	tcattgtccctgttgctgtcc	103
E2A	taccctccgccaagacc	ttgggggataaggcactg	535
Twist	cgggtcatggctaacgtg	cagcttgccatcttggagtc	190
GAPDH	accacagtccatgccatcac	tccaccaccctgttgctga	452

FITC-Phalloidin staining

After fixation in 3.7% fresh paraformaldehyde in PBS for 15 min, cells were washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 8 min. After treatment with blocking solution (1% BSA and 0.1% Triton X-100 in PBS) for 10 min, the cells were stained with FITC-phalloidin (1 μg/ml) in blocking solution for 20 min in a dark room at room temperature to localize F-actin. The slides were washed twice with PBS, each for 10 min. Incubation and washing were performed in parallel for all wells on a slide. A coverslip was mounted on the slide with Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Actin was visualized with a fluorescence microscope (Olympus BHT, Tokyo, Japan).

Electron microscopy

Cells were washed with 6.8% saccharose in 0.1 M cacodylate buffer, pH 7.4, at room temperature and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 30 min. The cells were rinsed three times in the same buffer with 6.8% sucrose solution and subsequently postfixed in 2% OsO_4/3% $K_4Fe(CN)_6$ in 0.2 M cacodylate buffer (pH 7.4) at 4°C for 1 h. After rinsing in 0.1 M cacodylate buffer, pH 7.4, and dehydration in a graded alcohol series, the cells were embedded in Epon 812 and polymerized at 58°C for 64 h. Finally, ultrathin sections (60 nm) were cut and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM 12 electron microscope operating at 80 kV, and micrographs were taken.

Fluorescence Microscopy

Cells cultured on glass coverslips were fixed with ice-cold methanol in PBS for 10 min at 4°C, followed by permeabilization with 0.1% Triton X-100 in PBS at room temperature for 5 min. Blocking incubations were performed in PBS containing 3% BSA at room temperature for 1 h. After extensive washes with PBS, cells were incubated with the first antibody at room temperature for 2 h. After washing with PBS, cells were then incubated with the corresponding secondary antibody at room temperature for 1 h. After another round of extensive washes in PBS, the coverslips were mounted in a drop of mounting medium (Vectashield). The antibodies used were as follows: mouse monoclonal anti-E-cadherin and mouse monoclonal anti-β-catenin antibody from BD Biosciences, and Alexa Fluor-596 goat anti-mouse from Molecular Probes, Eugene, OR.

Reporter Assays

For studying effect of STRAP on WT1-mediated E-cadherin promoter induction, NIH3T3 cells, wild type and STRAP null embryonic fibroblasts were plated in 12-well plates. After 30 h, luciferase constructs (0.5 μg/well where not mentioned) along with expression plasmids for WT1 (100 ng) and/or STRAP (2 doses of 150 and 450 ng) were transfected into the cells using Lipofectamine and Plus reagent following the manufacturers protocol. After approximately 48 hours, cells were lyzed and luciferase assays were performed using a luminometer (BD bioscience) according to the manufacturer's protocol. Transfection of each construct was performed in triplicate in each assay and a total of three assays were performed on three separate days. All wells were transfected with 25 ng of beta-galactosidase to serve as a control for the transfection efficiency. Ratios of luciferase readings to beta-gal readings were taken for each experiment and triplicates were averaged. Bars represent the averages of the normalized values with error bars indicating the standard deviation.

For understanding effect of STRAP on ICN3-mediated transcriptional activation, HeLa cells were transfected with HES1 promoter (0.5 μg/well) with different combinations of expression plasmids for ICN3 (50 and 200 ng) and/or STRAP (2 doses of 100 and 300 ng). Other procedure was exactly as stated above.

Microarray Analysis

To characterize the gene expression profile of wild type and STRAP null MEFs, RNA was isolated from these cells using the Trizol method and further purified using

Qiagen RNeasy kit according to the protocol from the manufacturer (Qiagen, Valencia, CA). Microarray was done using GeneChip 430 Mouse 2.0 Array from Affymetrix that contains 45,000 probes for analyzing 39,000 variants of 34,000 mouse genes and signal intensity was detected according to supplier's instructions.

Production of STRAP shRNA lentivirus

Second-generation VSV-G pseudotyped high titer lentivirus was generated by transient co-transfection of 293T cells with a three-plasmid combination as follows:
One 15 cm dish containing $1x10^7$ 293T cells was transfected using Lipofectamine2000 (Invitrogen) with 5 µg STRAP shRNA lentiviral vector (pGIPZ, Open Biosystems), 3.75 µg pCMV Δ8.91 and 1.25 µg pMD VSV-G. For vector control lentivirus, empty lentiviral vector was used instead of STRAP shRNA lentiviral vector. A shRNA construct targeting human and mouse STRAP was obtained from Open Biosystems. The 21 bp sequence was 5'-GCTCATGTACTCTCAGGACAT- 3'. Supernatants were collected every 12 hr between 36 to 96 hr after transfection, pulled together and frozen at -70ºC.

Lentiviral transduction

For lentiviral transduction, $1x10^5$ cells were seeded in 6 well tissue culture plates and infected the following day with STRAP or vector control lentivirus. The cells were then selected for 7 days with puromycin and when cultures reached near confluency, cells were trypsinized and processed for FACS analysis to separate cells with highest GFP expression. To generate stable STRAP knockdown clones, these cells were plated at high

dilutions in 10 cm Petri dishes and colonies obtained from single cells were screened for STRAP expression by Western blot analysis.

Immunohistochemical analysis

Tissue Microarray (TMA) slides containing duplicates of 42 samples of different lung carcinomas were obtained from the Lung SPORE project at Vanderbilt University. The slides were placed in the sodium citrate solution, microwaved for 45 sec at full power, and heated in a pre-warmed steamer for 25min. After cooling at room temperature for 15min, the slides were washed three times with PBS. After antigen retrieval, the specimens were treated with 3% H2O2 (DAKO) for 5 min to quench endogenous peroxidase activity and a protein block treatment (Dako, Inc.) was performed prior to primary antibody addition. Tissues were incubated with anti-STRAP antibody (BD Biosciences) at 1:400, anti-Notch3 antibody (Novus Biological) at 1:200. After primary antibody incubation, the slides were washed three times with PBS. The specimens were then incubated for 10 min at room temperature with biotin labeled goat anti-mouse immunoglobulin (DAKO). Slides were lightly counterstained with Mayer's hematoxylin (Mayer's, VWR) for nuclear staining. Afterwards, the slides were dehydrated by sequential incubation in 95% ethanol, 100% ethanol and 100% ethanol for 5 min each, before transferring to xylene and coverslipping.

In-vivo ubiquitination Assay

HEK-293T cells were transfected with appropriate combinations of plasmids expressing his6-tagged ubiquitin, STRAP-FLAG and HA-ICN3 as indicated. 43 hours after transfection, the cells were treated or not with 50 µM of MG132 for 5 hours. The cells were then lysed in highly denaturing conditions using 1ml lysis buffer with 8 M urea at pH 8. This inhibits the otherwise rapid deubiquitination of proteins by deubiquinases after using regular cell lysis conditions. Cell lysates were then sonicated and centrifuged at 13,000 rpm for 20 min. A fraction was saved for western analysis and remaining was incubated with 50 µl of 50 % slurry of Nickel-Nitrilo triacetic acid (Ni-NTA) agarose beads slurry for 4 hours on a rocker at room temperature. Ni-NTA beads bind to histidine residues of proteins effectively and strongly pull down hexa-histidine tagged proteins, his-ubiquitinated proteins in this case. The beads were washed 5 times in a buffer with 8 M urea, 20 mM Imidazole kept at a pH of 6.3. This helps to remove background of endogenous proteins binding within-NTA beads. His-ICN3 bound with the beads were finally eluted with a buffer containing 8 M urea, 250 mM β-mercaptoethanol (BME) and 200 mM Imidazole at a pH of 4.5. The proteins were eluted in 2 batches first with 50 µl and then with 30 µl of elution buffer. The eluates were then boiled at 95^{o} C for 6 min with 1X SDS sample buffer and stored at -80^{o} C until further analysis. 15 µl of this eluate was analyzed by SDS-PAGE followed by western blotting with anti-HA antibody to detect ICN3 species that were ubiquitinated with HA-tagged ubiquitin.

CHAPTER III

SERINE THREONINE RECEPTOR-ASSOCIATED PROTEIN (STRAP) PLAYS A ROLE IN THE MAINTENANCE OF MESENCHYMAL MORPHOLOGY

Introduction

Epithelial and mesenchymal cells represent two distinct cell phenotypes that have unique gene expression profiles and functions specific to that cell type. Compared to differentiated epithelial cells, mesenchymal cells do not establish intercellular junctions in a stable manner mostly through suppression of E-cadherin expression and this imparts them with a higher capacity to detach in response to low shear forces such as within lymphatic vessels and venules. A similar process also decreases the adhesive force in epithelial cells during normal embryonic development and during carcinogenesis. Epithelial cells located at the periphery of a tumor frequently exhibit a substantial downregulation of epithelial markers along with a loss of intercellular junctions and other features of epithelial cells, accompanied by expression of a mesenchymal set of genes (Lee et al., 2006). This switch is referred to as epithelial-to-mesenchymal transition (EMT). Practically, it is often difficult to classify cell phenotypes into either extremes such as mesenchymal or epithelial and a relative shift from one phenotype to the other holds more importance. A recent study reported phenotypic alterations of the metastatic T24/TSU-Pr1 bladder carcinoma line that could express markers of both epithelial and mesenchymal type (Chaffer et al., 2006). A new term ‘metastable phenotype’ has been coined by Savagner et al. for such cells that continue to express attributes of both

epithelial and mesenchymal phenotypes (Klymkowsky et a., 2009). Such mixed phenotypes are being reported more frequently now (Laffin et al., 2008).

EMT provides a mechanism for epithelial cells to overcome the physical barrier of intercellular junctions and thus switch to a more motile phenotype. In many types of carcinomas, presence of EMT correlates with poor histologic differentiation, loss of tissue integrity and metastasis. Diverse developmental signaling pathways such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), hedgehog, Wnt/β-catenin, Notch and integrin signaling may play a role in the events leading to EMT (Mimeault et al., 2007). Interestingly, a reverse process where mesenchymal cells acquire an epithelial phenotype has also been defined and termed as mesenchymal-to-epithelial transition (MET) (Hugo et al., 2007). MET occurs during normal embryogenesis as well as during re-establishment of metastatic cells at distant sites. E-cadherin is known to be upregulated or re-expressed in most of these instances (Wells et al., 2008; Baum et al., 2008 and Chaffer et al., 2007). In vitro, stable expression of E-cadherin alone has been shown to force the fibroblasts to adopt an epithelial morphology (Vanderburg et al., 1996). Expression of E-cadherin sequesters β-catenin and p120-catenin to the cell membrane and helps deactivate the mesenchymal cell program (Orsulic et al., 1999 and Nelson et al., 2004). Conversely, absence of E-cadherin frees up β-catenin that can translocate to the nucleus and activate transcription of a number of mesenchymal transcription factors like including c-myc, LEF1, CyclinD1, cdc2 (Shtutman et al., 1999; Vadlamudi et al., 2005 and Bryan et al., 2003) . Nuclear localization of β-catenin is therefore frequently used as a marker of EMT and indicates a poor prognosis in cancer (Lee et al., 2006).

E-cadherin thus acts as a master regulator of epithelial phenotype and a continuous downregulation of E-cadherin is required in mesenchymal cells (Rubinfeld et al., 1997). This can be achieved through transcriptional as well as post-transcriptional mechanisms. A number of transcription factors such as Snail, Slug, ZEB1, SIP1, E2A, and WT1 are known to regulate E-cadherin expression (Batlle et al., 2000; Comijn et al., 2001; Hosono et al., 2000; Savagner et al., 1997 and Perez-Moreno et al., 2001). Interestingly, many of these transcription factors that are important for EMT during embryonic development were later found to play a role in EMT during cancer progression (Moustakas, et al., 2007). Yet most of the reports regarding these factors have only studied their roles during EMT and little is known about the molecular mechanisms important for the maintenance of mesenchymal morphology of fibroblasts themselves. Apart from E-cadherin itself, overexpression of WT1 in NIH3T3 fibroblasts has been shown to induce epithelialization with E-cadherin upregulation and formation of adherens junctions (Hosono et al., 1999). Accordingly, absence of MET in kidney is one of the main features in the development of Wilm's tumor, a rare embryonal malignancy which often has deregulated WT1 expression (Li et al., 2002). More recently, overexpression of Versican, a type of proteoglycan, in NIH3T3 fibroblasts and deletion of Prkar1a from MEFs has been shown to induce MET (Sheng et al., 2006).

We have previously reported the identification of a novel WD domain protein, STRAP, that can bind with TGF-β type I and type II receptors (TβRI & TβRII) and inhibit downstream TGF-β signaling through interaction with Smad7 (Datta et al., 1998 and Datta et al., 2000). The common function of WD domain proteins is to provide a

suitable scaffold for coordinating multiprotein complex assemblies and thus, regulate a variety of cellular processes like signal transduction, transcriptional regulation, programmed cell death, RNA synthesis/processing, chromatin assembly, cell cycle progression and vesicular trafficking (Kashikar and Datta, 2007). Likewise, STRAP has been implicated in a wide array of cellular functions. Our previous studies suggest that STRAP is upregulated in several cancers and functions as an oncogene (Halder et al., 2006). Apart from its role in TGF-β pathway, STRAP interacts with PDK1 to positively modulate PDK1 activity (Halder et al., 2006). We have shown that STRAP induces extracellular signal-regulated kinase (MEK/ERK) activity in a TGF-β-independent manner (Halder et al., 2006 and Seong et al., 2005), and binds with EWS and inhibits the interaction between EWS with p300, resulting in a downregulation of EWS target genes (Anumanthan et al., 2006). In a different role, STRAP seems to be involved in the assembly of the SMN complex which is necessary for mRNA splicing, through its interaction with Gemin6 and Gemin7 as well as SmB, SmD2 and SmD3 components of the complex (Grimmler et al., 2005 and Carissimi et al., 2005). In a relevant interesting finding, STRAP along with EWS was shown to be present in the kinesin driven mRNA transport granules in the dendrites of murine neurons and also shown to bind with NFX1 and MAP1B to help export of mRNA out of the nucleus (Tretyakova et al., 2005). Homozygous deletion of STRAP gene in mice resulted in embryonic lethality between embryonic day (E) 10.5 and 12.5 due to the defects in angiogenesis, cardiogenesis, somitogenesis, neural tube closure and embryonic turning (Chen et al., 2004). A recent study suggests that STRAP may be a predictive marker of 5-fluorouracil (5-FU)-based adjuvant chemotherapy benefit in colorectal cancer (Buess et al., 2004). This wide

variety of functions of STRAP suggests a broader role for it in tumorigenesis and development. However, nothing is known about of the function of STRAP in morphological changes of cells and mechanisms involved in it. Here, we demonstrate, for the first time, that loss of STRAP expression induces a mesenchymal-to-epithelial transition through upregulation of E-cadherin. Furthermore, STRAP-mediated downregulation of WT1 may play a role in the regulation of E-cadherin and subsequently in the maintenance of mesenchymal morphology.

Results

STRAP Deletion Induces an Epithelial-like Morphological Conversion through upregulation of E-cadherin

We have previously reported a role for STRAP in inhibition of TGF-β signaling (Datta et al., 2000). We and others have also shown that STRAP is upregulated in lung, colon and breast cancer (Halder et al., 2006 and Matsuda et al., 2000). To determine the role of STRAP deletion in mouse embryonic fibroblasts (MEFs), we used STRAP null and wild type MEFs that were generated during microarray coupled gene trap mutagenesis to find out PDGF target genes. The gene trap insertion results in embryonic lethality between embryonic day E10.5 and E12.5 due to loss of STRAP (Chen et al., 2004). We first confirmed the loss of STRAP expression in STRAP null MEFs by western blot analysis (Fig 4A). Interestingly, we observed an obvious morphological change in all three STRAP null MEFs, whereas the wild type MEFs maintained mesenchymal morphology, as expected. STRAP null MEFs aggregated and formed

epithelial islands with an increased capacity for adhesion to the tissue culture plates (Fig 4B, top panel). Two other MEF cell lines isolated from different STRAP null embryos showed similar morphological changes (data not shown). To confirm that the cell adhesion and morphological change were due to STRAP loss, we generated and tested HA and Flag-tagged STRAP adenovirus as well as the GFP adenovirus (Fig 4A). Transient expression of adenoviral HA- or Flag-tagged STRAP reverted the morphology of these null MEFs back to the original fibroblastoid type (Fig 4B, bottom panel). The morphological changes in STRAP null MEFs suggested an alteration in intercellular adhesion. Then we analyzed the expression of different epithelial and mesenchymal markers including E-cadherin, β-catenin, fibronectin, cytokeratin and vimentin by western blot analyses. Our data showed that E-cadherin expression was significantly upregulated in STRAP null MEFs. Conversely, fibronectin and MMP9 were downregulated in STRAP null MEFs and total β-catenin and vimentin showed no change. These data suggest that absence of STRAP lead to a partial epithelial transition or a metastable phenotype in MEFs.

STRAP null MEFs show polarization at cell organelle level

Together with protein expression changes, changes at cell organelle level often accompany phenotypic alterations in cells and tend to have a functional impact on the cell characteristics. Therefore we decided to look at the cell-organelle level to understand more about the changes induced by loss of STRAP using transmission electron microscopy (TEM). TEM confirmed the formation of a tightly packed epithelial monolayer showing a close alignment of the lateral plasma membranes of adjacent

STRAP null MEFs. It further revealed that the STRAP null MEFs exhibited an apical-basal polarity, which is a characteristic of epithelial cells. STRAP null MEFs contained numerous electron-dense membranous structures that resembled adherens junctions at basolateral border as indicated by the black arrow. The cell-cell interface is indicated by white arrowheads (Fig 5A upper 2 panels). STRAP null MEFs have an apical distribution of the Golgi complex (shown by notched black arrowheads) relative to the cell nucleus (N) and exhibited microvilli along the apical surface as shown by black an asteric. These features were absent in wild type MEFs (Fig 5A middle 2 panels). TEM also showed the presence of multiple electron-dense cytoplasmic fibers running through the length of wild type MEFs, indicated by black arrowheads. These represent the cytoplasmic stress fibers that are typical for cells of mesenchymal origin. These fibers are contractile actomyosin bundles that are instrumental in the maintenance of the mesenchymal architecture inside cells and facilitate cellular motility. In contrast, these fibers had a more membranous localization in STRAP null MEFs which is typically observed in epithelial cells (Fig 5A lower 2 panels). To confirm this redistribution of actin organization, we stained the MEFs with FITC-phalloidin. As expected, in wild type MEFs, F-actin formed parallel cytoplasmic stress fibers whereas actin was mostly redistributed towards the cell membrane in the STRAP null MEFs (Fig 5 B). These data suggest that STRAP null cells underwent the process of MET not only in regards to changes in gene expression but also showed a consistent change in the cell architecture and distribution of cellular organelles.

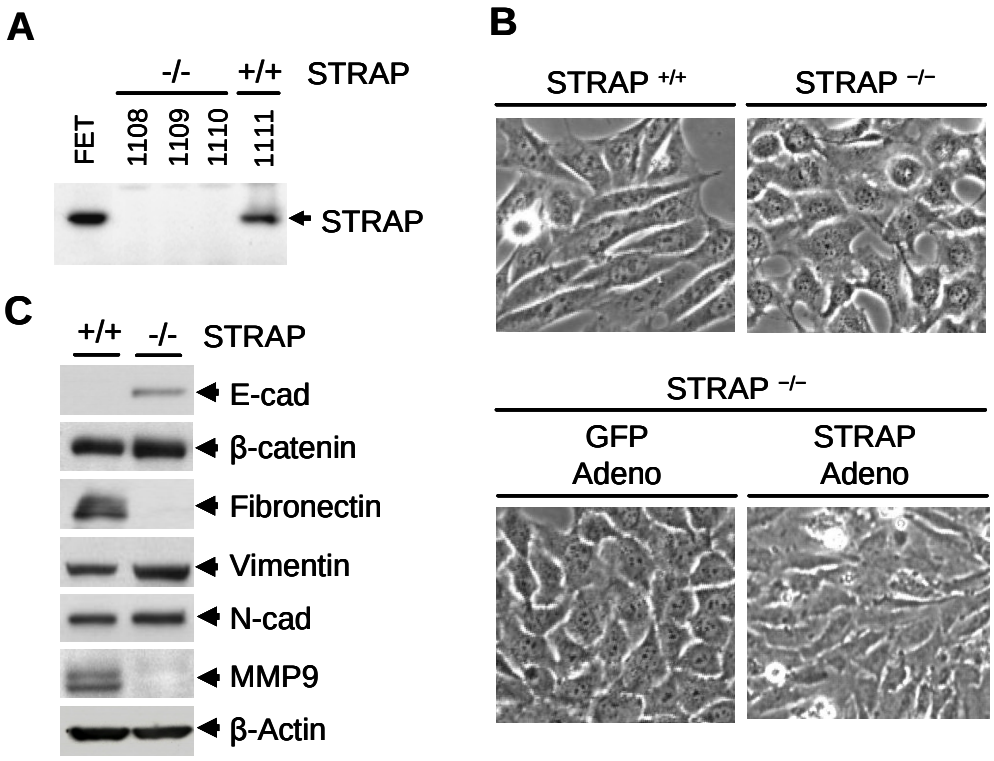


Figure 4: Effect of STRAP on cell morphology. (A) MEFs, isolated from wild type and STRAP null embryos were tested for STRAP expression. **(B)** Phase-contrast photomicrographs showing that loss of STRAP results in a loss of fibroblastoid morphology of the MEFs as cells acquire a epithelial morphology (upper panel). Adenoviral transient expression of STRAP can induce epithelial-to-mesenchymal transition (EMT) in STRAP null MEFs while a control GFP adenovirus fails to induce EMT (lower panel). Adenoviral titer: 200 MOI. **(C)** Lysates prepared from wild type and STRAP null MEFs were used to study markers of epithelial and mesenchymal differentiation. Western analyses shows upregulation of E-cadherin and downregulation of fibronectin and MMP-9 while expression of β-catenin, N-cadherin, vimentin does not change significantly.

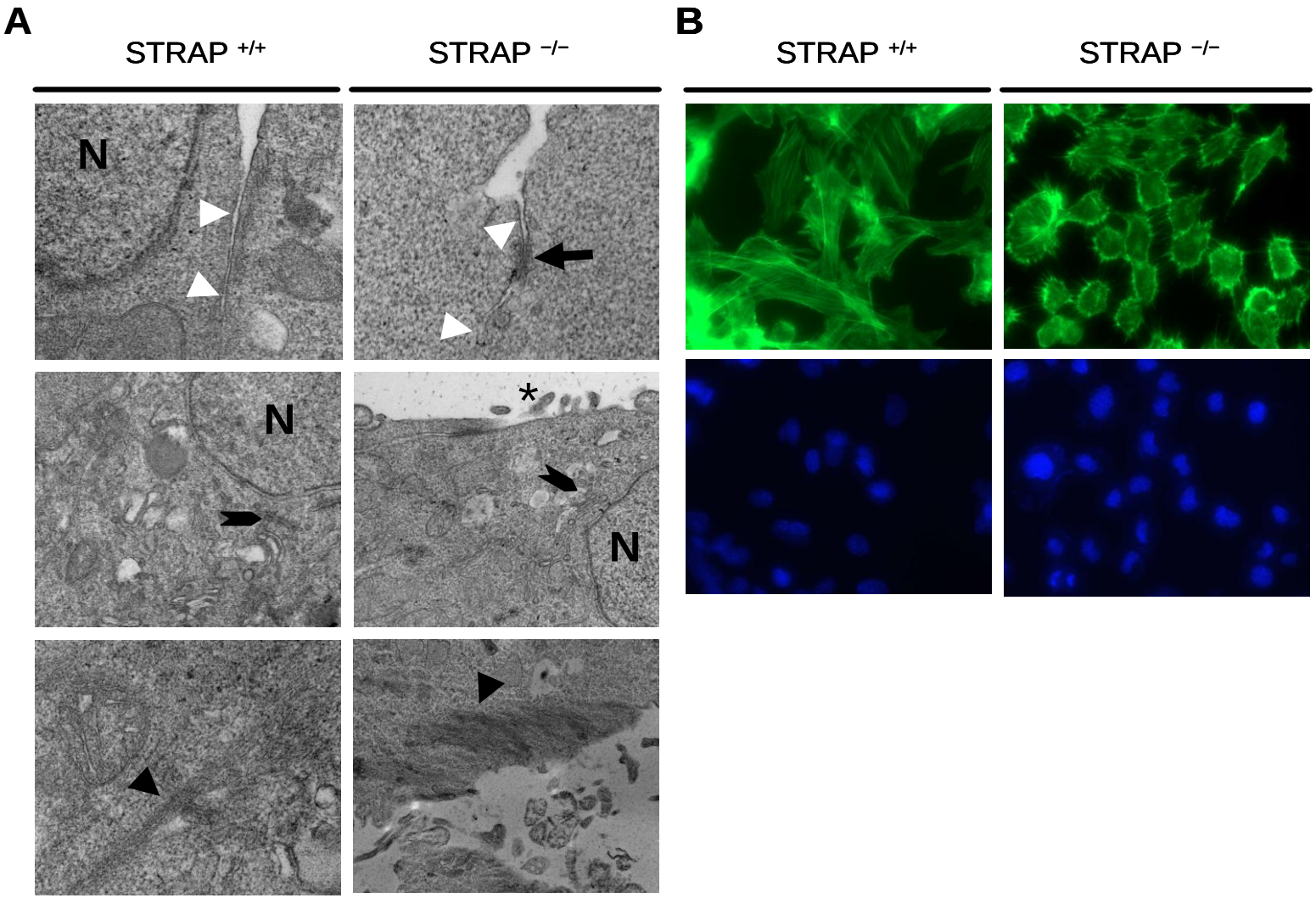
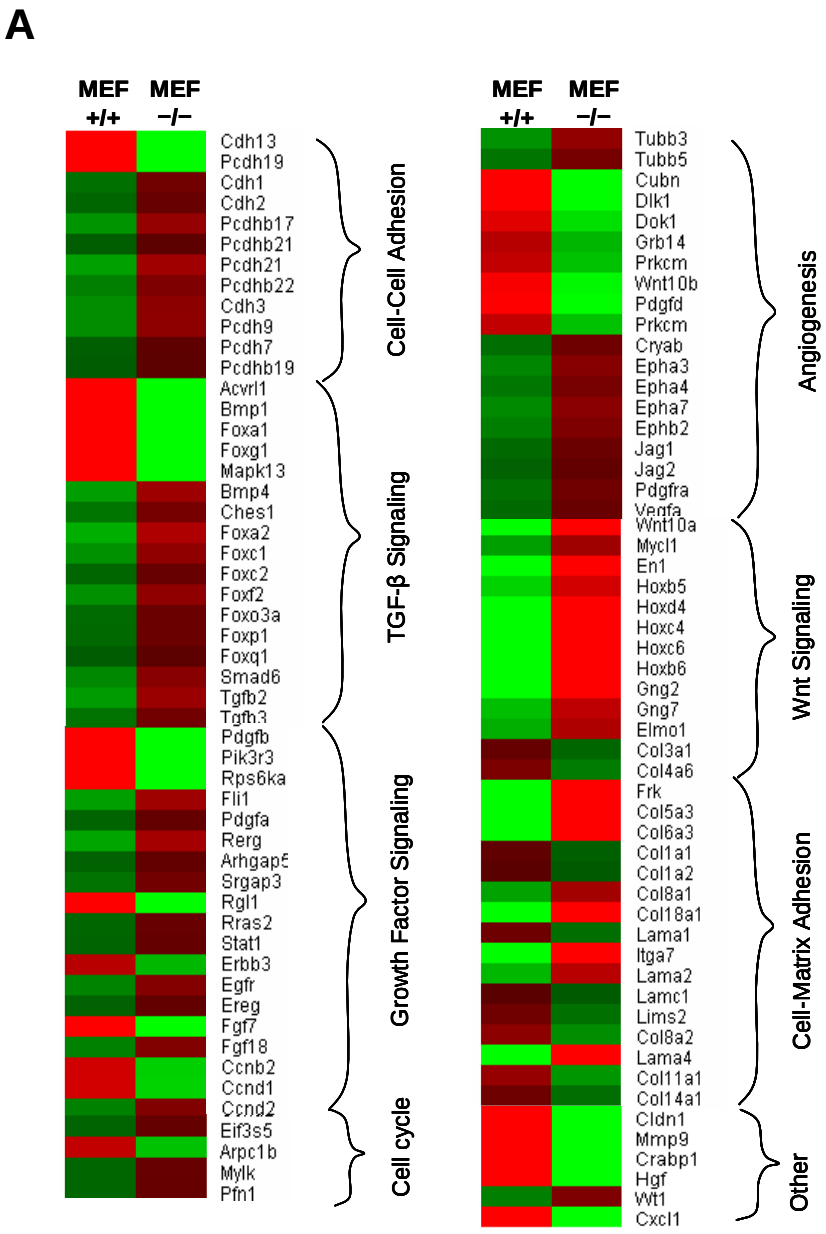

A
STRAP +/+
STRAP −/−
N
N
*
N
B
STRAP +/+
STRAP −/−

Figure 5. Electron microscopic examination indicates STRAP induces EMT through changes at the cell organelle level. (A) Wild type and STRAP null cells were grown in culture on formvar coated coverslips, then fixed with 2.5 % Glutaraldehyde and stained with lead citrate and geranyl acetate. Thin sections were cut using microtome and the sections were analyzed using electron microscopy. Cell-cell interface, indicated by white arrowheads, were observed for frequency of adherens junction formation indicated by black arrows (upper 2 panels). In the middle 2 panels, localization of the Golgi complex, indicated by notched arrows, was observed for polarization relative to the cell orientation. Cell surfaces were observed for microvilli, indicated by a black asterisk. Finally, in the lower panels, cells were analyzed for the pattern and localization of actin (black arrowheads). (N: nucleus). **(B)** Wild type and STRAP-null MEFs were grown in culture, washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1 % Triton X. The cells were then stained with 2 μg/ml of FITC-conjugated phalloidin to map the actin arrangement and localization (upper panel). Nuclei were stained with DAPI (lower panel).

STRAP regulates expression of genes critical for multiple cellular signaling pathways and biological processes

In previous studies, STRAP has been implicated to play role in diverse functions including TGF-β signaling, mRNA splicing, RNA transport, PDK1 activation etc. Here, we noted that STRAP deletion caused partial epithelialization of MEFs. So next we decided to study the overall impact of STRAP in terms of gene expression regulation in MEFs. Analysis of microarray data using PANTHER Prowler analysis tool confirmed that STRAP loss affected sets of genes important for cellular functions like TGF-β signaling, growth factor signaling, cell-cell adhesion, cell-matrix adhesion, cell cycle regulation and Wnt signaling (Fig 6A). Analyses of genes affected by STRAP deletion were also done after regrouping them according to the overall biological processes they are involved with. These results suggest that STRAP deletion affects biological processes such as developmental processes, cell adhesion, signal transduction, mesoderm development, cell motility, angiogenesis, oncogenesis etc (Fig 6B). The cellular functions

affected by STRAP, including TGF-β/other growth factor and Wnt signaling as well as cell-cell/cell-matrix adhesion are known to play diverse roles in the regulation of cell morphology.



B

Biological Process	Genes	P-value
Developmental processes	270	2.46E-28
Signal transduction	348	2.56E-21
Cell communication	167	4.94E-20
Mesoderm development	94	3.27E-16
Cell proliferation and differentiation	118	2.42E-09
Cell adhesion	79	5.38E-08
Cell adhesion-mediated signaling	55	1.47E-06
Ligand-mediated signaling	58	3.44E-06
Cell structure and motility	114	1.04E-05
Receptor mediated signal transduction	154	1.14E-05
Protein phosphorylation	73	2.53E-04
Oncogenesis	54	3.77E-04
Angiogenesis	14	1.72E-03
Cell motility	42	6.34E-03

Figure 6. Differential expression of important groups of genes in wild type and STRAP null MEFs. **(A)** Different gene groups important for diverse functions are altered in wild type and STRAP null MEFs. Wild type and STRAP null MEFs were used to isolate mRNA using Trizol method. Microarray was done using GeneChip 430 Mouse 2.0 Array from Affymetrix that contains 45,000 probes for analyzing 39,000 variants of 34,000 mouse genes. Relative expression of selected genes is shown as a heat diagram. Intense red and green colors indicate high and low expression respectively. **(B)** Biological processes most significantly affected after STRAP deletion were assessed based on the whole array of gene expression changes obtained form the microarray analysis using PANTHER Prowler analysis tool.

STRAP downregulates E-cadherin from cellular junctions

To test the specificity of the effect of STRAP on the regulation of E-cadherin expression, we generated Flag- and HA-tagged STRAP-expressing adenoviruses and tested its expression in wild type and STRAP null MEFs. We observed that exogenous STRAP expression is similar to or less than the endogenous level (Fig 7A). Next we used these adenoviruses to assess the effect of STRAP re-expression on E-cadherin expression in STRAP null MEFs. Adenoviral re-expression of STRAP led to

downregulation of E-cadherin in a time-dependent manner whereas β-gal adenovirus had no effect on E-cadherin (Fig 7B). To further determine whether endogenous STRAP can downregulate E-cadherin from the cellular membrane, we performed immunofluorescence staining and examined subcellular distribution of E-cadherin and β-catenin. The staining pattern showed that E cadherin was absent in wild type MEFs and was seen prominently at the cell-cell junctions in STRAP null MEFs. Hence we speculated that β-catenin would be lost from membranes of wild type MEFs but it would be localized to the membranes in STRAP null MEFs. Indeed, β-catenin was present mainly as a diffuse signal in the cytoplasm in wild type MEFs. By contrast, β-catenin was localized predominantly at the cell–cell contacts in STRAP null MEFs (Fig 7C). These results are consistent with the total levels of E-cadherin and β-catenin in these MEFs (Fig 4C). Collectively, these findings suggest that STRAP regulates E-cadherin expression, and in-turn regulates subcellular distribution of β-catenin. This is significant mainly because nuclear β-catenin is considered as an indicator of EMT in cancer cells. Since it is difficult to detect nuclear β-catenin by immunofluorescence, the functional impact of this E-cadherin loss on nuclear localization of β-catenin was assessed by luciferase assays using TOPFLASH and FOPFLASH reporters. The FOPFLASH is the control luciferase vector without any β-catenin binding sites, whereas TOPFLASH has three TCF/LEF/β-catenin complex binding sites [41]. These assays indicated that β-catenin mediated transcription was reduced by 40-50% in STRAP null MEFs when compared to wild type MEFs. Interestingly, transient re-expression of STRAP in null MEFs increased TOPFLASH reporter activity (Fig 7D) to levels comparable to wild type

MEFs. Taken together, these data suggest that STRAP induces loss of E-cadherin from the membrane that results in nuclear translocation of β-catenin.

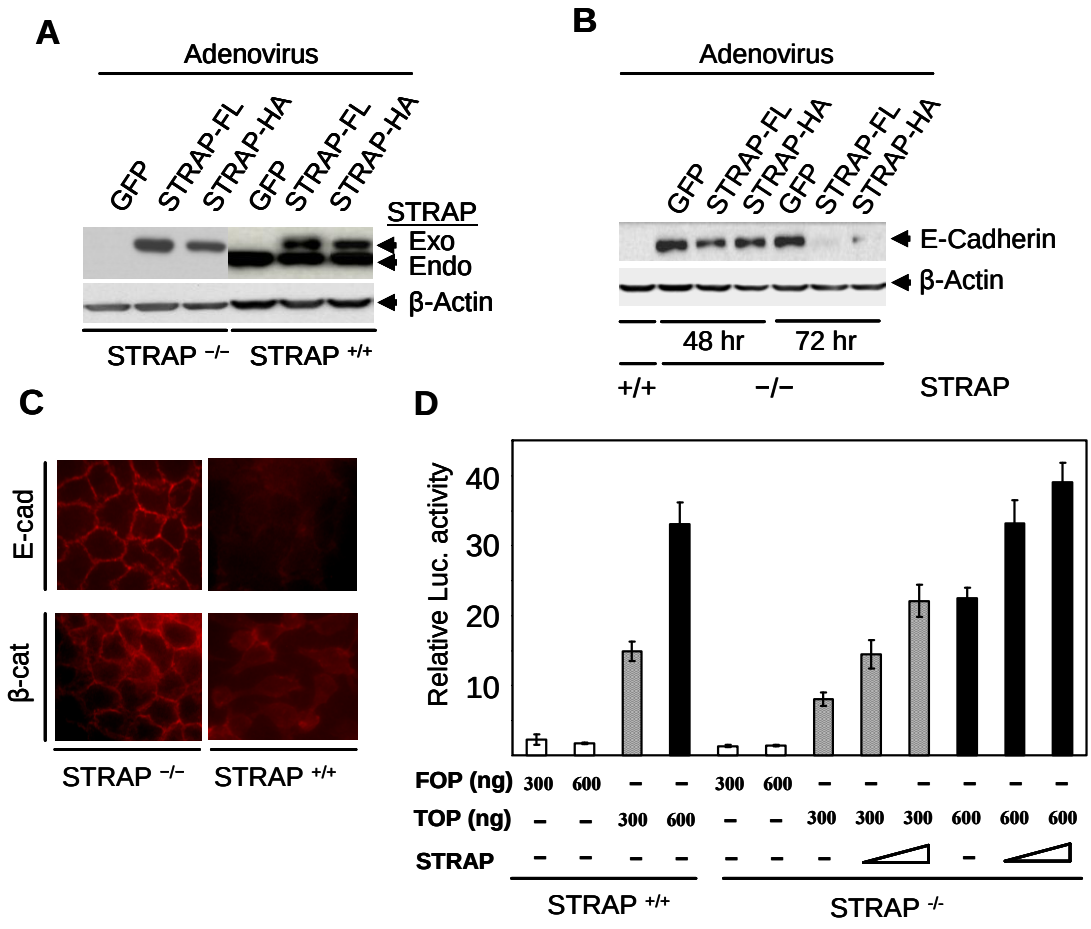


Figure 7. STRAP inversely regulates E-cadherin expression and induces transcriptional activity of β-catenin. **(A)** Replication deficient adenoviruses (RDA) that are able to transiently express either FLAG-tagged or HA-tagged STRAP were generated using AdEasy vector system. Exogenous expression of STRAP was comparable to endogenous expression in wild type MEFs. **(B)** Both HA-tagged and FLAG-tagged STRAP re-expression in STRAP null MEFs repressed E-cadherin expression in a time-dependent manner whereas GFP adenoviral infection did not affect E-cadherin expression. **(C)** STRAP deletion results in expression and localization of E-cadherin and β-catenin to the cell membrane. Wild type and STRAP null MEFs were grown in chamber slides, washed with PBS and after fixing with 4% paraformaldehyde, they were incubated first with either anti-E-cadherin or anti-β-catenin primary antibody and then with cy3-conjugated mouse secondary antibody. **(D)** Decrease in β-catenin

transcriptional activity by STRAP. Wild type and STRAP null MEFs were cotransfected with two doses (300 and 600 ng) of either wild-type (TOPFlash) or mutant (FOPFlash) reporter plasmid and β-galactosidase plasmid with or without increasing doses of STRAP expression plasmid. The total amount of DNA was kept equal. After 48 h of transfection, luciferase activity was determined and normalized to β-galactosidase activity. The mean of triplicate luciferase values was used for fold expression comparison.

Stable expression of STRAP in null MEFs rescued the mesenchymal phenotype

Earlier we showed (Fig 4B) a reversion of STRAP null MEFs from epithelial to a mesenchymal phenotype after transient expression of STRAP. In order to validate this data, we generated stable clones expressing STRAP in null MEFs. pBabe-Puro retroviral vector with mouse STRAP gene was used and the resulting clones were selected in 0.75 μg/ml puromycin The expression of STRAP in these stable clones is shown in Fig 10B. Three independent clones displayed a reversal from the cobblestone-like morphology of STRAP null MEFs to a mesenchymal phenotype (Fig 8). FITC-phalloidin staining revealed that F-actin was organized in parallel stress fibers in these clones (Fig 8) similar to wild type MEFs. Immunofluorescence studies confirmed that E-cadherin expression was almost absent and β-catenin was delocalized from the membrane in STRAP stable clones (Fig 8). No effect on E-cadherin and β-catenin and on morphology was observed in the vector control clone indicating that stable STRAP expression could specifically reverse the MET that occurred in STRAP null MEFs.

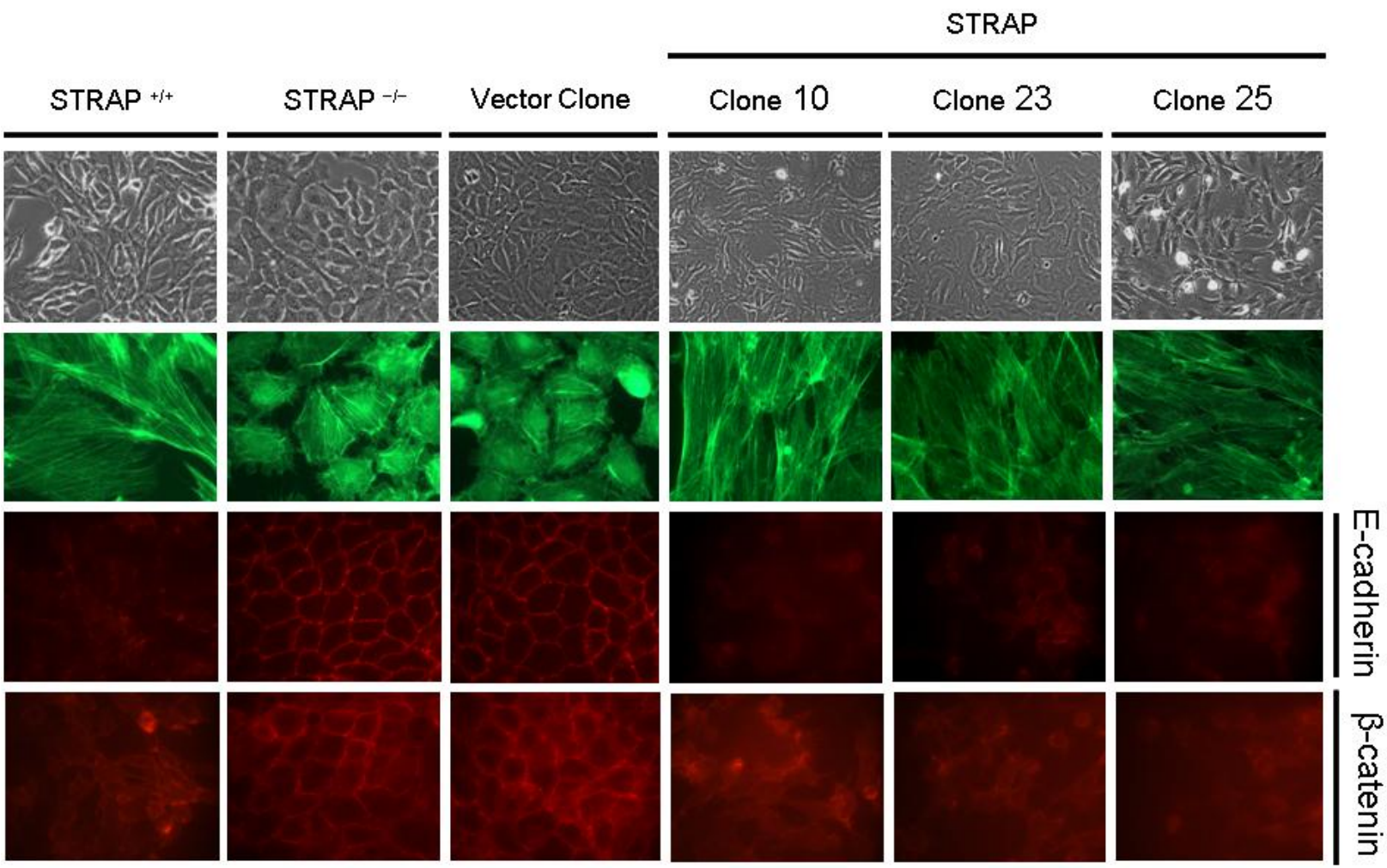

STRAP
STRAP +/+
STRAP −/−
Vector Clone
Clone 10
Clone 23
Clone 25
E-cadherin
β-catenin

Figure 8: Stable STRAP expression in null MEFs restores the mesenchymal phenotype. STRAP overexpressing clones (#10, 23 and 25) were generated from STRAP null MEFs using STRAP pBabe retrovirus, and selected in 0.75 μg/ml of puromycin. These clones reverted to a mesenchymal morphology whereas the vector control clone did not show any morphological alterations. E-cadherin expression went down from the membrane and β-catenin was delocalized in the STRAP stable clones. Loss of E-cadherin results in actin re-organization from a more cortical form in STRAP null MEFs to parallel stress fibers in STRAP stable clones.

Transcriptional upregulation of E-cadherin in STRAP null MEFs through upregulation of WT1

Regulation of the total E-cadherin pool in a cell is a complex process. It has been shown that E-cadherin can be regulated at multiple levels including synthesis, processing and stability of mRNA; synthesis and stability of protein; localization and posttranslational modification and also binding to the catenins. So we next decided to analyze the mechanism responsible for STRAP mediated regulation of E-cadherin. Reporter assays with a mouse E-cadherin promoter luciferase construct showed significant upregulation of E-cadherin promoter activity in the STRAP null MEFs compared to wild type MEFs. This upregulation was suppressed considerably when STRAP was expressed in STRAP null MEFs indicating that STRAP indeed regulates E-cadherin at transcriptional level (Fig 9A). During our analysis of the microarray data, we noticed that one of the known inducers of E-cadherin expression, Wilms tumor 1 (WT1) was significantly upregulated (3.33 fold) in STRAP null MEFs (Fig 6A). On the other hand, zinc finger transcription factors like Snail, Slug, E2A, Twist, SIP1, and ZEB1 are known repressors of E-cadherin expression. We used RT-PCR to analyze the status of the transcriptional regulators of E-cadherin in MEFs. RT-PCR analyses confirmed that

E-cadherin and WT1 mRNA were upregulated in STRAP null MEFs, whereas the expression of Snail, Slug, E2A, SIP1, and ZEB1 was not altered in STRAP null MEFs (Fig 9B). This suggests that WT1 might be involved in the upregulation of E-cadherin in STRAP null MEFs. Expression of other mesenchymal markers revealed that LEF1 was downregulated in STRAP null MEFs whereas FSP1 (S100A) remained unchanged (Fig 9B). Free β-catenin is known to go to the nucleus and activate transcription of target genes such as LEF1 together with co-factors like the TCF family transcription factors (Buess et al., 2004). This is consistent with our data that β-catenin was localized to the membrane of STRAP null MEFs due to upregulation of E-cadherin. This implies a decrease in the nuclear level of β-catenin that can lead to downregulation of LEF1. Western analyses confirmed upregulation of WT1 in STRAP null MEFs. Transient adenoviral STRAP expression in the STRAP null MEFs reduced expression of both WT1 and E-cadherin (Fig 9C). These data suggest that STRAP-mediated downregulation of WT1 may be involved in the regulation of E-cadherin.

Overexpression of STRAP in null MEFs reduces WT1 expression and WT1 activates E-cadherin promoter activity

WT1 has been suggested to induce mesenchymal to epithelial transition in the metanephric mesoderm during the formation of renal parenchyma. It has already been established that WT1 can induce E-cadherin expression in fibroblasts and that stable overexpression of WT1 in fibroblasts induced partial epithelialization (Hosono et al., 1999). Also, mice with Sertoli cells deficient in WT1 show a loss of adherens junctions (Rao et al., 2006). We used STRAP stable clones to test the specificity of the effect of

STRAP on the regulation of WT1. WT1 was downregulated in these clones both in mRNA (Fig 10A) and protein (Fig 10B) levels as seen in RT-PCR and western analyses. RT-PCR also showed transcriptional downregulation of E-cadherin and upregulation of LEF1 in the STRAP stable clones (Fig 10A). This is in accordance with the decreased level of membranous β-catenin that can transcriptionally activate LEF1 expression. In western analyses, total level of β-catenin and N-cadherin did not show any appreciable change but fibronectin, an extracellular matrix protein produced by fibroblasts, is re-expressed in STRAP stable clones as compared to parental STRAP null MEFs or the vector control clone (Fig 10B).

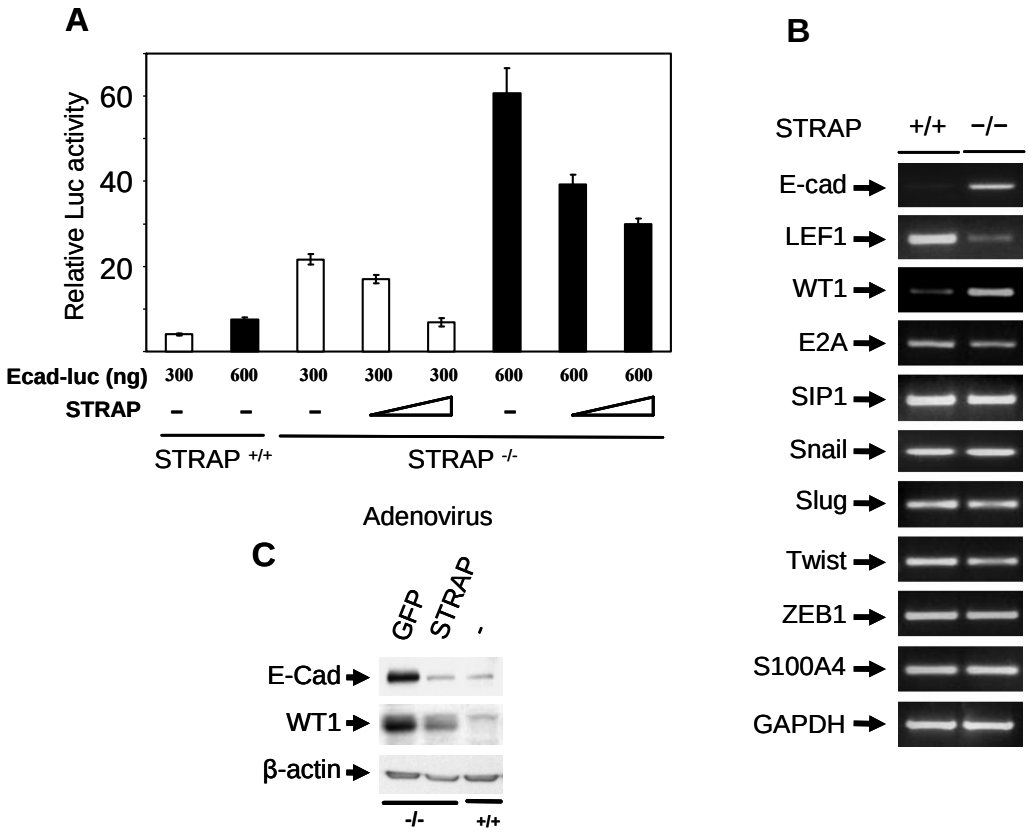
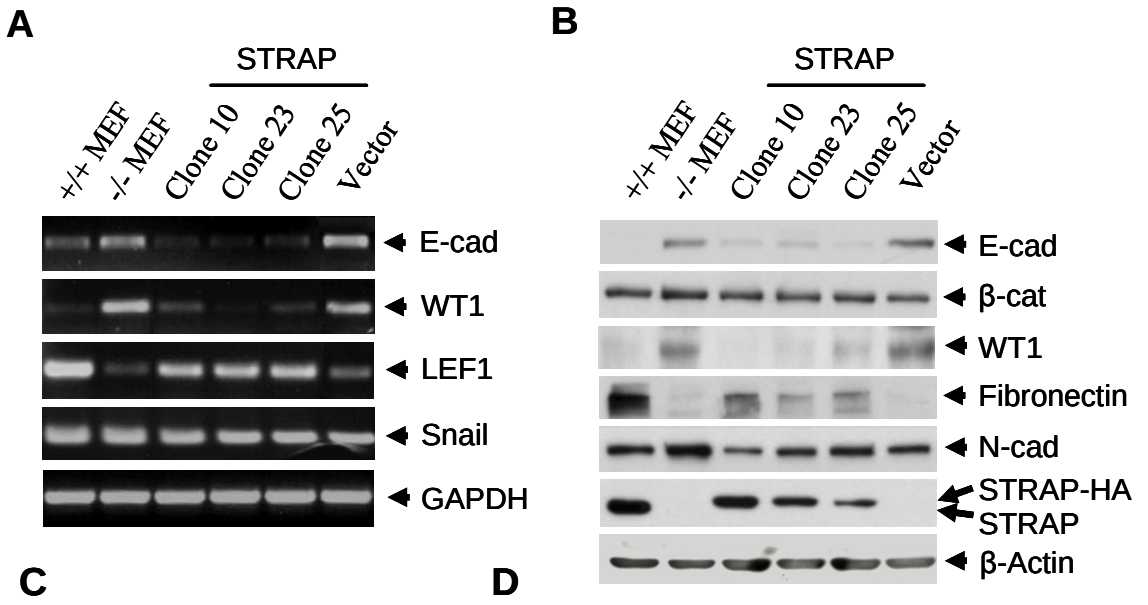


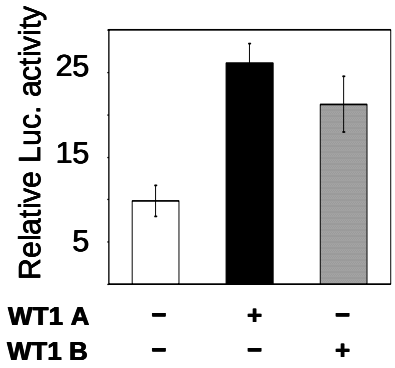
Figure 9. STRAP mediates transcriptional downregulation of E-cadherin through WT1. (A) STRAP represses E-cadherin promoter activity. Wild type and STRAP null MEFs were co-transfected with two doses (300 and 600 ng) of murine E-cadherin (-

178/+92) promoter reporter construct, increasing doses of STRAP expression plasmid and β-galactosidase construct. Luciferase assays were performed and represented as described above. **(B)** Transcriptional expression of E-cadherin and known regulators of E-cadherin was detected by RT-PCR. Total RNA was extracted from both STRAP null and wild type MEFs using Trizol. After treatment with DNAse 1, cDNA was synthesized from RNA and amplified by PCR for 30 cycles using the primers described in Materials and Methods. PCR products were analyzed by agarose gel electrophoresis. Up-regulation of E-cadherin and WT1 and downregulation of LEF1 was detected in STRAP null MEFs. **(C)** STRAP null MEFS were grown in culture and infected with STRAP or GFP adenovirus in a serum free media for 6 hours and lysed after another 72 hours. Immunoblotting was used to analyze for E-cadherin and WT1 expression along with appropriate controls.

The WT1 responsive site has been mapped to the GC rich region at about 40 bp upstream of the transcription start site in the E-cadherin promoter. In luciferase reporter assays, we observed that both WT1 isoforms A and B successfully induced the E-cadherin promoter activity (Fig 10C). This data suggests that STRAP may play a role in the downregulation of WT1 in wild type fibroblasts and this in turn affects E-cadherin expression. Interestingly, when microarray data from the wild type and STRAP null MEFs was analyzed, STRAP null MEFs showed upregulation of multiple WT1 inducible genes like amphiregulin, epiregulin, IGF2, podocalyxibn-like, SOX9 and TIMP3 suggesting that WT1 is transcriptionally active in these cells (Fig 10D). Taken together, our data indicates that STRAP downregulates WT1 expression in the wild type MEFs to suppress E-cadherin expression, and thus maintains the mesenchymal morphology of these cells.



C



D

WT1 Target Gene	Abbr	Fold change
Amphiregulin	Areg	4.8
Epiregulin	Ereg	5.9
E-cadherin	Cdh1	2.7
Podocalyxin-like	Podxl	2.2
Tissue inhibitor of Metalloprotease 3	Timp3	6.4
SRY-box containing protein-9	Sox9	3.5
Growth factor receptor bound protein 10	Grb10	3.1
GATA binding protein 4	Gata4	6.9
wingless-related MMTV integration site 4	Wnt4	2.7
Epidermal growth factor receptor	Egfr	3.6
insulin-like growth factor 2	Igf2	11.1
Connective tissue growth factor	Ctgf	16
Platelet derived growth factor, alpha	Pdgfa	4.5

Figure 10. WT1 can transcriptionally induce E-cadherin expression in fibroblasts. **(A)** The STRAP stable clones and a vector control clone were used to analyze expression status of E-cadherin and transcriptional regulators of E-cadherin using RT-PCR as described above. STRAP null and wild type MEFs served as negative and positive controls. **(B)** Western blot analyses were performed to study the expression of E-cadherin, β-catenin, WT1, fibronectin and N-cadherin in STRAP null and wild type MEFs, STRAP stable clones and a vector control clone. STRAP re-expression was verified in the stable clones and β-actin was used as a loading control. **(C)** Mouse E-cadherin promoter (-178/+92) was transfected in NIH3T3 fibroblasts with either WT1 A or WT1 B expression construct. Luciferase assays were performed and represented as described above. **(D)** WT1 regulated genes are expressed several fold higher in STRAP null MEFs as indicated by microarray analysis.

Discussion

Mesenchyme is a loose connective tissue of mesodermal origin. Fibroblasts, a major component of mesenchymal tissue, mainly function to provide structural support to all of the body tissues and organs. During embryogenesis, growth factors secreted by migrating fibroblasts as well as the epithelial-mesenchymal interactions in the developing tissues are proposed to be necessary for the optimal organ development. Other functions of fibroblasts include but are not limited to wound healing, inflammation, vasculogenesis, angiogenesis, fibrosis and regulation of self-tolerance (Haniffa et al., 2009). Most of these functions are in part due to the ability of these cells to effectively migrate to the sites of their function. In addition to their role in normal biological processes, fibroblasts can also promote pathological processes. For example, cancer associated fibroblasts are known to accelerate cancer growth by secreting growth factors, promoting neo-angiogenesis, and ECM remodeling. These fibroblastic functions are associated with increased rates of tumor invasion and metastasis.

The so-called mesenchymal morphology of fibroblasts is thought to be of central importance to the much higher migratory ability of these cells. During normal development, the process of EMT provides a means by which epithelial cells can move to their appropriate destination tissue by adopting a motile mesenchymal phenotype. Downregulation of E-cadherin appears to be a common mechanism for the acquisition of mesenchymal morphology, even though the exact mechanism used to downregulate E-cadherin can be context dependent. Some of the mechanisms regulating E-cadherin expression include transcription, stability of mRNA and protein, subcellular localization,

and posttranslational modification. Importantly, there is a strong clinicopathological correlation between decreased E-cadherin expression and tumor dissemination (Kopstein et al., 2006). This finding suggests that EMT is a critical step in progression towards invasive and metastatic cancer. A reverse process known as MET, in which cells with a mesenchymal phenotype gain a more adherent epithelial phenotype, is now being proposed as a mechanism for the re-establishment of metastatic cells in the distant organs (Wells et al., 2008). Although several studies have identified factors important for the EMT, not much is known about the molecular mechanisms vital for maintenance of the fibroblastoid morphology of the mesenchymal cells like fibroblasts. To the best of our knowledge, there have been no reports describing the role for STRAP in EMT. Apart from E-cadherin itself, only over-expression of a proteoglycan versican or WT1 is known to be able to induce features of epithelial morphology in fibroblasts. Very recently it has been shown that deletion of Prkar1a in MEFs induces MET through upregulation of E-cadherin expression in fibroblasts (Nadella et al., 2008).

Our present study suggests that expression of STRAP is vital for maintenance of fibroblastoid morphology as deletion of STRAP leads to a partial MET in fibroblasts though upregulation of E-cadherin (Fig 4B & 4C). Our electron microscopic studies showed that when compared to wild type MEFs, STRAP null MEFs showed increased adherens junctions, apically located Golgi apparatus, microvilli on their apical surface and a more cortical localization of actin fibers (Fig 5A). These changes are consistent with features of epithelial cells. Furthermore, re-expression of STRAP in null MEFs leads to downregulation of E-cadherin and to a reversal of the MET. Compared to wild type MEFs, expression of E-cadherin in STRAP null MEFs is elevated at both mRNA and

protein level (Fig 4C, 6A and 9C). The loss of a mesenchymal marker such as fibronectin was observed but other markers such as N-cadherin, FSP1 and vimentin did not show any change (Fig 4C). This suggests that the process of epithelialization of STRAP null MEFs may be a partial one and fits more with the idea of a 'metastable phenotype' described by Savagner et al. According to this study, cells can express both epithelial and mesenchymal markers when they are in transition.

In an effort to understand the mechanism of E-cadherin upregulation, we studied expression of known regulators of E-cadherin expression in wild type and STRAP null MEFs. RT-PCR analyses showed that both cell types expressed similar levels of Snail, Slug, ZEB1, SIP1, Twist and E2A mRNA. However, STRAP null MEFs consistently showed elevated expression of WT1 and reduced expression of the mesenchymal marker, LEF1 (Fig 9B and C). It is interesting to note that WT1 is crucial for induction of MET in metanephric mesoderm during embryonic development (Davies et al., 2004). Additionally, stable WT1 expression in NIH3T3 fibroblasts has been shown to induce partial epithelialization with formation of adherens junctions (Hosono et al., 1999). STRAP null MEFs demonstrated upregulation of WT1 expression at both mRNA (Fig 9C) and protein (Fig 10B) levels. Reporter assays using a murine E-cadherin promoter indicated that both WT1 isoforms A and B could induce E-cadherin promoter activity (Fig 10C), suggesting a role for WT1 in regulating E-cadherin expression in STRAP null MEFs. STRAP was able to repress E-cadherin promoter activity in STRAP null cells in a dose dependent manner (Fig 9A) and this is in agreement with the lower E-cadherin message and protein levels in STRAP null cells. Interestingly, microarray analysis of the STRAP null MEFs showed a robust upregulation of multiple WT1 regulated genes

including amphiregulin, epiregulin, podocalyxin-like, TIMP3, SOX9 and IGF2 in addition to E-cadherin (Fig 10D). Furthermore, transient adenovirus mediated re-expression of STRAP was able to downregulate WT1 and E-cadherin in STRAP null MEFs (Fig 9C) as well as rescue the mesenchymal phenotype (Fig 4B).

The role of STRAP in the maintenance of mesenchymal morphology was confirmed by generating stable clones re-expressing HA-tagged STRAP in STRAP null MEFs. In accordance with transient STRAP expression, stable STRAP expression results in a reversion to a mesenchymal morphology (Fig 8). This morphological change was accompanied by suppression of WT1 and E-cadherin expression (Fig 10A and B). These cells show delocalization of β-catenin from the cell membrane and reorganization of cortical actin into parallel stress fibers. These results are in agreement with nuclear translocation of β-catenin and with the increase in TOP-FLASH activity (Fig 7D). This may lead to downregulation of LEF1 in STRAP null cells (Fig 9B). This was further supported by the observation that stable expression of STRAP in null cells increased the level of LEF1 (Fig 10A). RT-PCR analyses also confirmed downregulation of both WT1 and E-cadherin mRNA in the STRAP stable clones. Chen et al. have found that deletion of STRAP in mice leads to an embryonic lethal phenotype between days (E) 10.5 to 12.5. The STRAP null embryos have defects in processes such angiogenesis, cardiogenesis, gut rotation, somitogenesis and neural tube closure. It is unclear whether these defects are due to some intrinsic defects in the cells of these tissues as a result of STRAP deletion or due to the absence of proper stroma and fibroblast function. Further work will be needed to arrive at a conclusion. However, analysis of the microarray data from wild type and STRAP null MEFs revealed an alteration of a number of genes important for functions

like cell-cell adhesion, cell motility and mesoderm development. Additionally, STRAP deletion also significantly alters the expression of genes (Fig 6A and B) important for embryonic development, signal transduction, cell communication and angiogenesis, which support the previously published biological functions of STRAP (Datta et al., 2000; Seong et al., 2005 and Chen et al., 2004). We also speculate that the balance of EMT versus MET in different tissues may be controlled by cell and tissue type–specific factors including STRAP and therefore, outcomes of such studies will depend on the exact tissue/cell type chosen for that study as previously suggested by the group showing MET in Prkar1a-/- MEFs (Nadella et al., 2008).

EMT allows cancer cells to become more motile and invasive. We reported that STRAP expression is increased in several cancers including 60% of colorectal, 78% of lung and 46 % of breast carcinomas (Halder et al., 2006 and Matsuda et al., 2000). Ectopic expression of STRAP in different cell lines promotes cellular proliferation, induces anchorage-independent growth and increases tumorigenicity during in vitro and in vivo experiments (Halder et al., 2006). It is possible that STRAP overexpression may help tumor cells downregulate E-cadherin in co-operation with other factors known to induce EMT, thereby contributing to the increased migratory and invasive ability of these cells. Further work would be needed in this area to determine whether STRAP can play such a role in tumor cells.

In summary, we have shown, for the first time, that deletion of STRAP in murine fibroblasts is sufficient to cause MET through upregulation of WT1 and subsequently E-cadherin. Re-expression of STRAP in these null cells leads to a loss of WT1 and E-

cadherin expression, and a reversal from epithelial to the mesenchymal morphology. Whether STRAP plays a role in EMT in epithelial cancer cells and whether the defects in STRAP null mice are from perturbation of cell phenotypes in local tissues or due to defect in stromal fibroblasts remains to be seen.

CHAPTER IV

NOVEL ROLE FOR STRAP IN GSK3β SIGNALING PATHWAY

Introduction

GSK3β signaling pathway

Originally, GSK3β was named for its ability to phosphorylate and thereby to inactivate the glycogen synthase (GS), which is a key regulator of glycogen metabolism. GSK-3 is a highly conserved protein kinase and in mammals, GSK-3 is encoded by two genes, termed GSK-3α and GSK-3β (Woodgett 1990). These isoforms share almost complete sequence identity between their protein kinase domains, but have significant sequence differences outside of this region [98% sequence identity in the catalytic domain and 84% overall] (Jope and Johnson 2004). The substrate specificity of the isoforms is similar but the isoform-specific functions are still unclear. GSK3β knockout mice exhibit embryonic lethality, indicating that GSK3α cannot compensate for the loss of GSK3β, whereas GSK3β can overcome the loss of GSK3α (Hoeflich et al., 2000).

GSK3β is ubiquitously expressed with a relatively higher level in brain tissue and is constitutively active in resting cells or tissues (Ferrer et al., 2002). In contrast to a typical kinase, it has been shown that a variety of signaling pathways acting on cells can result in a reversible inhibition of its enzymatic activity. Interestingly, most of the substrates of GSK3β are functionally inhibited after phosphorylation. This means that signals that inhibit GSK3β generally induce the function of GSK3β substrates. GSK3β

exhibits very unique substrate specificity. Almost all of the GSK3β substrates are required to have a priming phosphate at n + 4 (where n is the site of phosphorylation-a serine or threonine residue) to be in turn phosphorylated by GSK3β. Thus, in general, the substrate recognition site of GSK3β is -S-X-X-X-Sp-, where Sp is the priming pre-phosphorylated Serine residue. For example, glycogen synthase (GS) is phosphorylated at Ser-656 by casein kinase 2 (CK2) that in turn 'primes' it to be phosphorylated at Ser-652 by GSK3β, which in turn primes it again for phosphorylation at Ser-648 and so on until five serine residues have become phosphorylated (Cohen and Frame 2001). In a similar fashion, eukaryotic initiation factor-2B (eIF2B) is phosphorylated by dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) at Ser-539 prior to GSK3β phosphorylation at Ser-535. This however does not hold true for a very small subgroup of GSK3β substrates such as Axin that need not be primed to be phosphorylated by GSK3β. This is because Axin binds to a distinct site of GSK3β.

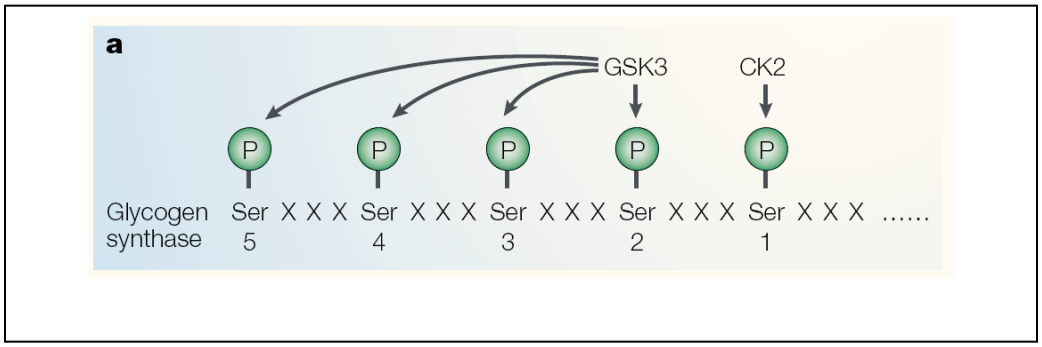


Figure 11. Mechanism of phosphorylation by GSK3β. This figure shows an example of glycogen synthase, which is primed by casein kinase 2 before multiple serial phosphorylations by GSK3β. (From Nat Rev Mol Cell Biol, Oct. 2001, vol 2)

The serine/threonine protein kinase glycogen synthase kinase 3 (GSK3β) is a key regulator of many transcription factors. GSK3β phosphorylates and thereby regulates the functions of many metabolic, signaling and structural proteins. According to Grimes and

Jope (2001), one of the most important roles of GSK3β is the regulation by phosphorylation of numerous transcription factors and thereby the control of the expression of the respective target genes. These transcription factors regulate genes involved in cell growth, cell proliferation, regulation of cellular differentiation and cell death such as AP-1, c-Myc, Notch and CCAAT/enhancer binding protein α (C/EBPα). The list of putative GSK3β substrates is given in table 2. Some including nuclear factor kappa B (NFκB), nuclear factor of activating T cells (NFAT) and heat shock factor-1 (HSF-1) are involved in the immune system. Data published to date suggest that GSK3β plays pivotal roles in the regulation of apoptosis, development, metabolism regulation, neuronal growth and differentiation, cell polarity, and cell fate. Since GSK3β is involved in such a variety of signaling pathways and cellular functions, it is thought that agents that target specific functions of GSK3β may be needed to selectively interfere with GSK3β signaling. Towards this, it is necessary to understand how GSK3β regulates its many roles in the cell.

Table 2. Putative substrates of GSK3β (Jope and Johnson 2004)

Metabolic and signaling proteins	Structural proteins	Transcription factors
AcetylCoA carboxylase	DF3/MUC1	AP-1 (Jun family)
Amyloid precursor protein	Dynamin-like protein	β-catenin
APC	Kinesin light chain	C/EBP
ATP-citrate lyase	MAP1B	CREB
Axin	MAP2	GATA4
Cubitus interruptus	Neural cell-adhesion Protein (NCAM)	Glucocorticoid receptor (rat)
Cyclic-AMP-dependent protein kinase	Neurofilaments	HIF-1
Cyclin D1	Ninein	HSF-1
Cyclin E	tau	Mash1
eIF2B	Telokin (KRP)	MITF
Glycogen synthase		c-Myb
hnRNP		c-Myc
Insulin receptor substrate-1		NeuroD
Myelin basic protein		NFAT
NGF receptor		NF-κB (p65 and p105)
Nucleoporin p62		Notch
P21		p53
Presenilin-1		TCF
Protein kinase A (RII subunit)		
Protein phosphatase 1		
Protein phosphatase inhibitor-2		
Pyruvate dehydrogenase		

Regulation of GSK3β activity

Since GSK3β has a predominant role in the control of several intracellular pathways, its activity needs to be carefully regulated. GSK3β is phosphorylated at Serine9 that inhibits its activity toward exogenous substrates (Cross et al., 1995). GSK3β is also tyrosine phosphorylated within its T-loop region at Y216 (Wang et al., 1994). This is considered to be an activating phosphorylation of GSK3β, but modification of this tyrosine appears to be the result of an autophosphorylation reaction. Additionally, the extent of phosphorylation of this tyrosine residue is always very high in most cells, indicating that there is no important regulatory function of this phosphorylation.

Phosphorylation of the N-terminus and subsequent inactivation of GSK3β by Protein Kinase B (PKB) in response to insulin signaling is one of the most studied out of a number of regulating protein kinases for GSK3β. Insulin signals through its receptor to induce the tyrosine phosphorylation of specific adaptor proteins bound to the receptor. These adapter proteins then recruit different SH2 and PTB domain proteins to the plasma membrane, including the phosphatidylinositol 3′ kinase (PI3K). The PI3K then phosphorylates the phosphatidylinositol 4, 5-phosphate, leading to the formation of 3, 4, 5 phosphatidylinositol (PIP3). This specific phospholipid is limited to the membrane and has a high affinity for proteins harboring pleckstrin homology domain such as phosphoinositide-dependent protein kinase-1 (PDK1) and PKB. PDK1 then phosphorylates PKB after their co-localization at the membrane. PKB in turn phosphorylates and inactivates GSK3β (Cross et al., 1995; Jope and Johnson 2004). Cyclic AMP-dependent protein kinase (PKA) (Fang et al., 2000), atypical protein kinase

C (PKC) (Fang et al., 2002) and p90Rsk (Sutherland et al., 1993) are other protein kinases that target the N-terminal domain of GSK3β.

Structural considerations for GSK3β

As discussed above, most of the substrates need to be primed by another kinase before being phosphorylated by GSK3β. For some substrates, the phosphate group added by GSK3β acts as a priming phosphate for subsequent phosphorylation by GSK3β, leading to serial phosphorylation by it. It is also possible to regulate the processing of any GSK3 substrate by regulating the priming kinase activity. GSK3β comprises an amino-terminal lobe composed mostly of β-sheets and a carboxy-terminal lobe that is predominantly α-helical (Dajani et al., 2001; ter Haar et al., 2001). As discussed above, the activation or T-loop of GSK3β is only phosphorylated at a single tyrosine residue, Y216. Phosphorylation at this tyrosine residue has little effect on the conformation of GSK3β. Consistent with this; removal of the phosphorylated tyrosine does not completely inactivate the enzyme. But the binding of the priming phosphate to the positively charged pocket made of R96, R180 and K205 residues of GSK3β leads to a favorable conformation of the catalytic domain.

As discussed above, phosphorylation of S9 of GSK3β creates a primed pseudosubstrate that occupies the catalytic groove and prevents phosphorylation of exogenous substrates. The binding affinity of the pseudosubstrate is low so that phosphorylated primed substrates can compete off the inhibitory polypeptide at higher concentrations (Frame et al., 2001). However, it is the dephosphorylation of the N-

terminal residue, possibly by a phosphatase termed Laforin that is generally needed to reverse the GSK3β inhibition in the cells (Lohi et al., 2005).

GSK3β substrate specificity

Though mitogens and hormones such as insulin negatively regulate GSK3β through Ser9 phosphorylation, this mechanism appears to play no role in the regulation of GSK3β in the Wnt pathway. It has been observed that cells maintain localized subpopulations of GSK3β in such a way that growth factors and hormones that inhibit GSK3β through Ser9 phosphorylation have no effect on β-catenin stabilization. Similarly, Wnt signaling does not cause changes in GSK3β substrates that are regulated by growth factors and hormones such as glucose/glycogen metabolism. This is certainly a very critical property of GSK3β as any cross-talk of these pathways can lead to ectopic or inappropriate stabilization of key GSK3β substrates such as β-catenin and c-Myc and drive cells towards oncogenesis. It seems that protein complex formation and intracellular localization are effective ways to regulate this enzyme. The idea that is gaining ground is that sequestration of distinct pools of signaling molecules occurs within organizing complexes such as A-kinase anchoring proteins (AKAPs) and other scaffolding proteins (Carnegie et al., 2009). For example, effective binding of key GSK3β substrates β-catenin and tau need docking of these substrates to GSK3β by scaffolding proteins like Axin and presenilin, respectively. Considering that any inhibitor of GSK3β will inhibit access of all substrates to GSK3β rather than just the one substrate critically deregulated in a certain disease state, it might be a useful to develop compounds that target specifically the 'priming phosphate' of specific substrates.

GSK3β: Role in Wnt signaling

Wnt proteins are secreted glycoproteins that play key roles in cell growth, differentiation, migration, and cell fate determination. N-terminal phosphorylation at Ser9 plays no role in regulating GSK3β function by the Wnt pathway. GSK3β plays a role only in the "canonical" Wnt signaling pathway to tightly control the cytoplasmic levels of β-catenin. GSK3β only interacts with β-catenin within the context of a large protein complex that comprises GSK3β, β-catenin, the scaffolding protein Axin, and the tumor suppressor, adenomatous polyposis coli (APC). In resting cells, GSK3β phosphorylates β-catenin at three residues S33, S37, and T41. This targets β-catenin for ubiquitination by the E3 ubiquitin ligase βTrCP (Lagna et al., 1999; Winston et al., 1999), and subsequent degradation via the 26S proteasome to maintain cytoplasmic and nuclear concentrations of β-catenin at extremely low levels. GSK3β mediated phosphorylation of Axin is not necessary for its binding with β-catenin but GSK3β mediated phosphorylation of APC does increase its binding affinity for β-catenin (Yamamoto et al., 1999; Rubinfeld et al., 1996). Wnt stimulation leads to a reduction in Axin phosphorylation and disrupts the complex formation with GSK3β. This may contribute to Wnt-mediated stabilization of β-catenin by inhibiting its phosphorylation, leading to its accumulation in the nucleus where it transactivates target genes by interacting with LEF/TCF family of transcription factors. This is a critically regulated pathway and mutations in scaffold proteins like APC occur in 70 % of sporadic colon cancers whereas Axin is mutated in hepatocellular carcinomas. Both of these events lead to chronic stabilization of β-catenin (Salahshor and Woodgett 2005).

Other functions of GSK3β

GSK3β plays a role in glucose homeostasis through its substrate glycogen synthase, the rate-limiting enzyme of glycogen synthesis. Insulin inhibits GSK3β through PDK to allow for glycogen synthesis. In neurons, GSK3β promotes increased microtubule polymerization and axonal outgrowth by inhibiting its substrate collapsing response mediator protein 2 (CRMP2) that is known to bind microtubules. Phosphorylation of APC by GSK3β acts to destabilize the microtubules as APC is also a microtubule binding protein (Zumbrunn et al., 2001). Several other brain-enriched structural proteins are GSK3β substrates, including microtubule-associated protein 1b (MAP1B) and Tau which is known for its possible role in Alzheimer's disease (AD) (Maccioni et al., 2001). Apart from β-catenin, GSK3β is shown to phosphorylate c-Myc, c-Jun, Cyclin E, Notch, Cyclin D1 and target them for proteolytic degradation through ubiquitin-proteasome system (Xu et al., 2008).

Notch Signaling Pathway

Notch signaling, conserved from flies to mammals, regulates cell fate decisions through direct cell-cell interactions (Lai, 2004). Notch was named so due to the indentations (notches) displayed in the wing of mutant flies. Notch signaling is now known to regulate a wide variety of developmental processes such as hematopoiesis, neurogenesis, miogenesis, wing formation and somite segregation (Lewis, 1998).

Notch Signaling in Brief

Notch signaling pathway relies only on a few key components to convey the signal from the cell surface to the transcriptional machinery. Binding of ligands from one cell to Notch receptors in neighboring cells triggers proteolytic cleavage of Notch receptor, which results in a release of the intracellular domain of Notch (ICN) from the plasma membrane. ICN then travels to the nucleus and associates with a CSL (CBF-1/Suppressor of Hairless/Lag-1) DNA-binding protein. This binding converts CSL-containing complexes from transcriptional repressors to transcriptional activators, and thereby changes the transcriptional program of these cells (Fig 12 A) (Lai, 2004). Apart from this, there exists a wide array of modulators of Notch signaling, most of which are specific for only a subset of Notch modes of action.

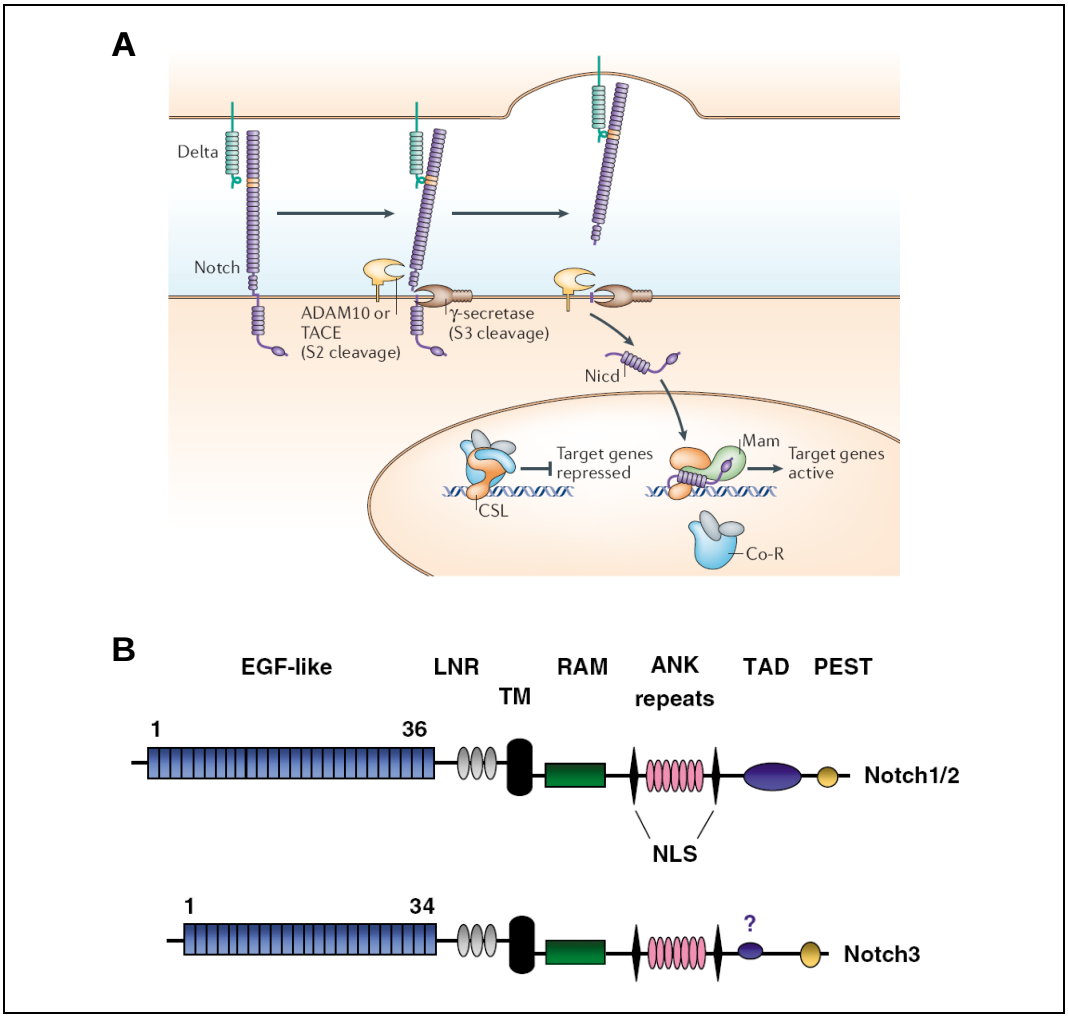


Figure 12. Notch Signaling. (A) Schematic view of Notch signaling. Binding of Delta ligand on one cell to the Notch receptor on another cell results in two proteolytic cleavages of the receptor, releasing the Notch intracellular domain (ICN). ICN enters the nucleus helping to recruit co-activator Mastermind and other transcription factors to the CSL complex while releasing co-repressors. (From Nat Rev Mol Cell Biol. Sep 2006 vol. 7) **(B)** Comparison between Nocth1/2 and Notch3. All three notches have the LIN, ANK and PEST domains but there is a controversy whether Notch3 has a functional transactivating domain (TAD) or not and therefore represented by a question mark. (From Oncogene Sep 2008 vol. 27)

Structure of the Notch receptors and ligands: The Notch receptor family encodes large single-pass transmembrane proteins that are present at the plasma membranes as heterodimers. They consist of an extracellular domain and a membrane-tethered

intracellular domain, which share some common characteristic features. On the extracellular side, the receptors contain a variable number of tandemly-arranged Epidermal Growth Factor (EGF)-like repeats and a family-specific LNR (Lin Notch Repeat) (Wharton et al., 1985). The intracellular domain of Notch has four main sub-domains: the RAM, ankyrin repeat, RE/AC and C-terminal region. The RAM domain is believed to mediate direct interaction with CSL, probably together with the ankyrin repeats (Roehl et al., 1996). The ankyrin repeats are known to mediate protein-protein interactions (Kopan et al., 1994). The C-terminal part is rich in Prolines (P), Glutamic acids (E), Serines (S), Threonines (T) and termed as the PEST domain. PEST sequences are thought to be important for the ubiquitination and stability of the protein (Greenwald, 1994). There are two types of Notch ligands, Delta and Serrate. Just like the Notch receptors, the ligands are also single-pass transmembrane proteins, with a large EC domain containing tandemly arranged EGF-like repeats. There are five characterized mammalian ligand homologs: Deltalike1, 3, 4 and Jagged 1 and 2, and four mammalian Notch receptors: Notch 1 to Notch 4.

Activation of Notch receptor

The Notch signaling mechanism is characterized by a series of proteolytic events referred to as S1, S2, and S3 cleavages (Aster et al., 1994). The first, S1 cleavage occurs in the trans-Golgi network in a constitutive manner by a furin-like convertase before receptor is transported to the membrane. Binding of ligand to the Notch extracellular domain results in S2 cleavage and releases the majority of the extracellular domain. This is mediated by the Tumor Necrosis Factor-Converting Enzyme (TACE), a disintegrin and

metalloprotease domain (ADAM) protein in vertebrates (Brou et al., 2000). S2 cleavage by ADAM is a necessary step for γ-secretase mediated S3 cleavage. Rapidly following S2 cleavage, the membrane anchored Notch receptor is constitutively cleaved at a third site (S3) and the signal mediator ICN is released from the membrane. S3 cleavage is executed in the transmembrane region of Notch receptor by a large enzymatic complex known as γ-secretase complex. Following S3 cleavage, the intracellular domain of Notch translocates to the nucleus and directly induces transcription of downstream target genes. ICN can't bind to cognate DNA by itself, but interacts with the protein CSL (CBF-1/Suppressor of Hairless/Lag-1, RBP-Jk) (Tamura et al., 1995), which is a highly conserved DNA-binding protein. In the absence of Notch ICD, CSL represses transcription (Zeng et al., 2005) by interacting with ubiquitous co-repressor proteins to form multi-protein transcriptional repressor complexes, which in turn recruits histone deacetylase complexes (HDACs) to the site and convert the local chromatin into a transcriptionally silent state. The effects downstream of canonical Notch signaling pathway are not completely understood, but two families of basic helix-loop-helix transcription factors, Hes and Hey have been well established to be primary downstream targets following Notch activation (Sasai et al., 1992; Zhong et al., 2000). These proteins are thought to keep the signal-receiving cells in an undifferentiated state. Microarray analysis is revealing new Notch target genes such as SKP2 (Dohda et al., 2007), interleukin-6, c-Myc (Sharma et al., 2007), Cyclin D1 (Ronchini et al., 2001), p21 (Rangarajan et al., 2001) and smooth muscle-actin (Noseda et al., 2006). The current opinion is that the expression of these target genes induced by Notch activation is highly

dependent on the cell context, suggesting that additional specific proteins may be involved in regulating their expression.

Regulation of Notch Signaling

To maintain sensitivity to new signaling input, cells need to have effective means to tightly control the signaling strength. For Notch signaling, MAML acts as both coactivator and terminator for ICN transcriptional activity. MAML directly recruits cyclin C:CDK8 complex to the CSL-ICN-MAML transcriptional coactivator complex in the nucleus. This is followed by phosphorylation of ICN within the TAD and PEST region (Fryer et al., 2004). It has also been demonstrated that Fbw7 (F-box and WD-40 domain-containing protein 7) is an E3 ubiquitin ligase that induces polyubiquitylation of phosphorylated ICN and triggers rapid degradation of ICN by proteasomes (Wu et al., 2001).

Notch as an Oncogene

Patients suffering from T-cell lymphoblastic leukemia (T-ALL) provided the first evidence for an oncogenic function of Notch. About 1% of the cases possess a specific chromosomal translocation, t(7;9), resulting in the fusion of the carboxy-terminal region from within the EGF-repeat 34 of Notch 1 to the enhancer sequences of the T cell antigen receptor subunit (Reynolds et al., 1987) that produces a truncated Notch 1 receptor that corresponds to ICN1, which behaves in a constitutively active fashion. More recently two types of activating mutations within Notch 1 were found in 55-60% of human T-ALL

cases (Weng et al., 2004). The first type occurs in the heterodimerization region and leads to ligand-independent activation of Notch receptor. Much more relevant to our work, the second type involves the C-terminal PEST region of Notch receptor, resulting in the stabilization of ICN.

Fbw7 is a WD40 domain protein that acts as an ubiquitin ligase implicated in ICN turnover (Gupta-Rossi et al., 2001; Wu et al., 2001)). Fbw7 specifically recognizes phosphorylated substrates. Sequences recognized by Fbw7 in c-Myc, c-Jun, SREBP1a and Notch are phosphorylated by GSK3β, resulting in the ubiquitylation and degradation of these proteins (Sundqvist et Al., 2003; Wei et al., 2005). Mutations that abrogate the binding of Fbw7 to Notch1 also abrogate its binding to another two characterized targets, c-Myc (Yada et al., 2004) and cyclin E (Minella et al., 2005). Stabilization of both ICN (intracellular notch) and its principle downstream target, c-Myc, may contribute to transformation in leukemias with Fbw7 mutations. ICN1 is also phosphorylated by GSK3β. In addition to T-ALL, the most compelling evidence for Notch oncogenic effect in other context stems from breast cancer and melanoma. Tissue microarray studies have shown that high expression levels of Jagged1 and/or Notch 1 in human breast cancer are associated with a more aggressive disease course (Bismar et al., 2006). Notch signaling has also been suggested to be required in the hypoxia-induced EMT and cell migration in tumor cells.

Just as other Notches, Notch3 plays a role in development indicated by it ability to alter cell fate in animals expressing gain-of-function mutants of Notch3 (Dang et al., 2003; Apelqvist et al., 1999). Most of the studies relating to the role of Notch in cancer

focus on Notch1 and little is known about the role of Notch3 in epithelial tumors, such as lung carcinomas. Recently, Notch3 was shown to be expressed in approximately 40% of resected human non-small cell lung cancers (NSCLC) (Haruki et al., 2005). This is significant as Notch3 expression in normal adult lungs is restricted only to the smooth muscle cells of blood vessels (Villa et al., 2001). Furthermore, inhibition of the Notch3 pathway using a dominant-negative receptor dramatically decreased the malignant potential of lung cancer cells as evidenced by reduced growth in soft agar and increase in growth factor dependence. Treatment of lung cancer cells with a γ-secretase inhibitor inhibited Notch3 signaling, reduced tumor cell proliferation and induced apoptosis (Konishi et al., 2007).

Recent reports have shown increased expression of Notch3 in T cell leukemias and epithelial malignancies arising from pancreas, ovary, breast and lung (Talora et al., 2003; Miyamoto et al., 2003; Dang et al., 2000; Lu et al., 2004 and Yamaguchi et al., 2008). It is suggested that Notch3 may play a causative role in these tumors. How Notch3 is upregulated in these cancers and what is the mechanism for the possible Notch3-mediated carcinogenesis is not known. Here we show that STRAP deubiquitinates ICN3 and may help to stabilize it. STRAP is already known to be upregulated in 78% lung cancers and if STRAP is found to be important for Notch stabilization, overexpression of STRAP may explain ICN stabilization in some of the cases.

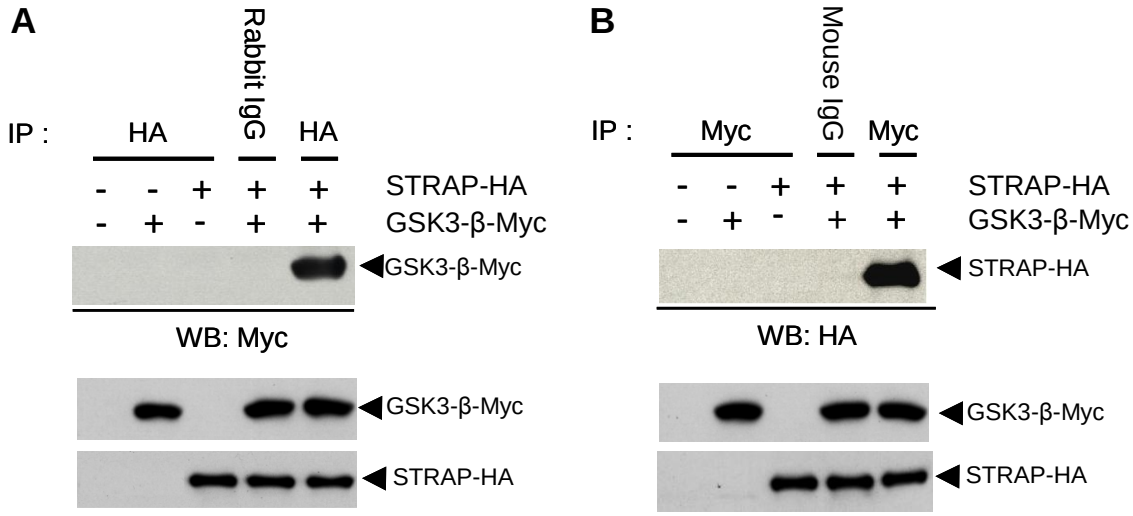
Results

STRAP binds to GSK3β through its WD40 domain region

Ewing et al. (2007) were the first to report the possibility of an interaction between the scaffold protein STRAP and GSK3β, the classic enzyme in the Wnt and insulin signaling pathways. During a large scale analysis of human protein-protein interactions using mass spectroscopy, they predicted that STRAP binds with GSK3β with a probability of only 0.5. Since they had not validated the binding between STRAP and GSK3β, we decided to determine whether STRAP actually interacts with GSK3β using co-immunoprecipitation experiments. 293T cells were transfected with myc-tagged GSK3β and HA-tagged STRAP using Lipofectamine and Plus reagent according to manufacturer's protocol. The cells were lysed and the lysates were incubated with either anti-HA, anti-Myc or appropriate control IgG antibody followed by incubation with protein G-sepharose beads. Figure 13 subpanels A and B show that GSK3β-myc was co-immoprecipitated along with STRAP-HA and vice versa. Corresponding negative controls with either transfection of single plasmid (second and third lanes) or immunoprecipitation with a pre-immune Rabbit or Mouse IgG (fourth lane of both panels) did not show any co-immunoprecipitated proteins indicating that the binding between STRAP-HA and GSK3β-myc was a specific one.

After validating the specific interaction between STRAP and GSK3β, we decided to do a preliminary mapping of the region of STRAP that mediates this interaction. We tested whether STRAP binds GSK3β through its WD domain region or the C-terminal region. We used a STRAP deletion construct that has only the WD40 region i.e. the N-

terminal 294 amino acids but lacks the C-terminal 57 amino acids (CT1-STRAP). When a co-immunoprecipitation assay was performed in a similar way as above, CT1-STRAP-HA was co-precipitated equally well with GSK3β as the wild type STRAP-HA indicating that GSK3β binds STRAP through the WD40 domain region (Fig 13C, lanes 2 & 4). Further search to find the exact STRAP region that binds to GSK3β was prohibited by the fact that any deletions in the WD40 region have a tendency to make STRAP unstable. Together, these results indicate that STRAP specifically associates with GSK3β though its WD40 domain region. It is widely known that WD40 domain proteins are involved in a range of diverse cellular functions including signal transduction pathways. This is achieved by using the WD40 domains as a platform for a stable association of multi-protein assemblies. We then speculated that STRAP being a WD40 scaffold protein can play a similar function in the GSK3β signaling pathway. It is possible that STRAP may either recruit an upstream signaling kinase to bind with GSK3β or it can recruit a substrate to GSK3β. It is also possible that STRAP may help further processing of the substrate after it is phosphorylated by GSK3β.



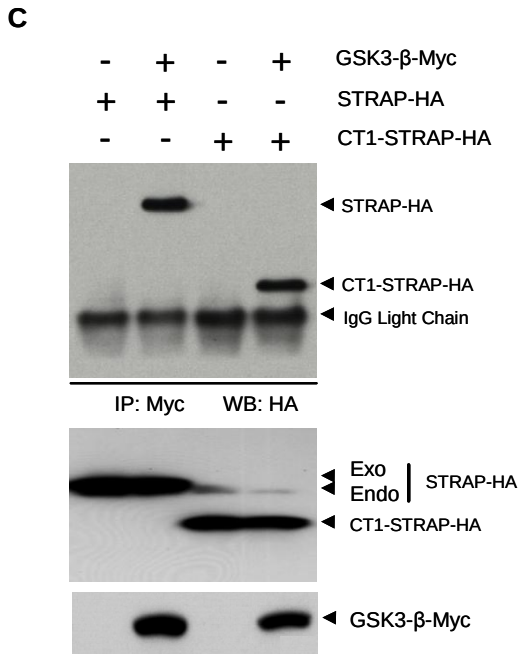


Figure 13. GSK3β and STRAP physically interact with each other. (A) STRAP-HA and GSK3β-Myc constructs were transiently transfected into 293T cells. Cells were subjected to lysis 48 hours after tranfection, immunoprecipitation using 1 μg of pre-immuneanti-rabbit IgG or 1 μg anti-HA antibody and immunoblotted with anti-myc antibody as indicated. Bottom panels show comparable expression of GSK3β-Myc and STRAP-HA in the lysates. **(B)** Same as above except immunoprecipitations were done with anti-mouse IgG and anti-myc antibodies and immunoblotting was done with anti-HA antibody. All antibodies are from Santa Cruz Biotechnology. Bottom panels show comparable expression of GSK3β and STRAP in the lysates. **(C)** GSK3β interacts with the WD40-domain region of STRAP. STRAP-HA, CT1-STRAP-HA and GSK3β-Myc constructs were transiently transfected into 293T cells. Immunoprecipitation was done with anti-myc and immunoblotting with anti-HA antibody as indicated. Light chain of the myc antibody used for immunoprecipitation is visible just below the CT1-STRAP-HA band. Bottom panels show comparable expression of STRAP-HA, CT1-STRAP-HA and GSK3β-Myc in the lysates.

Effect of GSK3β inhibitors on STRAP/ GSK3β binding

EGF, insulin and Wnt signaling regulate GSK3β through the inhibitory Ser9 phosphorylation medicated by PKA or PKB. However, the mechanism of GSK3β regulation in response to other signals is less clear. It is also proposed that different pools

of GSK3β exist within the cell to integrate upstream signals with specific downstream targets.

To understand if STRAP has any preference towards binding activated or inhibited state of GSK3β, we repeated the co-immunoprecipitation assay between GSK3β and STRAP in presence of the upstream inhibitor of GSK3β, lithium chloride and three other small molecule inhibitors, namely AR-A01441, SB415286 and SB216763. LiCl is an ATP noncompetitive inhibitor of GSK3β activity (Ki 2 mM) that has been used extensively in studies investigating the functional role of GSK3β (Hong et al., 1997; Klein et al., 1997; Stambolic et al., 1996). Lithium ion competes with Mg++ ion that is necessary for GSK3β activity. LiCl has also been reported to acutely elevate phosphatidylinositol 3-phosphate levels in some cell types, thereby activating PKB (Chalecka-Franaszek et al., 1999). Activated PKB phosphorylates and inhibits GSK3β, suggesting that LiCl has the potential to inhibit this kinase both directly and indirectly in cells. AR-A01441, SB415286 and SB216763 are among the new potent, highly selective and cell permeable small molecule inhibitors of GSK3β. These compounds inhibit their target protein kinase in an ATP competitive manner (Coghlan et al., 2000). These small molecule inhibitors bind to the Val135 and Asp133 residues in the catalytic domain of GSK3β and inhibit GSK3β in vitro at 0.01 mM ATP with IC50s less than 100 nM. These compounds are highly GSK3 specific and also mimic actions of external inhibitors of GSK3β like Insulin as they stimulate glycogen synthesis in human liver cells and also mimic Wnt signaling in their ability to induce expression of a β-catenin-LEF/TCF regulated reporter gene in HEK293 cells. Figure 14 shows results of the

coimmunoprecipitation-western blot experiment between STRAP and GSK3β in presence of LiCl and the small molecule inhibitors.

Comparison between lane 2 and lane 6 of figure 14 indicated that lithium did not have any notable effect on binding of STRAP with GSK3β suggesting that STRAP may not have any dependence on the active or inactive state of GSK3β to bind with it. In contrast to this, STRAP binding with GSK3β was reduced moderately in presence of AR-A014418 and reduced considerably in presence of the other two small molecule inhibitors, SB216763 and SB415286 (Fig 14). Since these inhibitors directly bind with the catalytic domain of GSK3β, this domain or more likely, the region surrounding the catalytic site seems to play a role in the binding of GSK3β with STRAP. There are so far no reports showing any unfavorable effect of the small molecule inhibitors on Axin and GSK3β interaction. This is expected as Axin binds GSK3β through the C-terminal region of GSK3β at a site distant from the catalytic domain.

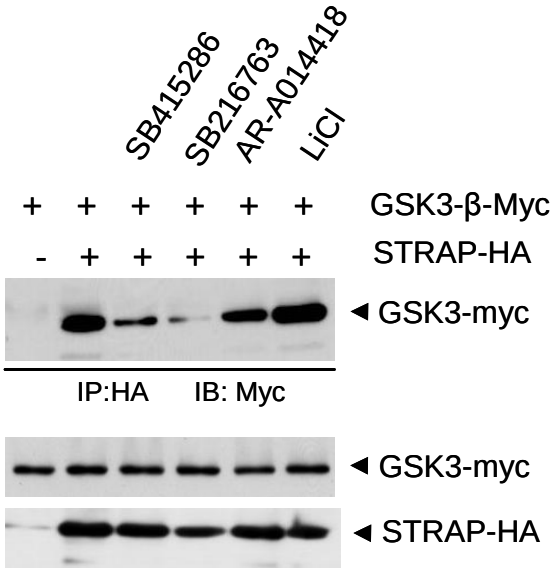


Figure 14. Effect of lithium chloride and small molecule inhibitors of GSK3β on STRAP and GSK3β binding. STRAP-HA and GSK3β-Myc constructs were transiently transfected into 293T cells. 35 hours after transfection, cells were treated with SB415286 (20 μM), SB216763 (25 μM) and AR-A014418 (20 μM) as shown in figure. 48 hours after tranfection, cells were subjected to lysis, immunoprecipitation using 1 μg anti-HA antibody and immunoblotted with anti-myc antibody as indicated. Bottom panels show comparable expression of GSK3β-myc and STRAP-HA in the lysates

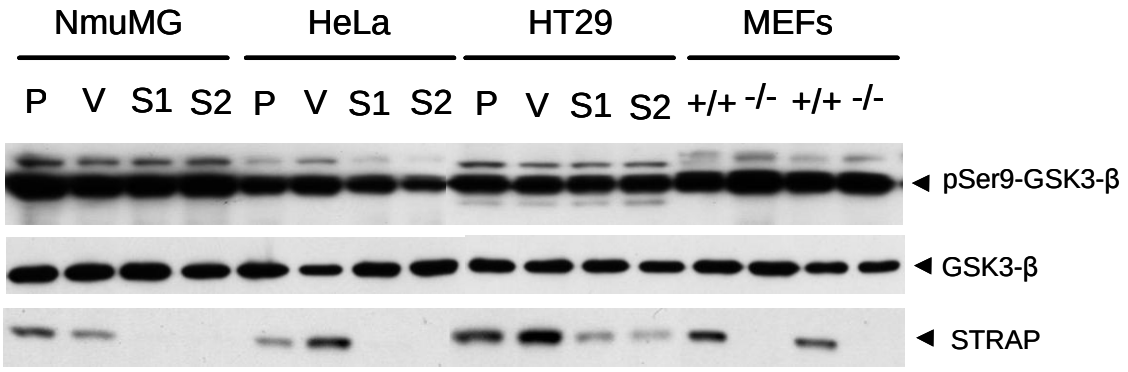


Figure 15. STRAP has no effect of the phosphorylation/activation status of GSK3β in a panel of cell lines. MEFs from wild type and STRAP null mice were used and STRAP was also knocked down in NmuMG, HeLa and HT29 cells using a lentiviral

shRNA construct (Open Biosystems). Lysates were prepared and total proteins (30 µg) were analyzed for phospho-Ser9-GSK3β, total GSK3β (Cell Signaling) and also β-actin as a loading control. (P: parental cells; V: vector control cells; S1 and S2: two STRAP knockdown clones, +/+: wild type MEFs and -/-: STRAP null MEFs)

STRAP does not alter phosphorylation/activation status of GSK3β

STRAP binds GSK3β through WD40 domain region. One possible outcome of this interaction is that STRAP may regulate signaling upstream to GSK3β. This might be achieved by STRAP acting as a scaffold protein to recruit an upstream inhibitory regulator of GSK3β such as PKB (Akt) to GSK3β. This will result in phosphorylation of GSKβ at Ser9 residue in the N-terminal region. The N-terminal free loop with phospho-Ser9 can now bind the catalytic site of GSK3β resulting in self inhibition. Although GSK3β is also phosphorylated at Y216 that seems necessary for GSK3β activity, this phosphorylation is always present constitutively in most cells and does not seem to play a significant role in the regulation of GSK3β activity (Cohen and Frame, 2001). We decided to test whether STRAP affects activation status of GSK3β in a range of human and mouse cell lines. We predicted that a significant change in the total pool of intracellular STRAP may alter the activation status of GSK3β if STRAP was crucial for mediating signaling upstream of GSK3β. We used the wild type and STRAP null MEFs and STRAP knockdown clones derived from HeLa, HT29 and NmuMG cells. Lysates from these cell lines were analyzed, as seen in figure 15. Western analyses using the phospho-Ser9 specific antibody showed no difference in Ser9 phospohorylation of GSK3β in these cell lines. This mostly ruled out that STRAP may affect signaling upstream of GSK3β or activation status of GSK3β.

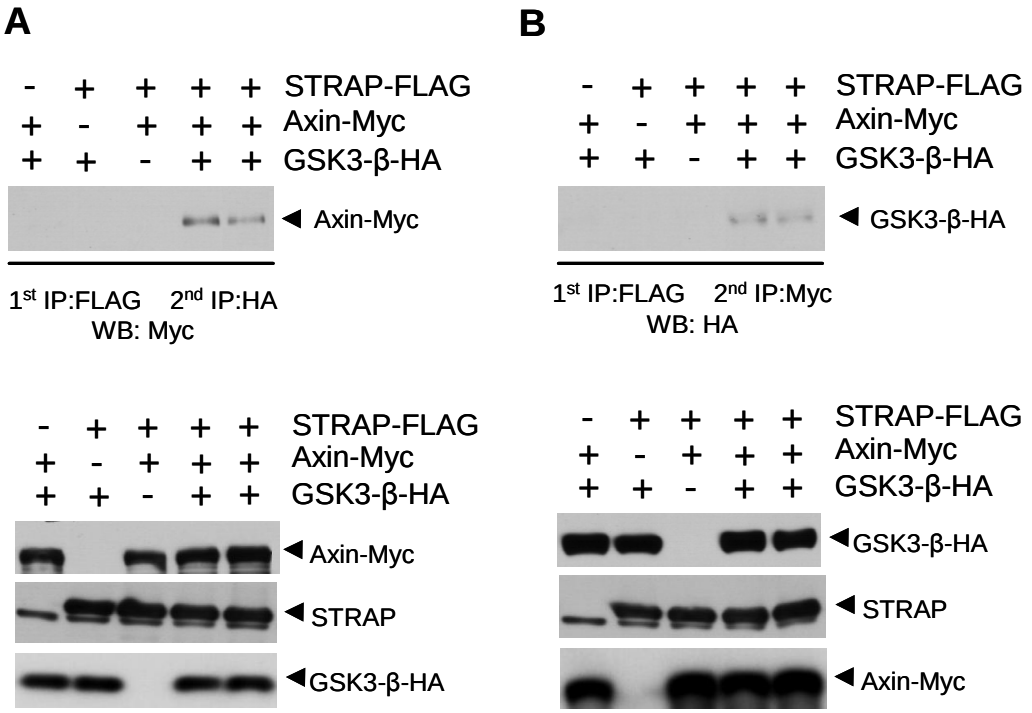


Figure 16. STRAP and GSK3β form a ternary complex together with Axin. (A) 293T cells were co-transfected with STRAP-FLAG, GSK3β-HA and Axin-Myc in different combinations as indicated. Cell lysates were prepared and co-immunoprecipitated with 2.5 µg of anti-Flag antibody. After 5 washes with the wash buffer, bound proteins were eluted using the FLAG peptide (Sigma). The eluate was diluted in the lysis buffer and subjected to a second immunoprecipitation with the anti- HA antibody. After washes, the bound proteins were eluted and analyzed by western blotting with anti-myc antibody. Bottom panels show comparable expression of STRAP-FLAG, GSK3β-HA and Axin-Myc in the lysates. **(B)** Same as above except the second immunoprecipitation was done using anti-myc antibody and western analysis was done using anti-HA antibody.

STRAP forms a ternary complex with GSK3β and Axin

GSK3β is responsible for constitutive phosphorylation β-catenin that leads to ubiquitination and proteasomal degradation of β-catenin. Scaffolding proteins like Axin and APC mediate this recruitment of β-catenin to GSK3β. Deregulation of this pathway in colon cancer, where mutations in APC disrupt this complex formation leading to the

stabilization of β-catenin and progression of colon cancer. Considering that STRAP may play a similar role in recruiting substrates into a complex with GSK3β, we tried to determine whether STRAP is present in a complex together with Axin. 293T cells were transfected as described above either with all Myc-tagged Axin, HA-tagged GSK3β and FLAG-tagged STRAP together or in combinations of two of them together. Lysates were prepared similarly and STRAP was immunoprecipitated with anti-FLAG antibody (Sigma). After final wash, the protein complexes bound with the beads were eluted with 300 µl of 1X FLAG peptide (Sigma). The eluants were then subjected to a second immunoprecipitation with either anti-HA (Fig 16 left panel) or anti-Myc antibodies (Fig 16 right panel) to pull down GSK3β or Axin respectively. The proteins eluted after second immunoprecipitation were subjected to western analysis for the other protein. The results indicate that STARP, GSK3β and Axin formed a ternary complex with each other. The role of Axin to recruit β-catenin to GSK3β has been extensively studied. Only recently Axin has been shown to aid recruitment of substrates other than β-catenin, such as Smad3 to GSK3β (Guo et al., 2008). APC is the other scaffold protein that helps Axin to recruit β-catenin to GSK3β. It is possible that STRAP may help Axin in recruiting substrates like β-catenin, Smad3 or some yet unknown substrate to GSK3β.

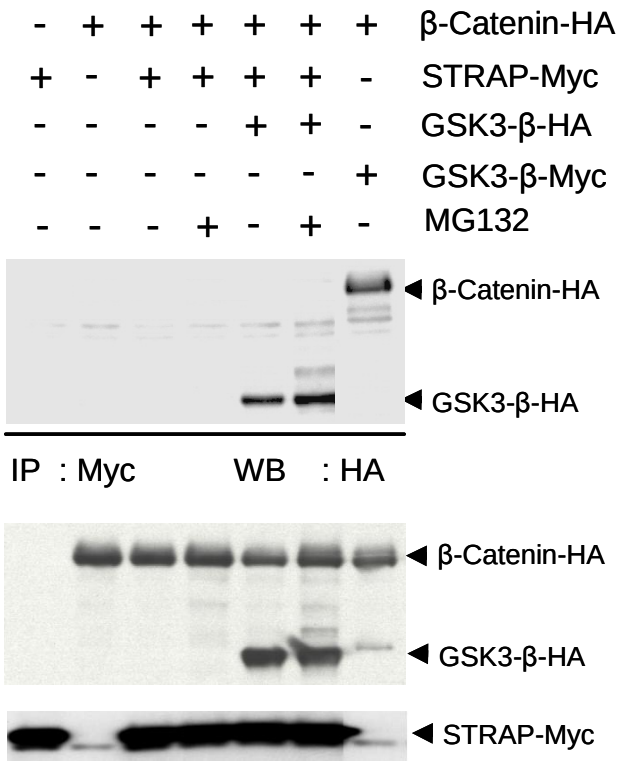


Figure 17. STRAP does not interact with β-catenin even in presence of GSK3β and/or the proteasomal inhibitor MG132. STRAP-Myc, β-catenin-HA, GSK3β-HA and GSK3β-myc constructs were transiently transfected into 293T cells. Where indicated, cells were treated with 40 μM of the proteasomal inhibitor MG132 (Sigma) for 5 hours before lysis. After this, cells were lysed and subjected to immunoprecipitation using 1 μg anti-myc antibody and immunoblotted with anti-HA antibody. Bottom panels show comparable expression of STRAP-Myc, β-catenin-HA, GSK3β-Myc and GSK3β-HA in the lysates.

STRAP does not appear to be involved in β-catenin recruitment to GSK3β

Axin binds directly to GSK3β and β-catenin, through distinct domains at amino acids 477–561 and 561–630, respectively (Hsu et al., 1999). Interestingly, apart from recruiting β-catenin to GSK3β, Axin also recruits the priming kinase for β-catenin, casein kinase 1 (CK1) to the same complex (Liu et al., 2002). This process seems to be highly

regulated as other large scaffold proteins like APC are also involved. As STRAP forms a complex with GSK3β and Axin, we hypothesized that STRAP may serve as another scaffold protein that may help recruitment or processing of β-catenin in the complex with GSK3β. MG132 is a peptide aldehyde that inhibits ubiquitin-mediated proteolysis by binding and inactivating both the 20S and 26S proteasomes. This is useful in case interaction of a particular form of β-catenin with STRAP quickly targeted β-catenin for ubiquitin-mediated proteolysis. For example, the half-life of β-catenin is approximately 100 minutes. This might render the STRAP-binding pool of β-catenin too small to be determined by co-immunoprecipitation assays. In such situation, a short treatment of the cells before lysis ensures that appreciable amount of this particular form of β-catenin is available in the cells for co-immunoprecipitation assays. A similar strategy is often used for protein-protein interaction assays of molecules of substrates like c-Myc or c-Jun with short half-lives. In co-immunoprecipitation experiments, β-catenin showed binding with GSK3β (Fig 17, lane 7) but failed to show any interaction with STRAP in absence or presence of a proteasomal inhibitor MG132 or GSK3β (Fig. 17, lanes 3-6) indicating that STRAP is unlikely to play any role in recruiting β-catenin to GSK3β.

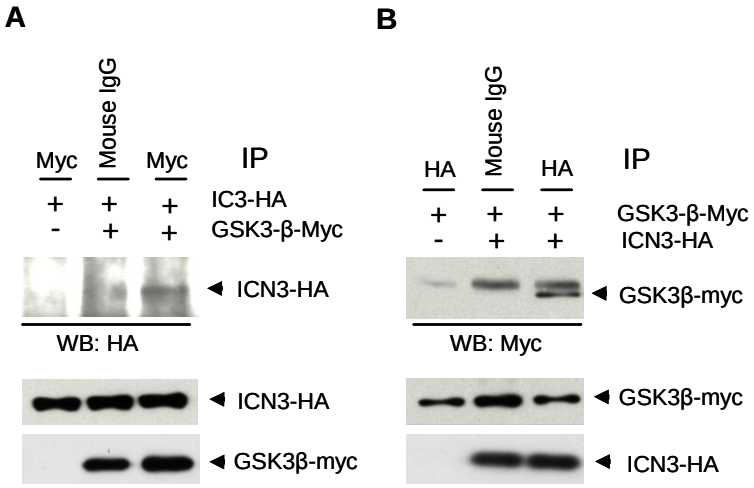


Figure 18. GSK3β binds specifically with ICN3. **(A)** ICN3-HA and GSK3β-Myc constructs were transiently transfected into 293T cells. Cells were subjected to lysis 48 hours after tranfection, immunoprecipitation using 1 µg pre-immune mouse IgG or 1 µg anti-Myc antibody and immunoblotted with anti-HA antibody as indicated. Bottom panels show comparable expression of GSK3β-myc and ICN3-HA in the lysates. **(B)** Same as above except immunoprecipitations were done with pre-immune rabbit IgG and anti-HA antibodies and immunoblotting was done using anti-myc antibody. The band above the GSK3β band is the heavy chain. All antibodies are from Santa Cruz Biotechnology. Bottom panels show comparable expression of GSK3β-Myc and ICN3-HA in the lysates.

GSK3β binds with Intracellular fragment of Notch3

Earlier reports have shown that GSK3β binds and phosphorylates intracellular fragments of Notch1 and Notch2 (Espinosa et al., 2003, Foltz et al., 2002; Jin et al., 2009). There is no report yet of GSK3β interacting or phosphorylating Notch3 or Notch4. Though Notch3 shares an overall good homology with Notch1 and Notch2, they differ in certain regions like the transactivating domain (TAD). We used co-immunoprecipitation assays in 293T cells after transient transfection to assess interaction of HA-tagged ICN3 with GSK3β-myc. We successfully showed for the first time that GSK3β interacts with

ICN3. This is evident from the 3rd lanes of panels A and B of figure 18. Binding of Notch3 with GSK3β predicts that Notch3 is also a substrate for GSK3β. There are conflicting reports about the possible outcome of GSK3β mediated phosphorylation of Notch1 and Notch2. While some reports indicate that this phosphorylation can stabilize Notch, others have claimed that this can accelerate degradation of Notch1 and Notch2. For example, a previous report by Foltz et al. (2002) showed that ICN1 interacts with GSK3β and was phosphorylated by GSK3β. Their report shows that this phosphorylation decreased proteasomal degradation of ICN1 leading to its stabilization. Another report by Espinosa et al. (2003) showed that GSK3β binds and phosphorylated ICN2. But in contrast, this phosphorylation inhibited the activity of ICN2. Again, a recent report by Yun et al. (2009) suggests that ICN1 is negatively, not positively regulated by GSK3β as far as stability of protein is concerned. Interestingly, Notch3 stabilization has been reported to occur and contribute to the progression of lung cancer. Recent studies also suggest that increased Ser9 phosphorylation that inhibits GSK3β, predicts a good prognosis for lung cancer patients. It is possible to hypothesize that GSK3β phosphorylation may lead to stabilization of Notch3. It will need further work to find out the exact role of GSK3β in Notch3 mediated signaling pathway.

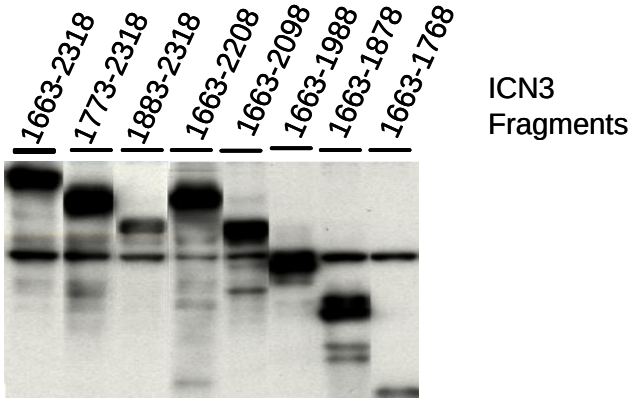


Figure 19. Generation of HA-tagged ICN3 deletion constructs. Using PCR, we generated DNA fragments coding for ICN3 deletion constructs. We added XhoI and XbaI endonuclease restiction sites at their ends and subcloned these fragments into the pCDNA3.1vector after digesting with XhoI and XbaI. All primers were carefully designed to add a HA tag in frame to the C-terminus of the ICN3 fragments. These plasmids were used to transiently transfect HEK-293 cells and lysates were analyzed using anti-HA antibody.

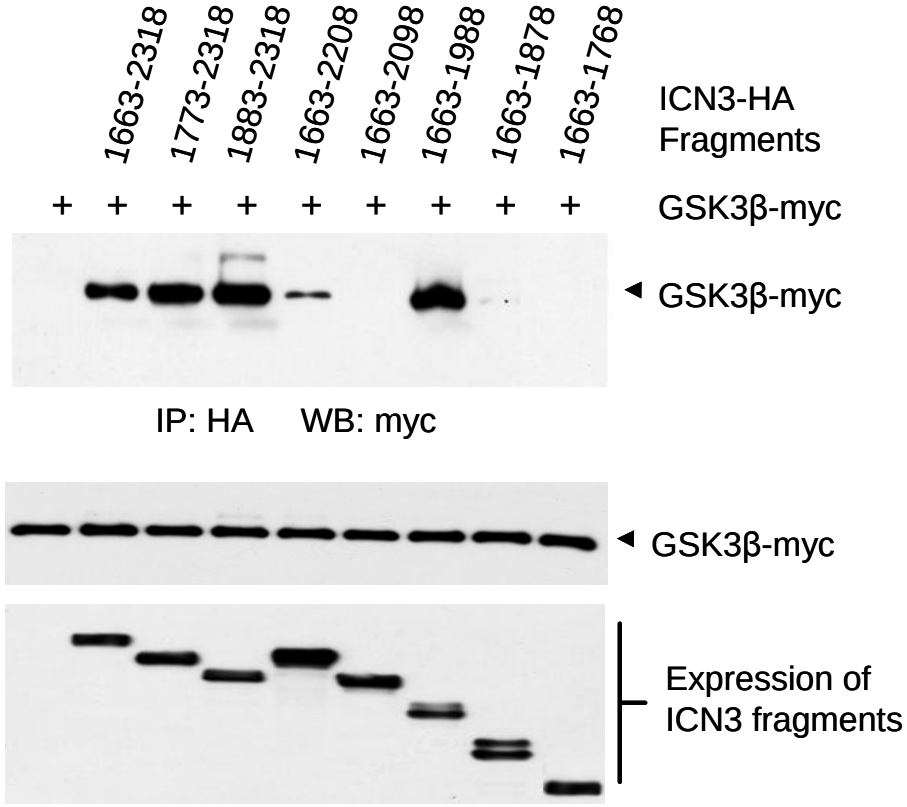


Figure 20. The ANK domain 1863- 2000 aa region of Notch3-IC physically interacts with GSK3β. A. HEK-293T cells transfected with 1 µg of HA- GSK3β and various deletion constructs of ICN3 as indicated. The lysates from these cells were incubated with anti-HA antibodies for 3 hours and then with G-sepharose beads for 1 hour. Complexes were precipitated anti-HA antibody and analyzed by Western blot with anti-myc antibody to detect GSK3β-myc. Bottom panels show equal expression of the ICN3 fragments and GSK3β.

1880-2000 aa region of Notch 3 is important for GSK3β binding

GSK3β binds to ICN2 through the ankyrin repeat domain, ANK (Espinosa et al., 2003). The ANK domain of ICN2 has 6 ankyrin repeats and ankyrin repeat 6 is most crucial for this interaction. Notch3 has a high homology with Notch2 in the ANK domain region. The ANK domain of ICN2 extends from 1824 to 2064 aa and the ANK domain of ICN3 extends from 1790 to 2000 aa. The high homology in this region is evident from figure 21. We decided to study the region of Notch3 that is necessary for binding with GSK3β. For this, we decided to generate serial deletion constructs of the intracellular portion of Notch3 from the pCDNA3 mICN3-HA, kindly gifted by Dr. Jon Aster (Brigham and Women's Hospital, Harvard University).

The fragments were amplified by PCR, gel purified and digested with restriction enzymes XbaI and XhoI. The fragments were ligated in pCDNA3.1 digested with the same enzymes. All constructs have a c-terminal HA tag. The expression was verified by western analysis (Figure 19). The fragments N1 (1663-2318), N2 (1773-2318), C1 (1663-2208), C2 (1663-2098), C3 (1663-1988) and C4 (1663-1878) showed comparable expressions but the expression from similar amounts of N3 (1883-2318) and C5 (1663-

1768) fragments was less compared to others. Other N-terminal deletion fragments did not show any expression indicating that they might be very unstable.

To study the binding pattern between ICN3 fragments and GSK3β, 293T cells were transfected with 1 µg of myc-tagged GSK3β and various amounts of HA-tagged deletion fragments of ICN3 to achieve comparable expression level with each other. Results from the co-immunoprecipitation assays suggest that the region of ICN3 from 1883 to 2000 aa is vital for binding with GSK3β (Fig 20). Looking at the high homology between ICN2 and ICN3 in the ANK domain, it is possible to predict that the ankyrin repeat 6 of ICN3 (1972-2001 aa) might be crucial for the interaction. At the same time, the 2010-2120 region seems to have an inhibitory effect on ICN3 and GSK3β binding. This inhibitory effect seems to be amplified when this region is freely mobile as C-terminal tail in the C2 construct.

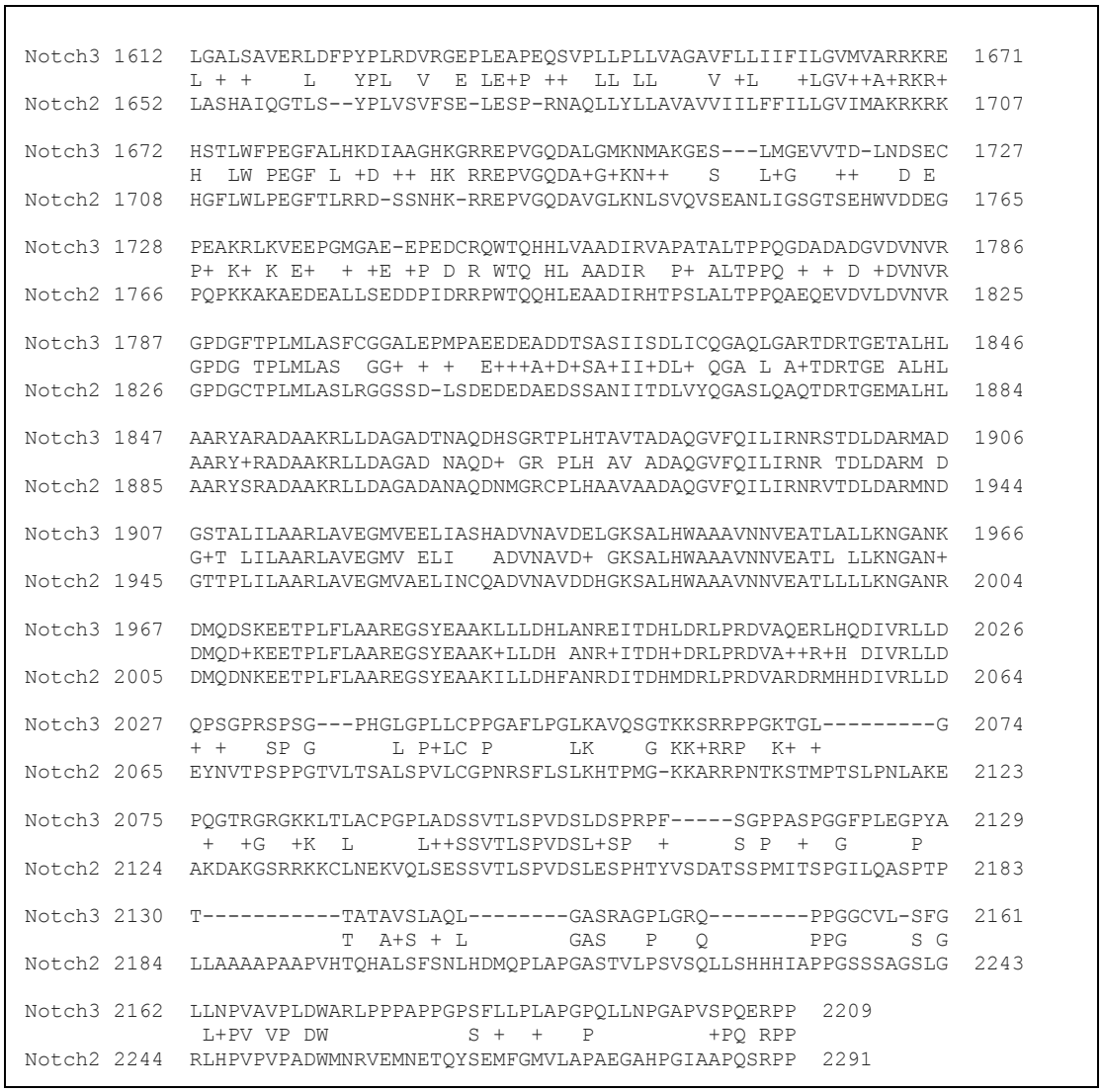


Figure 21. Homology between mouse ICN3 and ICN2. Protein sequences of mouse ICN3 and ICN2 were compared and homology is indicated by the common sequence placed between the Notch2 and Notch3 sequences.

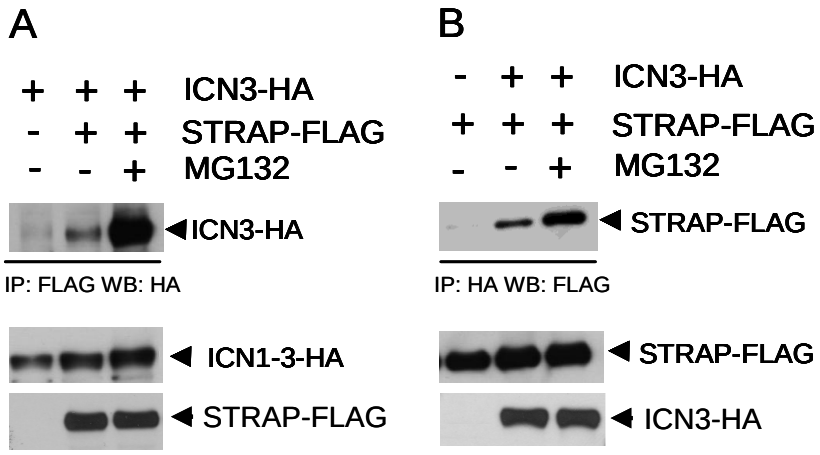


Figure 22. STRAP binds ICN3 and this binding is significantly upregulated in presence of MG132. **(A)** 1 µg of STRAP-FLAG and ICN3-HA constructs were transiently transfected into HEK-293T cells. Where indicated, cells were treated with 40 µM of the proteasomal inhibitor MG132 (Sigma Biotechnology) for 5 hours before lysis. 48 hours after transfection, cells were subjected to lysis, immunoprecipitation using 1 µg anti-FLAG antibody and immunoblotted with anti-HA antibody. **(B)** Same as above except immunoprecipitation was done using anti-HA antibody and western analysis with anti-FLAG antibody. For both A and B, bottom panels show comparable expression of STRAP-FLAG and ICN3 in the lystaes.

STRAP binding to ICN3 is enhanced in a proteasome inhibition dependent manner

Apart from β-catenin, c-Myc, c-Jun, Notch family proteins and Cyclin E are targeted for ubiquitination and proteolysis after GSK3β mediated phosphorylation. Axin acts a as a docking protein that allows substrates like β-catenin, Smad3 and even some priming kinases like CK1 to be in a complex with GSK3β. Even if STRAP was not involved in β-catenin processing, we decided to determine whether STRAP was involved in the GSK3β mediated processing of the other GSK3β substrates. It is possible that the

abundance of particular docking proteins might, thus, specify different GSK3β functions in a cell.

Co-immunoprecipitation assays were done in 293 T cells transfected with FLAG-tagged STRAP and HA-tagged ICN3 as described above. The cells were treated with MG132 or not 43 hours after transfection and lysed 5 hours later. Immunoprecipitations were carried out using either anti-FLAG (panel A) or anti-myc (panel B) antibodies. Bound proteins were analyzed after running SDS-PAGE and western blotting with opposite antibody. Results are shown in figure 22. It is evident from the second lanes of both panels that STRAP effectively binds to ICN3. Interestingly, short treatment with MG132 significantly enhanced the interaction between STRAP and ICN3. (lane 3 of both panels A and B). As the amount of ICN3 or STRAP present in lysates used for the immunoprecipitation is comparable to each other, this finding indicates that the form of ICN3 that binds with STRAP might be unstable or rapidly degraded in absence of proteasomal inhibition.

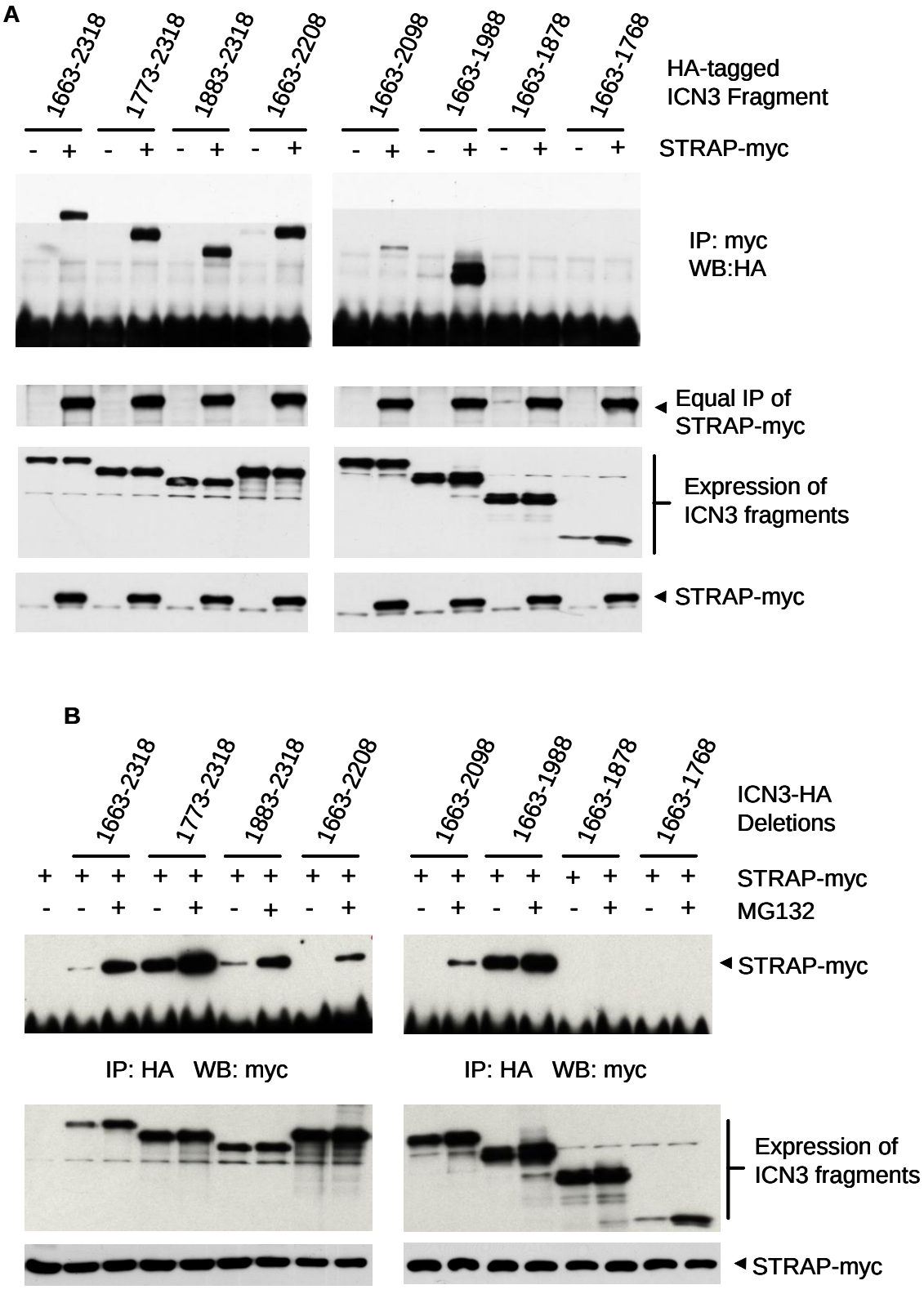

A
1663-2318
1773-2318
1883-2318
1663-2208
1663-2098
1663-1988
1663-1878
1663-1768
HA-tagged
ICN3 Fragment
STRAP-myc
IP: myc
WB:HA
Equal IP of
STRAP-myc
Expression of
ICN3 fragments
STRAP-myc
B
1663-2318
1773-2318
1883-2318
1663-2208
1663-2098
1663-1988
1663-1878
1663-1768
ICN3-HA
Deletions
STRAP-myc
MG132
STRAP-myc
IP: HA WB: myc
IP: HA WB: myc
Expression of
ICN3 fragments
STRAP-myc

Figure 23. STRAP binds ICN3 through the same ANK domain region as GSK3β. **(A)** ICN3 deletion fragments C4 and C5 do not bind with STRAP. HEK-293 cells were transiently transfected with 1 µg of STRAP-myc and the HA-tagged ICN3 deletion constructs in different combinations as indicated. All cells were treated with the proteasomal inhibitor MG132 (40 µM) for 5 hours before cell lysis. Cells were lysed 48 hours after transfection, subjected to immunoprecipitation with 1.5 µg of anti-myc antibody and immunoblotted with anti-HA antibody to detect co-immunoprecipitated ICN3 deletion fragments. The middle panel indicates the same western blot as in the top panel, after stripping and immunoblotting with anti-myc antibody to reveal equal immunoprecipitation of STRAP-myc. The bottom 2 panels indicate comparable expressions of the ICN3 deletion constructs and STRAP-myc in the lysates. **(B)** This is a reverse of the experiment in panel A. HEK-293 cells were transiently transfected with 1 µg of STRAP-myc and HA-tagged ICN3 deletion constructs. Lane 1 is a negative control transfected only with STRAP-myc. Cell in lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19 were treated with 40 µM of MG132 for 5 hours. Cells were lysed 48 after transfection and subjected to immunoprecipitation with 1.5 µg of anti-HA antibody. Western analysis of the bound proteins was done using anti-myc antibody. Lower panel indicates comparable expressions of STRAP-myc and ICN3 deletion constructs in the lysates.

STRAP interacts with ICN3 through the same ANK domain region as GSK3β

STRAP interacts strongly with GSK3β. Since ICN3 binds to both GSK3β and STRAP, there is a possibility that STRAP, GSK3β and ICN3 are present in a single complex in the cells. To consider this possibility, we decided to determine the region of ICN3 that binds with STRAP. We performed coimmunoprecipitation experiments in 293T cells as discussed above. HA-tagged ICN3 fragments were expressed either alone or together with STRAP-myc in presence of MG132. The proteins were pulled down with anti-myc antibody and analyzed with anti-HA antibody. We found that ICN3 fragments N1 (1663-2318), N2 (1773-2318), N3 (1883-2318), C1 (1663-2208) and C3 (1663-1988) demonstrated strong interaction with STRAP in presence of proteasomal inhibitor MG132 as evident from lanes 2, 4, 6, 8 and 12 of the figure 23. In contrast, the C2 (1663-2098) fragment bound STRAP with a relatively lesser affinity (lane 10) whereas fragments C4 (1663-1878) and C5 (1663-1768) failed completely to bind with STRAP

(lane 14 and 16). This suggested that the 1663-1883 aa region of ICN3 was not important for binding with STRAP and loss of 1768-1988 aa region also did not abolish binding of STRAP with ICN3. This data suggests that STRAP also appears to bind ICN3 through the 1883-2010 aa region which is the same highly conserved ANK domain of Notch3. This is the same region that mediates ICN3 binding with GSK3β. Again this is expected as this ankyrin repeat region is one of the most adapted motifs for protein-protein interactions in Notch3 (Mosavi et al., 2004). Which of the ankyrin repeats in the ANK domain mediates this interaction remains to be seen. It also appears that the region from 2010-2120 aa may have an inhibitory effect on STRAP-ICN3 binding just as the case with GSK3β. Taken together, this indicates that STRAP binds with ICN3 through a region of 1883 to 2010 which is similar to the overall region of ICN3 crucial to its binding with GSK3β.

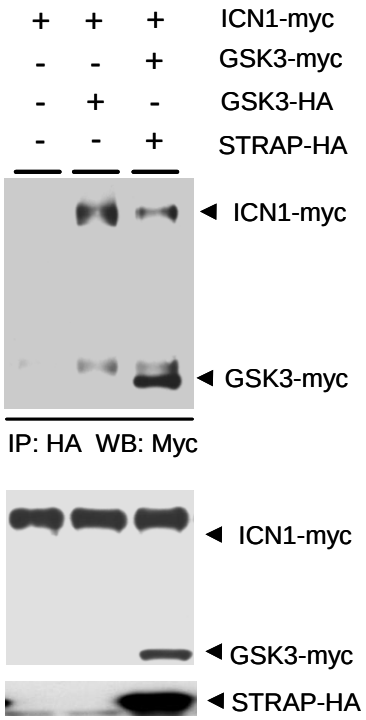


Figure 24. STRAP binds weakly with ICN1. 1 µg of STRAP-HA, GSK3β-HA and ICN1-myc constructs were transiently transfected into HEK-293T cells as indicated. 48 hours after transfection, cells were subjected to lysis, immunoprecipitation using 1 µg anti-HA antibody and immunoblotted with anti-myc antibody. Heavy chain band is visible just above the GSK3β-myc band. Bottom panels show comparable expression of STRAP-HA, GSK3β-HA and ICN3-myc in the lysates.

STRAP binds weakly with ICN1

STRAP binds ICN3 through a conserved region. Notch1 and Notch2 are the more studied members of the notch family that are already known substrates of GSK3β. Since all the Notch family members share a high overall homology in the ANK domain, it needed to be seen whether STRAP binds only with ICN3 in an exclusive manner or in a more generalized manner with other notch members too. In a co-immunoprecipitation assay when STRAP-HA was overexpressed in 293 T cells along with ICN1-myc, it was seen that STRAP binds with ICN1-myc as evident from the lane 3 of the figure 24. Lane 2 in the figure is a positive control where ICN1-myc co-immunoprecipiated with HA-tagged GSK3β. Lane 1 is a negative control with only ICN1-myc expression. Interaction with ICN1 may suggest that STRAP plays a more generalized role in notch signaling. Further experiments would be needed to delineate the effects of STRAP on ICN1 and other notch members like Notch2 and Notch4.

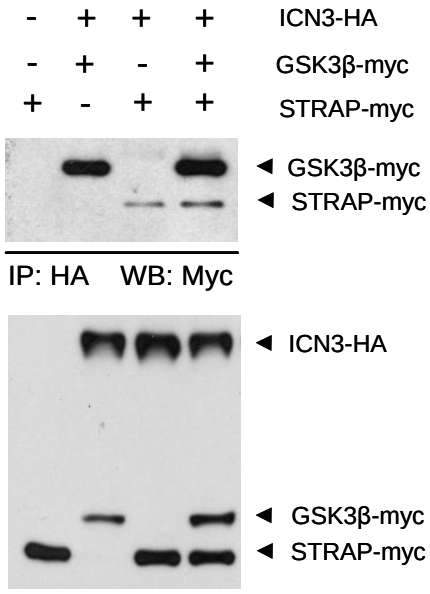


Figure 25. GSK3β co-expression does not affect binding between ICN3 and STRAP. STRAP-myc, GSK3β-myc, GSK3-HA and ICN3-HA constructs were transiently transfected into HEK-293T cells in different combinations as indicated. 48 hours after transfection, cells were subjected to lysis, immunoprecipitation using 1 μg anti-HA antibody. Immunoblotting was done with anti-HA antibody. Lanes 3 and 4 show the co-immunoprecipitation of STRAP with ICN3 in presence and absence of GSK3β-myc respectively. Similarly, lanes 2 and 4 show the co-immunoprecipitation of GSK3β-myc with ICN3-HA in presence and absence of STRAP-myc. Bottom panels indicate comparable expressions of STRAP-myc, GSK3β-myc and ICN3-HA in the lysates.

GSK3β overexpression does not significantly affect binding between ICN3 and STRAP

We have hypothesized that STRAP may act as scaffold protein and may have a role in the pre- GSK3β phosphorylation or post- GSK3β phosphorylation processing of ICN3. It means that STRAP may actively help to recruit ICN3 to GSK3β in a manner similar to Axin helping recruitment of β-catenin to GSK3β. Conversely, if STRAP was more important for the post-phosphorylation processing of ICN3, it would be possible that presence of GSK would lead to an elevation in the binding affinity of ICN3 towards

STRAP. To test this, we conducted coimmunoprecipitation studies between GSK3β and ICN3 in the presence or absence of STRAP overexpression. Similarly we also studied the binding pattern of ICN3 with STRAP in the presence or absence of GSK3β overexpression. To our surprise, we did not observe any effect of either protein on the binding of ICN3 with the other partner. The amount of GSK3β-myc co-immunoprecipitated with ICN3-HA in absence of STRAP-myc (lane 3 Fig 25) or in presence of STRAP-myc (lane 4) seems unaltered and similarly the amount of ICN3-HA co-immunoprecipitated with STRAP-myc in absence of GSK3β-HA (lane 3) or in presence of GSK3β-HA (lane 4). It is possible that the abundant overexpression of ICN3 compared to the relatively much lower levels found in cells may obscure these studies. Or more likely, STRAP can be a part of the multi-protein assembly needed for the recruitment of ICN3 to GSK3β.

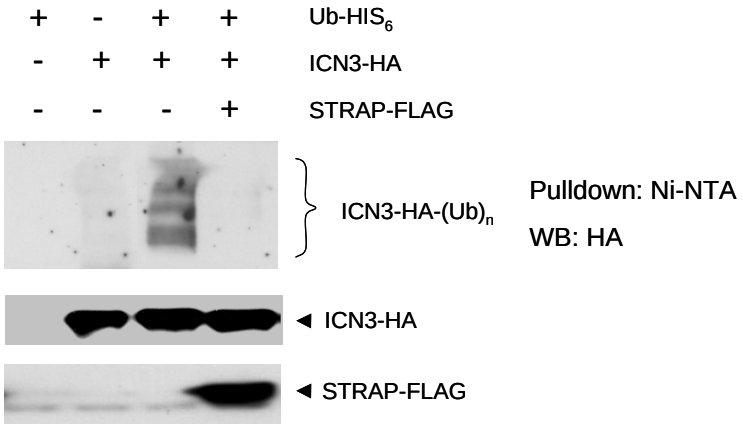


Figure 26. STRAP decreases ubiquitination of ICN3. HEK-293 cells were transfected with 0.8 µg of ICN3 and His_6-tagged ubiquitin and 1 µg of STRAP-FLAG in combinations as indicated. The cells were lysed in a modified lysis buffer as detailed the materials and methods. Proteins tagged with His_6-ubiquitin molecules were pulled down with Nickel-Nitrilo Tri-Acetic Acid (Ni-NTA) agarose beads. Eluted proteins were subjected to electrophoresis and immunoblotting with anti-HA antibody to specifically

detect ubiquitinated species of ICN3. Lower panels indicate equal expression of ICN3 in the lysates.

STRAP decreases ubiquitinated ICN3

STRAP binds with ICN3 and this binding appears to be enhanced in the presence of proteasomal inhibition. This suggested that STRAP may preferably bind with the form of ICN3 that tends to accumulate when 26S proteasomes are inhibited. This in turn may indicate two possibilities. First is that STRAP binds with post GSK3β phosphorylated and/or ubiquitinated form of ICN3 and targets it to proteasomes. The other possibility is that STRAP may bind ubiquitinated form of ICN3 and help it to be docked to some deubiquitinating proteins that can take off the ubiquitin residues of ICN3 to return it to the total cellular pool of ICN3. Plasmids expressing FLAG-tagged STRAP, HA-tagged ICN3 and hexa-histidine-tagged ubiquitin were expressed in 293T cells in combinations as indicated in figure 26. Ubiquitinated proteins in the cell lysates were pulled down with Ni-NTA (Nickel-nitrilo triacetic acid) agarose beads. The Nickel binds the hexa-histidine residues and pulls down approximately 4 ubiquitinated protein molecules per Nickel. The lysates were then washed and eluted proteins were analyzed by western blotting with anti-HA antibody. The western analysis shows only ubiquitinated forms of ICN3 as only ubiquitinated proteins were pulled down and HA-antibody detects only overexpressed ICN3 protein. Lane 3 shows ICN3 was poly-ubiquitinated in absence of STRAP. When STRAP was co-expressed, this ubiquitination of ICN3 was significantly inhibited. The total expression of exogenous ICN3 remained comparable in the cells as can be seen from the bottom panel indicating that the decrease in the ubiquitinated form of ICN3 was not

due to a decrease in the total level of overexpressed ICN3. Decrease in ubiquitination can have several effects on the functional aspects of a protein, but most commonly it will lead to stabilization of the protein in the cell.

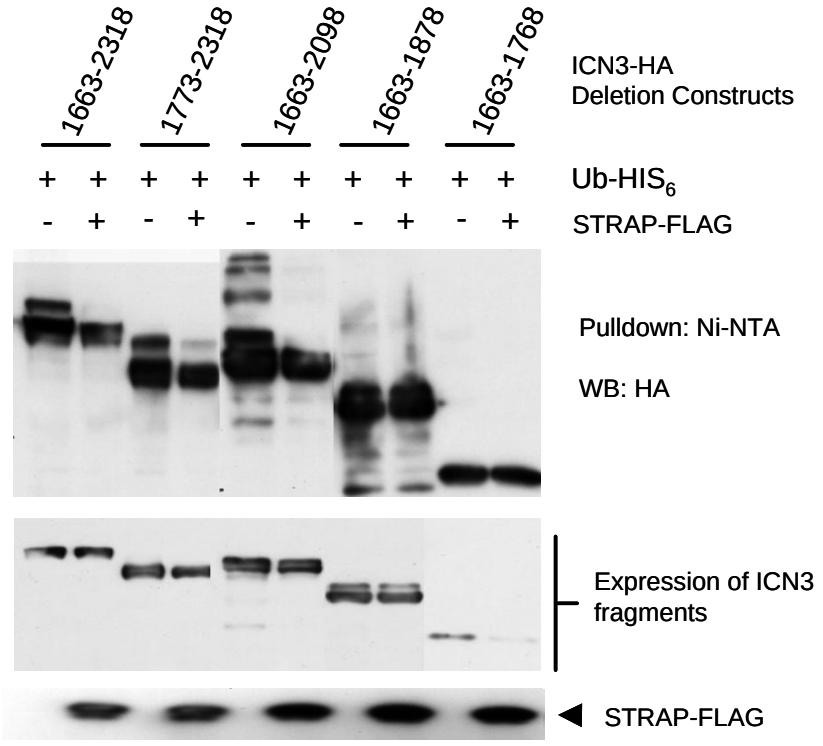


Figure 27. STRAP does not alter ubiquitination of ICN3 fragments C4 (1663-1878) andC5 (1663-1768). HEK-293 cells were transfected with 0.8 µg of ICN3 deletion constructs and His_6-tagged ubiquitin and 1 µg of STRAP-FLAG in combinations as indicated. Rest of procedure was as described above. Top panel shows ubiquitination pattern of the Notch3 fragments in the absence and presence of STRAP-FLAG and lower panel shows the expression of STRAP-FLAG and Notch3 deletion constructs in the lysates.

When a similar experiment was repeated with a few select fragments of ICN3, it was observed that STRAP expression decreased ubiquitination of the ICN3 (1663-2318), fragment 1773-2318 and fragment 1663-2098 but did not have much effect on the

ubiquitination of fragments 1663-1878 and 1663-1768 (Fig 27). This further supports the specificity of the deubiquitinating effect of STRAP as it does not decrease ubiquitination of ICN3 fragments it does not bind to. Taken together, this data suggests that STRAP may play a role in stabilization of ICN3 inside cells leading to a decreased turnover or longer half life of ICN3 in the cells. This will need a set of careful future experiments like S35 labeled pulse chase assays to determine the half life of ICN3 in presence and absence of STRAP in the cells.

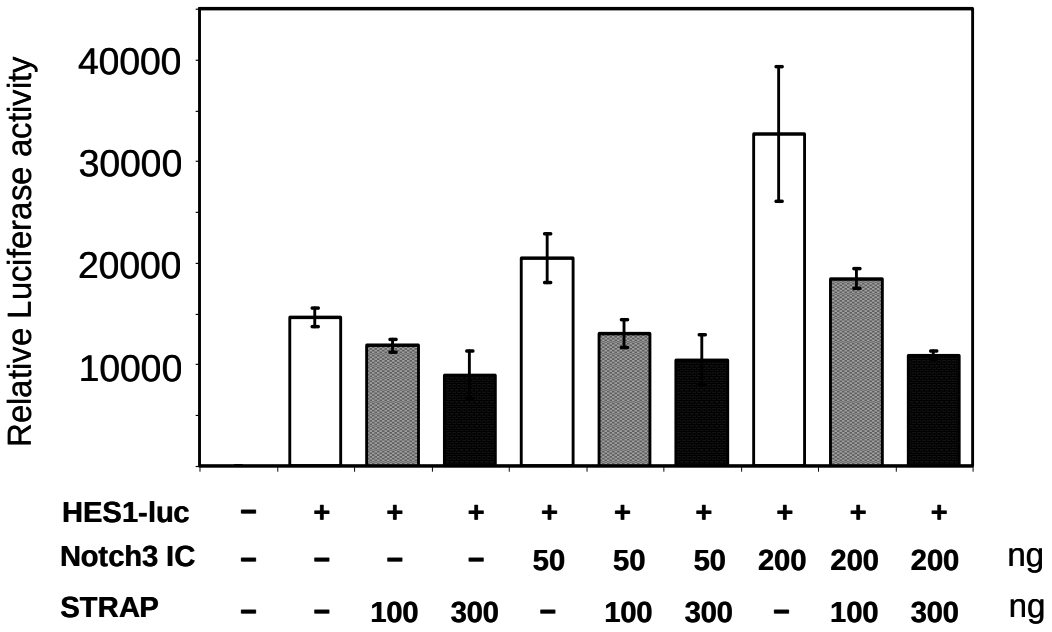


Figure 28. STRAP inhibits Notch3 mediated transactivation. HEK-293 cells were plated in 12 well plates, transfected with 0.5 µg of the HES1-promoter luciferase reporter construct and different combinations of ICN3-HA and STRAP-FLAG. All wells were also transfected with 20 ng of beta-galactosidase construct. Cells were lysed, luciferase activity was normalized using beta-galactosidase activity and averaged for triplicates before representing here. The experiment was replicated three times.

STRAP has an inhibitory effect on Notch3 mediated transcriptional activity

STRAP decreases ubiquitination of ICN3 and may stabilize it. An increase in the half life of the ICN3 protein by STRAP will lead to a larger intracellular pool of ICN3. This can possibly lead to an increase in the transcriptional activity by ICN3. We used the Hes1-luciferase reporter construct to study the effect of STRAP on ICN3 induced transcriptional activity. ICN3 was able to induce the reporter activity in HeLa cells and STRAP inhibited this induction in a dose-dependent manner when co-expressed with ICN3 (Fig 28). This is a paradoxical effect compared to our initial expectations. But there are increasing reports indicating that ubiquitination of some transcription factors helps them for certain protein-protein interactions with other transcriptional activators. Ubiquitination of Notch IC may facilitate formation of such a transcriptional activation complex and may explain why STRAP can decrease ICN3 ubiquitination by and at the same time reduce the transcriptional activity of ICN3.

NOTCH3 STRAP

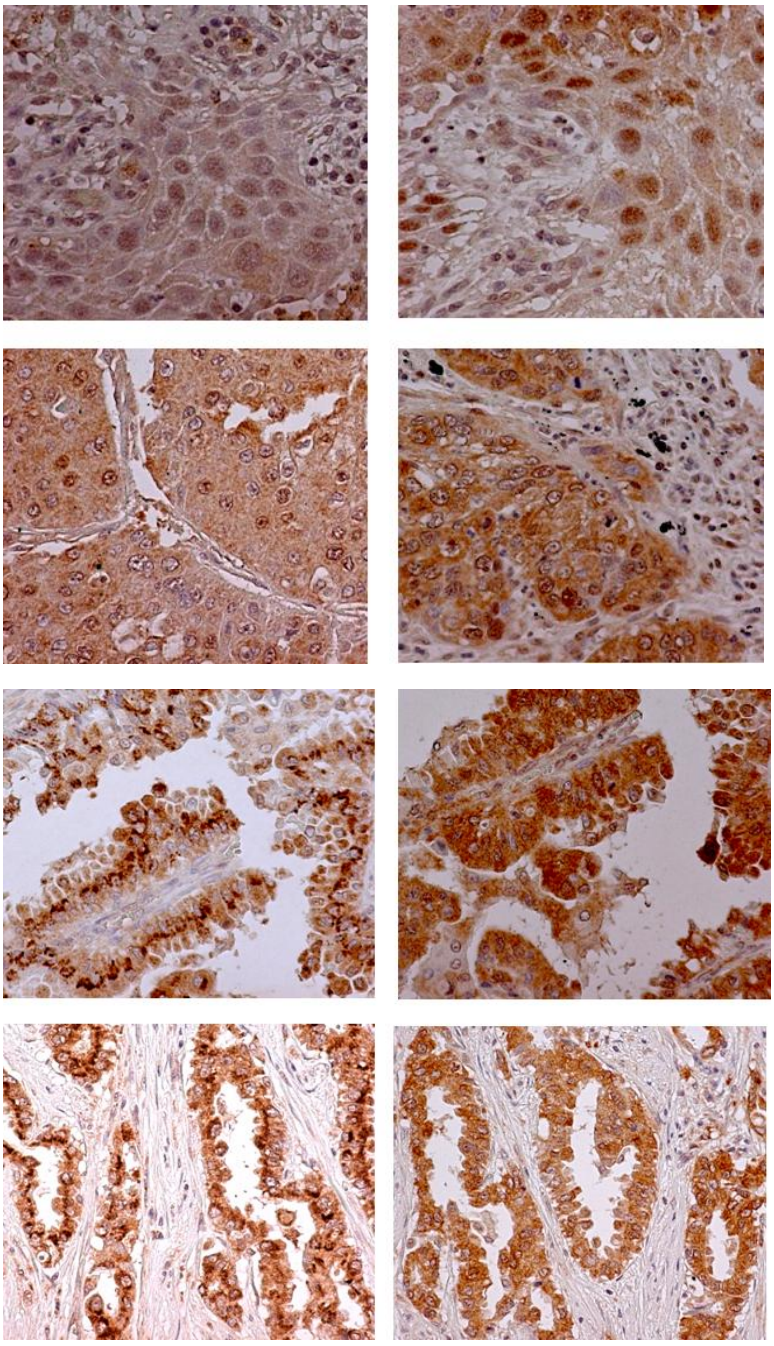


Figure 29. Immunohistochemical analysis of Notch3 and STRAP expression in lung cancer TMA. Left hand columns show expression of Notch3 in lung cancer TMA (Novus anti-Notch3 antibody). Right hand column shows expression of STRAP in the serial section of the same lung cancer samples. All of the positively stained pulmonary adenocarcinomas show a predominantly cytoplasmic localization of Notch3 and STRAP, whereas the squamous carcinomas exhibit a predominantly nuclear localization of both Notch3 and STRAP.

STRAP and ICN3 show significant co-overexpression in Lung Cancer

Previous reports by Haruki et al. (2005) have shown that ICN3 is upregulated to a significant degree in lung cancers. STRAP is also overexpressed in 60 % of lung cancers. Here we have shown that STRAP binds with Notch in a proteasomal inhibition-dependent manner and furthermore, STRAP seems to decrease ubiquitination of ICN3. These facts when taken together, suggested that STRAP may be one of the factors that help stabilization of ICN3 in lung cancer. To test this concept at a preliminary level, we stained serial sections of a lung tissue microarray (TMA) with an anti-Notch3 and anti-STRAP antibodies. The TMA contained duplicate samples from 42 lung cancer patients. Sample staining patterns for both STRAP and Notch3 are shown in a pair wise manner in figure 29. Each sample on the TMA was scored for the percentage of tumor cells showing staining (N) and also the intensity of staining (I). These two numbers were then multiplied to get the staining score for each spot. The score for the duplicate spots was averaged and then compared pair wise between STRAP and Notch3 staining. Using the Pearson's pairwise comparison ratio we obtained an overall correlation of 59 % for STRAP and Notch3 in the lung cancer TMA. This indicates a highly significant correlation between STRAP and ICN3 levels in lung cancer and strengthens the idea that STRAP may help to stabilize Notch3 in lung cancer. Interestingly, both STRAP and

ICN3 showed a nuclear localization in the two samples form patients suffering from squamous cell carcinomas. Conversely, all other samples, mostly adenocarcinomas, showed an intra-cytoplasmic localization for both STRAP and ICN3. This favors a cell-type specific role for the STRAP-ICN3 interaction. This may also result in a differential effect of STRAP on ICN3 function in a cell type-dependent manner as far as induction of transcriptional activity is concerned.

Discussion

In addition to the roles of STRAP in TGF-β signaling, mRNA splicing and transport, PDK1 signaling, EWS signaling, MAPK pathway regulation, maintenance of mesenchymal morphology, regulation of GSK3 signaling and Notch signaling are newly identified functions of STRAP that are discussed here. It is likely due to the WD40 domains based rigid scaffold platform that allows STRAP to mediate such a diverse protein functions.

We, for the first time, have validated that STRAP interacts with GSK3β, a kinase that regulates phosphorylation of a great variety of proteins including but not limited to enzymes and transcription factors (Fig 13). Looking at the list of its validated substrates, GSK3β appears to be at the cross roads of diverse cellular signaling pathways. Considering the limited pool of GSK3β that would be available in a cell at a given time, it is not well understood how GSK3β would regulate certain pathways selectively in a cell and time-dependent manner and be able to insulate other pathways at the same time. The emerging theme is that the abundance of various scaffold or docking proteins in a

cell will decide what substrates and subsequently pathways are being regulated by GSK3β in that particular cell. Axin/APC mediated docking of β-catenin and presenilin-mediated docking of tau protein are very well known examples of such mechanisms (Hart et al., 1998; Takashima et al., 1998). Based on the fact that STRAP being a novel scaffold protein that binds GSK3β through WD40 domains raises a possibility that STRAP may have a similar role in processing of one or a few of the GSK3β substrates. The ability of STRAP to form homo-oligomers can further enhance the possibility of such a role for STRAP.

Known scaffold proteins in GSK3β signaling namely Axin, APC and presenilin are all phosphorylated by GSK3β. Possibility of GSK3β mediated phosphorylation of STRAP needs to be tested with careful kinase assays. Though STRAP is phosphorylated through the C-terminal free tail by a hitherto unknown kinase, this phosphorylation is unlikely to play any role in STRAP-GSK3β binding as STRAP binds GSK3β through its WD40 domain and C-terminus of STRAP is dispensable for this. Use of eukaryotic linear motif (ELM) functional site prediction software to predict specific phosphorylation sites suggests that STRAP does have six probable GSK3β phosphorylation sites at 69-76, 102-109, 137-144, 172-179, 198-205 and 281-288, all in the WD40 domain region. Point mutational studies would be needed to confirm their phosphorylation by GSK3 in vivo. Even with the possibility of such a phosphorylation, it may or may not play a role in binding of STRAP with GSK3β. It is known that phosphorylation of Axin does not affect its binding ability with β-catenin but phosphorylation of APC does enhance APC's binding with β-catenin several fold (Yamamoto et al., 1999; Rubinfeld et al., 1996).

In another completely novel finding, we observed that STRAP, GSK3β and Axin can form a triple complex together (Fig 16). Interaction of STRAP with these two classic mediators of Wnt signaling pathways would indicate a possible role for STRAP in β-catenin processing. But our assays do not support this possibility as STRAP failed to interact with β-catenin in absence or presence of GSK3β or the proteasomal inhibitor MG132 (Fig 17). Recently, however, the role of Axin in GSK3β signaling has been shown to be more versatile as it also recruits Smad3 to GSK3β (Guo et al., 2008). Smad3 is phosphorylated and degraded as an effect of GSK3β phosphorylation. This finding can imply that Axin may play a similar role for other known and yet unknown substrates of GSK3β. Just as APC acts as an additional scaffold to recruit β-catenin to GSK3β, STRAP may play a similar role in recruiting or processing of Smad3 or other yet unknown substrates to GSK3β together with Axin. Consistent with this, previous studies have already shown that STRAP interacts with Smad3 and MAP1B, another known substrate of GSK3β (Datta et al., 1998; Iriyama et al., 2001).

Notch1 and Notch2 are among the validated substrates of GSK3β. Though Notch3 shares a high homology with Notch1 and Notch2 in some regions, the N-terminal and C-terminal regions of ICN3 are considerably different from ICN1 and ICN2. We for the first time show that ICN3 also interacts strongly with GSK3β, raising a possibility that it is another substrate of GSK3β (Fig 18). Using serial deletion constructs of ICN3 we have mapped the 1880 to 2000 aa region of ICN3 to be crucial for mediating the interaction with GSK3β (Fig 20). This region falls within the ANK domain of ICN3 that is made of 6 ankyrin repeats. Ankyrin repeat is one of the most widely existing protein motifs in nature (Mosavi et al., 2004). It consists of 30-34 amino acid residues and

exclusively functions to mediate protein-protein interactions, further validating the region we mapped in our studies. The Notch family members exhibit a very high homology with each other in the ANK domain compared to other regions. Our finding is also consistent with a previous study showing that the ankyrin repeat number 6 in the ANK domain of ICN2 mediates the interaction of ICN2 with GSK3β (Espinosa et al., 2003). Further studies with targeted deletions in ICN3 will be needed to see if the same ankyrin repeat is involved in GSK3β and ICN3 binding.

Similarly, further work in the form of kinase assays and point mutational studies will be needed to confirm that ICN3 is a bona fide substrate of GSK3β. But considering the very high homology of Notch3 with Notch2 in this region and the fact that ICN2 is phosphorylated by GSK3β in the same region; it seems highly probable that ICN3 might be phosphorylated by GSK3β. GSK3β being a serine threonine kinase, phosphorylates Notch2 in the STR (serine threonine rich) domain that immediately follows the ANK domain on the carboxy terminal side. Ser-2093, Thr-2074 and Thr-2068 or Ser-2070 are the three residues phosphorylated by GSK3β in ICN2. Similarly, ICN1 has been known to be phosphorylated by GSK3β and a recent report proposes that GSK3β phosphorylates ICN1 through the same STR region at residues Thr-1852, Thr-2123 and Thr-2125 (Jin et al., 2009). Analysis of ICN3 using eukaryotic linear motif (ELM) functional site prediction software revealed four putative GSK3β phosphorylation sites in the same STR domain of ICN3. These consensus sequences are MQDSKEET (1968-1975), DQPSGPRS (2026-2033), ADSSVTLS (2094-2101) and VTLSPVDS (2098-2105). Which and how many of these sites are actually phosphorylated by GSK3β will need

further work in the form of precise kinase assays with ICN3 point mutations and deletion constructs.

A previous study found that GSK3βmediated phosphorylation destabilizes ICN1 while another study found that the same phosphorylation seems to have a stabilizing effect on ICN1, inducing its transcriptional activity (Foltz et al., 2002; Jin et al., 2009). In the light of conflicting data about the outcome of GSK3β-mediated phosphorylation of Notch1 protein, the exact effect of this interaction of ICN3 with GSK3β and the probable outcome GSK3β-mediated phosphorylation may be complicated to predict. But considering that the GSK3β-mediated phosphorylation of other transcription factors including β-catenin, c-Myc, c-Jun, Snail, Notch2, HIF-1α etc. and also Notch1, destabilizes and inhibits them, it can be predicted that GSK3β may have a similar inhibitory effect on ICN3. In other words, GSK3β-mediated phosphorylation would probably lead to ubiquitination and proteasomal degradation of ICN3.

After finding that STRAP did not seem to bind with β-catenin, we looked at any possible interaction of STRAP with ICN3 as it was one of the new probable substrates of GSK3β we had found. Our results suggest that STRAP interacts with ICN3 (Fig 22). This might add STRAP to the list of known WD40-domain proteins like β-TRCP and Fbw7 that help to process substrates of GSK3β. These WD40 proteins bind these substrates only after they are phosphorylated by GSK3β. This is achieved through the WD40 domains that are considered to be very efficient for recognizing post-translational modification and especially phosphorylation. On a similar note, it is conceivable that STRAP may bind ICN3 only after specific residues have been phosphorylated by

GSK3β. Currently we do not have any evidence for or against this possibility. Further understanding may come after confirming the phosphorylation sites for GSK3β and performing co-immunoprecipitation experiments with the specific ICN3 point mutants.

But in an interesting observation, we noted that the interaction between STRAP and ICN3 was significantly enhanced when the cells were pretreated with the proteasomal inhibitor MG132 for a short period of time (Fig 22). Treatment with MG132 is usually employed when a particular form of the protein that may be more relevant to certain investigation rather than the total protein pool, is being degraded rapidly. The half life of Notch3 was calculated to be 0.7 days or approximately 17 hours (Takahashi et al., 2009). So a pretreatment of the cells for 5 hours may not lead to a significant elevation in the total ICN3 protein but it can possibly lead to a relatively higher accumulation of the fraction of the total level of ICN3 protein that is being rapidly degraded by proteasomes after ubiquitination. This data may just indicate that the phosphorylated form of ICN3 is degraded rapidly after phosphorylation. Accumulation of this phosphorylated form of ICN3 in an ubiquitinated or non-ubiquitinated form seems to have significantly enhanced ability for binding with STRAP. There is still a chance that this data may suggest that STRAP can also preferentially bind to a form of ICN3 that was ubiquitinated. In this case, whether STRAP may bind to a mono-ubiquitinated form or a poly-ubiquitinated form of ICN3 is difficult to predict. Ubiquitination of a protein substrate involves a cascade of enzymatic reactions. First, Ub, a highly conserved 76-amino acid polypeptide, is activated by an Ub-activating enzyme (E1), leading to ATP-dependent formation of a high energy thiol ester bond between the C terminus of Ub and E1; the activated Ub is then transferred to an E2 enzyme (Ub-conjugating enzyme or Ubc). E2 enzymes then

mediate the transfer of Ub to the target protein directly or to E3 Ub protein ligases, which are responsible for substrate recognition and for promoting Ub ligation to substrate. A substrate may be repetitively ubiquitinated by sequential linkage of additional Ub molecules to form a poly-(Ub) chain (Hochstrasser, 2009). This marks the protein substrate for recognition and consequent degradation by the 26 S proteasome. But considering that ubiquitination is a relatively very large post-translational modification and also that there are no reports about a WD40 domain being able to recognize an ubiquitinated protein, it is more likely that STRAP may bind with the phosphorylated form of ICN3 rather than an ubiquitinated form. Ubiquitination of this phosphorylated form of ICN3 may not have any effect on its binding ability with STRAP. As in case of GSK3β, the binding between STRAP and ICN3 appears to be through the 1883-2010 aa region that is known as the ANK domain (Fig 23). This can be expected since the ankyrin repeats in this domain are one of the most ideal modules for protein-protein interactions. The ANK domains of Notch proteins have 6 ankyrin repeats. GSK3β binds Notch2 through the 6^{th} ankyrin repeat. Since the first 2 repeats occur before 1883 aa and GSK3β itself binds to the 6^{th} repeat, STRAP may possibly bind Notch3 through the ankyrin repeats 3-5.

Notch3 ANK region retains a high homology between the members of Notch family. This was reflected in the ability of STRAP to bind ICN1, indicating that STRAP may play a more generalized role in notch signaling rather than just Notch3-mediated signaling (Fig 24). But our initial assays have also indicated that binding of STRAP with Notch1 was somewhat weaker than Notch3. How this might affect the role of STRAP in Notch1-mediated signaling when compared to Notch3 signaling is not known. To

understand the overall impact of STRAP on notch signaling will need additional studies between STRAP and Notch1, Notch2 and Notch4.

STRAP seems to interact with a form of ICN3 that may be rapidly degraded. So when we looked at the effect of STRAP on the ubiquitination of ICN3, STRAP consistently decreased Notch3 ubiquitination (Fig 26). We hypothesize that STRAP can either carry a phosphorylated ICN3 to a phosphatase to remove the phosphate group and avoid ubiquitination or later in the process dock it to an ubiquitin specific protease after ICN3 has already been ubiquitinated. This has been found to be the case for β-catenin. It was shown that PR55α, the regulatory subunit of protein phosphatase 2A (PP2A), controls β-catenin dephosphorylation and degradation (Zhang et al., 2009). Very surprisingly, PR55α is a WD40 domain protein with 7 WD40 repeats just like STRAP. Same report also showed that PR55α interacted with both β-catenin and Axin. This strengthens a chance of a similar role for STRAP and raises a possibility that STRAP may act as a regulatory subunit for a phosphatase like PP2A. A more detailed knowledge of Nocth3 phosphorylation sites and availability of phospho-specific antibodies will be necessary for additional experiments in this regard. On a similar note, c-Myc is also targeted to PP2A to remove the primed phosphate group but not the GSK3β mediated phosphorylation. PP2A regulatory subunit, B56α has been identified to play this role (Arnold and Sears, 2006). B56α, though is not a WD40 domain protein. Targeting the substrates to the respective phosphatases can be a rate limiting step in such a process, thus driving majority of the substrate towards ubiquitination and only a fraction of phosphorylated substrate might be returned back to the cellular pool in the absence of the scaffold protein to dock the substrate to the phosphatase. If STRAP helps such targeting

of the phosphorylated ICN3 to a specific phosphatase, it can inhibit the ubiquitination and proteasomal degradation of ICN3.

Another plausible explanation appears to be that STRAP can recruit the ubiquitinated ICN3 to a deubiquitinating enzyme (DUB). Ubiquitination of target proteins is reversible process and the removal of Ub can rescue proteins from degradation or re-modulate their activity. The deconjugation of ubiquitin involves removing the covalently linked ubiquitin molecules and is accomplished by the deubiquitinating enzymes (DUBs). The majority of the approximately 100 DUBs in the human genome belong to the ubiquitin specific protease (USP) subclass of DUBs and others belong to the ubiquitin C-terminal hydrolase (UCH) subclass. Structurally, USPs contain a common catalytic domain that consists of two short well-conserved motifs, called Cys and His boxes. Deubiquitination by USPs has been established as an important aspect of many cellular processes (Hurley et al., 2006). Though such a deubiquitinating protein has not been identified for Notch proteins yet, there are several reports demonstrating such a role for USPs. An example for such case is c-Myc, a vastly studied substrate of GSK3β. c-Myc is phosphorylated by GSK3β on Thr-58 after it has been phosphorylated by ERK on Ser-62 (Gregory et al., 2003). This doubly phosphorylated form of c-Myc is then carried by Fbw7 to the E3 ubiquitin ligase complex SCF (Skp, Cullin, F-box containing complex) to be ubiquitinated and then destroyed by proteasomes (Welcker et al., 2006). But there are additional levels of regulation even after c-Myc is phosphorylated by GSK3β. The same E3 ubquitin ligase component Fbw7 can also dock ubiquitinated c-Myc to an ubiquitin specific protease, specifically USP-28 (Popov et al., 2007). This leads to de-ubiquitination of c-Myc and it is returned back to the cellular pool. STRAP

might play a similar role for Notch3, though STRAP may not regulate Notch3 in a dual manner as Fbw7 regulates c-Myc.

Yet another possibility is that STRAP may act just by the mechanism of steric hindrance. In this scenario, binding of STRAP with ICN3 will not allow ICN3 to bind with its two known E3 ubiquitin ligases namely, Fbw7, a component of an SCF-class ubiquitin ligase (E3) complex and Itch, a Hect-type E3 ubiquitin ligase (Qui et al., 2000). Interaction of ICN3 with these substrate specific components of E3 ubiquitin ligases is crucial for docking ICN3 to these complexes for ubiquitination. Consistent with this idea, in the case of c-Myc, it was shown that when only the WD40 domain region of Fbw7 was overexpressed without the remaining c-terminal 350 aa, it acted in a dominant negative fashion to inhibit Fbw7 activity and stabilized c-Myc (Welcker et al., 2006). STRAP has 7 WD40 domains just as Fbw7 but naturally lacks the large c-terminal region observed in Fbw7 and thus may act in a dominant negative fashion to inhibit ubiquitination and subsequent degradation of ICN3 by Fbw7. Future experiments will be needed to resolve these issues.

Irrespective of the mechanism, deubiquitination of ICN3 mediated by STRAP may lead to stabilization of ICN3 in the cells. This will reflect in a longer half life for ICN3 in presence of STRAP compared to when STRAP is absent in the cells. Future experiments will include radioactive S35 and cycloheximide pulse chase assays after knocking down STRAP in some lung cancer cell lines like HCC2429, H1418, H1435 or H1993 that have detectable levels of ICN3.

Dang et al. (2000) first showed that level of ICN3 was significantly upregulated in samples of human lung carcinomas when compared to the surrounding normal lung tissue from the same patients. Overexpression of Notch3 later was shown to contribute towards the tumorigenic behavior of the lung cancer cell lines (Haruki et al., 2005; Konishi et al., 2007). The exact mechanism behind this Notch3 upregulation was not known. We know that STRAP is upregulated in 78% of lung cancers. Since our current work indicates that STRAP may stabilize ICN3, we hypothesized that STRAP upregulation may be one of the possible causes behind the stabilization and subsequent upregulation of Notch3 observed in lung cancer patients. Immunohistochemical staining of tissue micro array (TMA) with duplicate serial sections of lung tumors showed that STRAP and ICN3 were co-overexpressed in about 60% of all samples using the Pearson's pairwise correlation ratio (Fig 29). This is a very interesting but yet a preliminary finding. Further work will be needed to assign a causative role for STRAP in upregulation of ICN3 in lung cancer.

The localization pattern for both STRAP and ICN3 was mostly cytoplasmic and only slightly nuclear in the majority of the stained lung tumors. But in an interesting observation we found that only in the squamous carcinomas, both ICN3 and STRAP had a distinct nuclear localization. The reasons behind this are unclear at this time but emphasize a cell type-dependent role for STRAP in Notch3 signaling. It remains to be seen whether STRAP can stabilize ICN3 in the nucleus. A previous report has shown that squamous carcinomas of the cervix had a much higher nuclear distribution of ICN3 when compared with cervical adenocarcinomas (Yeasmin et al., 2010). Nuclear ICN3 was shown to be associated with adverse clinical outcome. Another study reported that immunohistological staining of pancreatic adenocarcinomas showed both cytoplasmic

and nuclear staining patterns. Again, statistical analysis suggested that nuclear localization of ICN3 was associated with a more aggressive tumor phenotype and a poorer prognosis (Doucas et al., 2008). It would be interesting to find the STRAP localization pattern in these cervical and pancreatic cancers.

The possibility of ICN3 stabilization by STRAP suggested that STRAP might be able to affect Notch3-mediated transcriptional activity. We expected that STRAP will be able to induce the transactivating ability of ICN3. Surprisingly, we found that STRAP was able to inhibit Notch3-induced activation of the HES-1 promoter in a dose-dependent manner (Fig 28). A deeper look into the newer emerging roles of protein ubiquitination enables us to explain our observation. Apart from novel functions like membrane trafficking, endocytosis, cell-cycle control, protein kinase activation and DNA repair, ubiquitination of some transcriptional factors is now being reported to be crucial for their transactivating ability (Chen and Sun, 2009). For example, during tumorigenesis, Lys-63-linked polyubiquitination of the transcription factor Myc by the E3 ubiquitin ligase HectH9 is required for the transactivation of multiple Myc target genes (Adhikary et al., 2005). Salghetti et al (2001) showed the studies on the activity of a transcription factor containing the VP16 transactivating domain (TAD) is regulated through ubiquitination. It is known that VP16 TAD signals ubiquitination through the Met30 ubiquitin-ligase and that Met30 is also required for the VP16 TAD to activate transcription. The requirement for Met30 in transcription can be bypassed by fusion of ubiquitin to the VP16 activator, demonstrating that activator ubiquitination is essential for transcriptional function. More recently, ubiquitination of Gal4 protein was shown to be essential for its binding to a promoter in vivo during transcriptional assays (Archer et al., 2008). Also the

ubiquitination of Cdk9 by Skp2 facilitated the formation of the ternary complex between P-TEFb, Tat, and transactivation response element (Barboric et al., 2005). These interactions are being achieved through the ubiquitin-binding domains (UBDs) present in the interacting proteins. The list of proteins with these domains is constantly growing and now there are 16 known classes of UBD domains. Ubiquitin-associated (UBA) domains are one of the more studied classes of UBDs. Classes 1, 2 and 4 of UBDs also bind to mono-ubiquitin, but in all cases with much lower affinity than to polyubiquitin. It is proposed that Lys-48-linked polyubiquitin chains may be important for protein degradation while Lys-63-linked polyubiquitin chains may play a role in signal transduction and transcriptional activation (Hurley et al., 2006). Ubiquitination of Notch IC through Lys-63-linked chains may facilitate formation of such a transcriptional activation complex. STRAP can possibly help de-ubiquitination of both Lys-48 and Lys-63 linked polyubiquitin chains and thus may prolong the half life but yet reduce the transcriptional activity of ICN3. Future experiments will include elaborate studies to find ubiquitination sites on ICN3 and the type of polyubiquitin chains on these residues.

As mentioned earlier STRAP knockout mice had multiple defects including angiogenesis, cardiogenesis, somitogenesis, neural tube closure and embryonic turning. Though Notch3 mice were viable, fertile, and developed normally, they have some defects in vasculogenesis (Domenga et al., 2004; Krebs et al., 2003). Reports have indicated that notch–Dll4 signaling is essential for vascular development in the embryo as well as during tumor angiogenesis (Ridgway et al., 2006; Hellstrom et al., 2007; Duarte et al., 2004). Notch signaling is also supposed to play a major role during somitogenesis (Weinmaster and Kintner, 2003). Interestingly, notch signaling in Drosophila

Melanogaster was implicated in tubulogenesis of the tracheal tree during development whereas Drosophila homolog of STRAP, named pterodactyl was also shown to be crucial for tubulogenesis (Ikeya and Hayashi, 1999; Khokhar et al., 2008). Tubulogenesis and branching morphogenesis are vital for the formation of substructure of numerous tissues and organs, including the neural tube, kidney, lung, breast, and circulatory system. Additional experiments will be needed to know whether notch plays any role in the phenotype observed in STRAP knockout mice or the tubelogenesis defect observed in pterodactyl knockout in Drosophila. This may be a challenging task considering the very diverse range of functions STRAP has been implicated in.

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

Our current work highlights a fact that for versatile scaffold proteins in the cells, it often seems difficult to assess their overall impact on different cellular functions and final contribution towards cell behavior. As discussed above, STRAP has been already implicated in functions such as TGF-β signaling, PDK1 signaling, ASK1 signaling, spliceosomal assembly, mRNA transport, p53 signaling to name a few. In all of these cases, STRAP performs its functions through binding with other proteins. Our studies have also uncovered two novel roles for STRAP in the cells.

In the first part of the study, we have shown, for the first time, that deletion of STRAP in murine fibroblasts is sufficient to cause MET through upregulation of WT1 and subsequently E-cadherin. Re-expression of STRAP in these null cells leads to a loss of WT1 and E-cadherin expression, and a reversal from epithelial to the mesenchymal morphology. Whether STRAP plays a role in EMT in epithelial cancer cells is not clear yet.

In the second part of the study, we show that STRAP interacts with another versatile enzyme, GSK3β. We also, for the first time, show that GSK3β binds with ICN3, possibly adding it to the list of substrates phosphorylated by GSK3β. Next, we found that STRAP also interacts with ICN3. Both STRAP and GSK3β bind with Notch3 through the wide ANK domain, containing specialized ankyrin repeats for protein-protein interaction.

Although, WD40 domains specialize in recognizing proteins that are post-translationally modified, mostly phosphorylated, whether this is the case regarding STRAP and Notch3 interaction is not known. Interestingly, STRAP expression lead to a decrease in Notch3 ubiquitination indicating that it may stabilize a specific pool of ICN3 but reporter assays indicated that STRAP decreased Notch3-mediated transcriptional activity. These contrasting results can be explained by the recent reports indicating role of ubiquitination in transcriptional activity of some transcription factors.

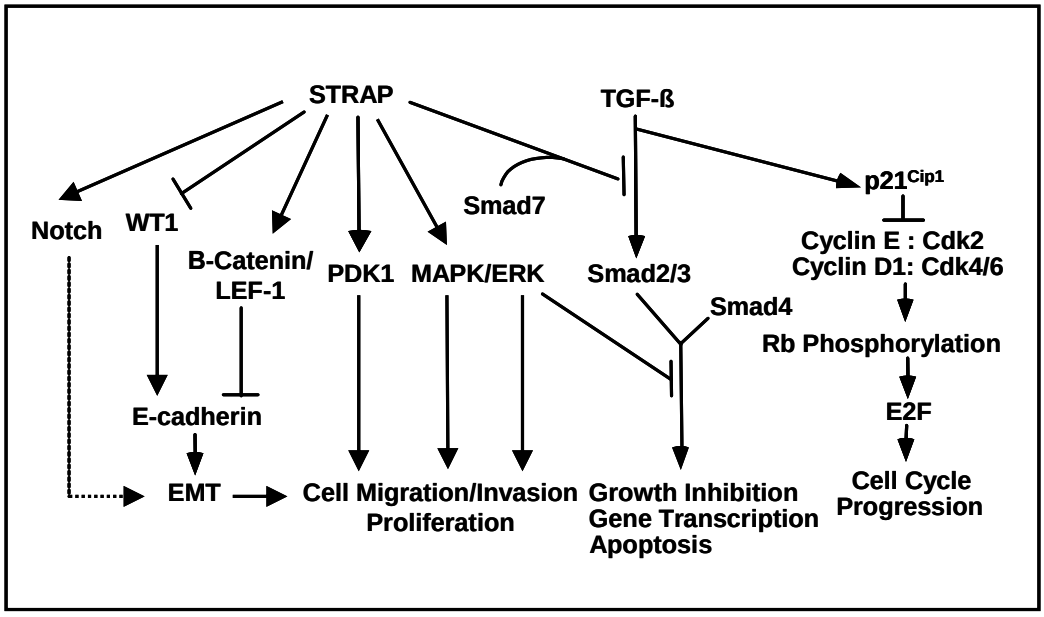


Figure 30. STRAP can promote oncogenesis through involvement of multiple pathways.

STRAP is a relatively small protein of 39 kDa, composed mostly of the WD40 domain and a short low complexity C-terminal chain. STRAP, even though not an enzyme, can still affect signaling pathways enumerated above though interaction with other enzymes in most cases. This reflects the versatility of the WD40 domain. We believe that STRAP may interact with additional proteins, transcription factors or

enzymes, and affect yet more cellular processes. Cancer development is a multi-step process and cells acquire a cancerous phenotype through the accumulation of multiple mutations. The level of STRAP is found to be altered in different cancers. The protein level is elevated in 60% of colorectal, 78% of lung and 46 % of the breast carcinomas. Ectopic expression of STRAP in different cell lines promotes cellular proliferation, induces anchorage-independent growth and increases tumorigenicity in in-vitro and in-vivo experiments. Based on our current understanding, STRAP can influence many cellular processes to influence this outcome. This is represented in the schematic of figure 30. STRAP may be a future possible target for the development of anti-cancer therapeutics if small molecule inhibitors can be developed that target specific regions of STRAP that will disrupt some specific protein-protein interactions and interfere only certain cellular pathways.

Taking the present literature into account, very little is known about STRAP. Experiments in a variety of fields will be required to gain a more complete understanding of how STRAP affects normal cellular processes/ tumorigenesis and to uncover yet unknown functions of STRAP. Here, we plan to do following experiments to further our knowledge of functions of STRAP we have uncovered in this work.

1. Effect of STRAP on Notch expression during embryonic development. STRAP seems to stabilize Notch3 and some phenotypic features like defective angiogenesis and somitogenesis of both STRAP and Notch (1,2 and 3) knockout overlap. We would like to generate STRAP knockout mice and obtain tissue samples from the STRAP knockout and corresponding wild type mouse embryos. Expression and all Notches will be checked

with western blotting. This will give us insight whether some of the features of STRAP knockout mice could possibly be attributed to deregulation of Notch signaling. The targeting construct will be prepared according to published protocols (Conquet et al., 1995). The study will be conducted after approval by the Animal Care and Use Committee at Vanderbilt University.

2. We will determine the effect of STRAP on Notch3 half life. ICN3 is expressed in lung cancer cell lines like HCC2429, H1435, H1418, H1999 etc. (Konishi et al., 2007). Since STRAP can possibly stabilize ICN3, we will use the STRAP shRNA lentivirus to knock down STRAP in these cells lines and observe any alteration in ICN3 half life. The control and STRAP knockdown cells will be incubated for 24 h with poly-L-lysine. The cells will be then starved for 3 h in cysteine/methionine-free DMEM (Invitrogen) supplemented with 1% L-glutamine and 10% fetal bovine serum and labeled for 2 h by the addition of 0.1 mCi/ml Pro-Mix L-[35S] in vitro labeling mix (GE Healthcare). The culture medium will be changed to fresh medium containing cold cysteine and methionine and chased for the indicated times. At each time point, cells will be harvested and lysed. The cell lysates were immunoprecipitated with anti-Notch3 antibody (Cell Signaling) and immunocomplexes will be subjected to SDS–PAGE. The separated proteins were visualized by autoradiography. A previous report indicates that half life of ICN3 is approximately 17 h or 0.7 days (Takahashi et al., 2010). We expect the half life of ICN3 to be decreased following decreased STRAP level.

3. We will study the effect of STRAP on transforming ability of Notch1. Stable overexpression of ICN1 in HC11 mouse mammary epithelial cells in vitro with the

pBabe/Notch1 retrovirus induced puromycin resistant cells which had a transformed morphology and were able to form colonies in soft agar (Diévart et al., 1999). The ankyrin-repeats are required for this transforming ability of Notch1. STRAP and GSK3β bind ICN3 through the same region. We will use the Notch1 overexpressing HC11 cells to knock down STRAP using the lentiviral STRAP shRNA (Open Biosystems). For soft agar growth assay, clones of vector control and STRAP knockdown Notch1 overexpressing HC11 cells will be plated in a 35 mm Petri dish at $2x10^4$ cells/ml in RPMI-1640 with 10% FCS, EGF and insulin, mixed vol/vol with 0.6% agar (Sigma). Colonies will be observed for 3 weeks, and counted under the microscope. Experiments will be repeated three times.

4. MAP1B and Smad3 are validated substrates of GSK3β that have also been shown to bind with STRAP. Though the role of STRAP in these interactions has been proposed to be quite different from what we have found for ICN3, we would like to see if STRAP has any effect on the ubiquitination and stability of these two proteins. Smad3 being a transcription factor compared to MAP1B which is a structural protein, there is a greater possibility of STRAP playing a similar role for Smad3. We will either obtain plasmids expressing these proteins and conduct ubiquitination experiments in 293 T cells in presence and absence of STRAP as outlined in materials and methods. STRAP knockdown clones from various cells lines made in our lab will be used to check Smad3 and MAP1B expression status and pulse chase analysis will be done to determine any alteration in half life of these proteins. Positive results will indicate a more general role of STRAP towards GSK3β substrates and negative results would indicate that the role of STRAP towards ICN3 deubiquitination is a specific one.

REFERENCES

Adhikary S, Marinoni F, Hock A, Hulleman E, Popov N, Beier R, Bernard S, Quarto M, Capra M, Goettig S, Kogel U, Scheffner M, Helin K, Eilers M. (2005) The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell **123** pp. 409-421.

Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund. (1999) Notch signalling controls pancreatic cell differentiation. Nature **400** pp. 877-881.

Archer CT, Delahodde A, Gonzalez F, Johnston SA, Kodadek T. (2008) Activation domain-dependent monoubiquitylation of Gal4 protein is essential for promoter binding in vivo. J Biol Chem **283** pp. 12614-12623.

Arnold HK, Sears RC. (2006) Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. Mol Cell Biol **26** pp. 2832-2844.

Aster J, Pear W, Hasserjian R, Erba H, Davi F, Luo B, Scott M, Baltimore D, Sklar J. (1994) Functional analysis of the TAN-1 gene, a human homolog of Drosophila notch. Cold Spring Harb Symp Quant Biol **59** pp. 125-136.

Anumanthan G, Halder SK, Friedman DB, Datta PK. (2006) Oncogenic serine-threonine kinase receptor-associated protein modulates the function of Ewing sarcoma protein through a novel mechanism. Cancer Res **6**6 pp. 10824-10832.

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Artega C. (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor ß-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem **275** pp. 36803–36810

Barboric M, Zhang F, Besenicar M, Plemenitas A, Peterlin BM. (2005) Ubiquitylation of Cdk9 by Skp2 facilitates optimal Tat transactivation. J Virol **79** pp. 11135-41.

Batlle E, Sancho E, Francí C, Domínguez D, Monfar M, Baulida J, García De Herreros A. (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol **2** pp. 84-91.

Battaglia G, Princivalle A, Forti F, Lizier C, Zeviani M. (1997) Expression of the SMN gene, the spinal muscular atrophy determining gene, in the mammalian central nervous system. Hum Mol Genet **11** pp. 1961-1971.

Baum B, Settleman J, Quinlan MP. (2008) Transitions between epithelial and mesenchymal states in development and disease. Semin Cell Dev Biol **19** pp. 294-308.

Bismar TA, Demichelis F, Riva A, Kim R, Varambally S, He L, Kutok J, Aster JC, Tang J, Kuefer R, Hofer MD, Febbo PG, Chinnaiyan AM, Rubin MA. (2006) Defining aggressive prostate cancer using a 12-gene model. Neoplasia **8** pp. 59-68

Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, Cumano A, Roux P, Black RA, Israël A. (2000) A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol Cell **5** pp. 207-216.

Bray SJ. (2006) Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol **9** pp. 678-689.

Bryan RT, Nicholls JH, Harrison RF, Jankowski JA, (2003) Wallace DM. The role of beta-catenin signaling in the malignant potential of cystitis glandularis. J Urol **170** pp. 1892-6.

Buess M, Terracciano L, Reuter J, Ballabeni P, Boulay JL, Laffer U, Metzger U, Herrmann R, Rochlitz C. (2004) STRAP is a strong predictive marker of adjuvant chemotherapy benefit in colorectal cancer. Neoplasia **6** pp. 813-820

Carissimi C, Baccon J, Straccia M, Chiarella P, Maiolica A, Sawyer A, Rappsilber J, Pellizzoni L. (2005) Unrip is a component of SMN complexes active in snRNP assembly. FEBS Lett **579** pp. 2348-2354

Carnegie GK, Means CK, Scott JD. (2009) A-kinase anchoring proteins: from protein complexes to physiology and disease. IUBMB Life **61** pp. 394-406.

Chaffer CL, Brennan JP, Slavin JL, Blick T, Thompson EW, Williams ED. (2006) Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. Cancer Res **66** pp. 11271-11278.

Chaffer CL, Thompson EW, Williams ED. (2007) Mesenchymal to epithelial transition in development and disease. Cells Tissues Organs **185** pp. 7-11.

Chalecka-Franaszek, E. & Chuang, D.M. (1999). Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. Proc. Natl. Acad. Sci. USA **96** pp. 8745-8750.

Chen RH, Su YH, Chuang RL & Chang TY (1998) Suppression of transforming growth factor-ß-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. Oncogene **17** pp. 1959–1968

Chen WV, Delrow J, Corrin PD, Frazier JP, Soriano P. (2004) Identification and validation of PDGF transcriptional targets by microarray-coupled gene-trap mutagenesis. Nat Genet. **36** pp. 304-12.

Chen Z, Seimiya H, Naito M, Mashima T, Kizaki A, Dan S, Imaizumi M, Ichijo H, Miyazono K, Tsuruo T. (1999) ASK1 mediates apoptotic cell death induced by genotoxic stress. Oncogene **7** pp. 173-80

Chen ZJ, Sun LJ. (2009) Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell **13** pp. 275-86.

Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD, Holder JC. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chem Biol **7** pp. 793-803.

Cohen P. and S. Frame. (2001) The renaissance of GSK3. Mol Cell Biol **2** pp. 759-776.

Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell **7** pp. 1267-1277

Cross DA., Alessi, P. Cohen, M. Andjelkovich, BA. Hemmings. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378** pp. 785-789.

Dajani R., Fraser E., Roe S.M., Young N., Good V., Dale T.C., Pearl L.H. (2001). Crystal structure of glycogen synthase kinase 3 beta: Structural basis for phosphate-primed substrate specifi city and autoinhibition. Cell **105** pp. 721–732.

Dang TP, Eichenberger S, Gonzalez A, Olson S, Carbone DP. (2003) Constitutive activation of Notch3 inhibits terminal epithelial differentiation in lungs of transgenic mice. Oncogene **22** pp. 1988–1999.

Dang TP, Gazdar AF, Virmani AK, Sepetavec T, Hande KR, Minna JD, Roberts JR, Carbone DP. (2000) Chromosome 19 translocation, overexpression of Notch3, and human lung cancer. J Natl Cancer Inst **92** pp. 1355-1357

Datta PK, Chytil A, Gorska AE, Moses HL. (1998) Identification of STRAP, a novel WD domain protein in transforming growth factor-beta signaling. J Biol Chem **273** pp. 34671-34674.

Datta PK, Moses HL. (2000) STRAP and Smad7 synergize in the inhibition of transforming growth factor beta signaling. Mol Cell Biol **20** pp. 3157-67.

Davies JA, Ladomery M, Hohenstein P, Michael L, Shafe A, Spraggon L, Hastie N. (2004) Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the Wt1 tumour suppressor is required for nephron differentiation. Hum Mol Genet **13** pp. 235-246.

Dohda T, Maljukova A, Liu L, Heyman M, Grandér D, Brodin D, Sangfelt O, Lendahl U. (2007). Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines. Exp Cell Res **313** pp. 3141-3152.

Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, Klonjkowski B, Berrou E, Mericskay M, Li Z, Tournier-Lasserve E, Gridley T, Joutel A. (2004) Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev **18** pp. 2730-5.

Doucas H, Mann CD, Sutton CD, Garcea G, Neal CP, Berry DP, Manson MM. (2008) Expression of nuclear Notch3 in pancreatic adenocarcinomas is associated with adverse clinical features, and correlates with the expression of STAT3 and phosphorylated Akt. J Surg Oncol **97** pp. 63-68.

Duarte, A, Hirashima, M, Benedito, R, Trindade, A, Diniz, P, Bekman, E, Costa, L, Henrique, D. and Rossant, J. (2004) Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev **18** pp. 2474 -247

Engel ME, McDonnell MA, Law BK, Moses HL (1999) Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J Biol Chem **274** pp. 37413-3720.

Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A. (2003) Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. J Biol Chem **278** pp. 32227-32235.

Ewing RM, Chu P, Elisma F, Li H, Taylor P, Climie S, McBroom-Cerajewski L, Robinson MD, O'Connor L, Li M, Taylor R, Dharsee M, Ho Y, Heilbut A, Moore L, Zhang S, Ornatsky O, Bukhman YV, Ethier M, Sheng Y, Vasilescu J, Abu-Farha M, Lambert JP, Duewel HS, Stewart II, Kuehl B, Hogue K, Colwill K, Gladwish K, Muskat B, Kinach R, Adams SL, Moran MF, Morin GB, Topaloglou T, Figeys D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol Syst Biol **3**

Fan Z, Beresford P, Oh D, Zhang D, Lieberman J. (2003) Tumor suppressor NM23-1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell **112** pp. 659–672.

Fang X Yu S, Tanyi JL, Woodgett JR, Mills GB. (2002) Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein

kinase C-dependent intracellular pathway. Mol. Cell. Biol **22** pp. 2099–2110

Fang X, Yu SX, Lu Y, Bast RC Jr, Woodgett JR, Mills GB. (2000) Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc. Natl. Acad. Sci. USA, **97** pp. 11960–11965.

Frame S, Cohen P, Biondi RM (2001) A common phosphate binding site explains the unique substrate specificity of GSK-3 and its inactivation by phosphorylation. Mol. Cell, **7** pp. 1321–1327.

Fryer, CJ, White JB, Jones KA. (2004) Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell **16** pp. 509-520.

Ferrer I, Barrachina M, Puig B. (2002) Glycogen synthase kinase-3 is associated with neuronal and glial hyperphosphorylated tau deposits in Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. Acta Neuropathol **104** pp. 583-591

Foltz DR, Santiago MC, Berechid BE, and Nye JS. (2002) Glycogen synthase kinase-3β modulates notch signaling and stability. Curr. Biol. **12** pp. 1006-1011

Frame S. and P. Cohen. (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J **359** pp. 1-16.

Greenwald, I. (1994) Structure/function studies of lin-12/Notch proteins. Curr Opin Genet Dev **4** pp. 556-562.

Gregory MA, Qi Y, Hann SR. (2003) Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. J Biol Chem **278** pp. 51606-12.

Grimes CA. and RS. Jope. (2001) The multifaceted roles of glycogen synthase kinase 3ß in cellular signalling. Progress in Neurobiology **65** pp. 391-426.

Grimmler M, Otter S, Peter C, Müller F, Chari A, Fischer U. (2005) Unrip, a factor implicated in cap-independent translation, associates with the cytosolic SMN complex and influences its intracellular localization. Hum Mol Genet **14** pp. 3099-3111.

Guo X, Ramirez A, Waddell DS, Li Z, Liu X, Wang XF. (2008) Axin and GSK3- control Smad3 protein stability and modulate TGF- signaling. Genes Dev **22** pp. 106-20

Gupta-Rossi N, Le Bail O, Gonen H, Brou C, Logeat F, Six E, Ciechanover A, Israël A. (2001) Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. J Biol Chem **276** pp. 34371-8.

Halder SK, Anumanthan G, Maddula R, Mann J, Chytil A, Gonzalez AL, Washington MK, Moses HL, Beauchamp RD, Datta PK. (2006) Oncogenic function of a novel WD-domain protein, STRAP, in human carcinogenesis. Cancer Res **66** pp. 6156-6166.

Haniffa MA, Collin MP, Buckley CD, Dazzi F (2009) Mesenchymal stem cells: the fibroblasts' new clothes? Haematologica. **94** pp. 258-63.

Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P. (1998) Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol **8** pp. 573-581.

Haruki N, Kawaguchi KS, Eichenberger S, Massion PP, Olson S, Gonzalez A, Carbone DP, Dang TP. (2005) Dominant-negative Notch3 receptor inhibits mitogen-activated protein kinase pathway and the growth of human lung cancers. Cancer Res **65** pp. 3555-3561.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A **95** pp 2509-14.

Hellström M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalén M, Gerhardt H, Betsholtz C. (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature **445** pp. 776-780.

Hochstrasser M. (2009) Origin and function of ubiquitin-like proteins. Nature **458** pp. 422-428.

Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O and Woodgett JR. (2000) Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation. Nature **406** pp. 86-90.

Hong M, Chen DC, Klein PS, Lee VM. (1997) Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. J Biol Chem **272** pp. 25326-25332.

Hosono S, Gross I, English MA, Hajra KM, Fearon ER, Licht JD. (2000) E-cadherin is a WT1 target gene. J Biol Chem **275** pp. 10943-1095

Hosono S, Luo X, Hyink DP, Schnapp LM, Wilson PD, Burrow CR, Reddy JC, Atweh GF, Licht JD. (1999) WT1 expression induces features of renal epithelial differentiation in mesenchymal fibroblasts. Oncogene **18** pp. 417-427.

Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW. (2007) Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. J Cell Physiol **213** pp. 374-83.

Hurley JH, Lee S, Prag G. (2006) Ubiquitin-binding domains. Biochem J **399** pp. 361-72

Hsu S, Huang F, Wang L, Banerjee S, Winawer S, Friedman E. (1994) The role of nm23 in transforming growth factor beta 1-mediated adherence and growth arrest. Cell Growth Differ **5** pp. 909-17.

Hsu W, Zeng L, Costantini F. (1999) Identification of a domain of Axin that binds to the serine/threonine protein phosphatase 2A and a self-binding domain. J Biol Chem **274** pp. 3439-3445.

Ikeya T, Hayashi S. (1999) Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the Drosophila trachea. Development **126** pp. 4455-4463.

Jin YH, Kim H, Oh M, Ki H, Kim K. (2009) Regulation of Notch1/NICD and Hes1 expressions by GSK-3alpha/beta. Mol Cells **27** pp. 15-19

Jope RS. and Johnson GVW. (2004) The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci **29** pp. 95-102.

Jung H, Seong HA, Manoharan R, Ha H. (2010) Serine-threonine kinase receptor-associated protein inhibits apoptosis signal-regulating kinase 1 function through direct interaction. J Biol Chem **285** pp. 54-70.

Kanai Y, Dohmae N, Hirokawa N. (2004) Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. Neuron **43** pp. 513-525

Kashikar ND and Datta PK. In: M. Schwab (Ed.), (2008) Encyclopedia of Cancer, second ed., Springer-Verlag, pp. 2710–2713.

Khokhar A, Chen N, Yuan JP, Li Y, Landis GN, Beaulieu G, Kaur H, Tower J. (2008) Conditional switches for extracellular matrix patterning in Drosophila melanogaster. Genetics **178** pp. 1283-1293.

Kim CJ, Choi BJ, Song JH, Park YK, Cho YG, Nam SW, Yoo NJ, Lee JY, Park WS. (2007) Overexpression of serine-threonine receptor kinase-associated protein in colorectal cancers. Pathol Int. **57** pp. 178-182.

Klein PS and Melton DA. (1996) A molecular mechanism for the effect of lithium on development. Proc. Natl. Acad. Sci. USA **93** pp. 8455-8459.

Konishi J, Kawaguchi KS, Vo H, Haruki N, Gonzalez A, Carbone DP, Dang TP. (2007) Gamma-secretase inhibitor prevents Notch3 activation and reduces proliferation in human lung cancers. Cancer Res **67** pp. 8051-8057.

Klymkowsky MW, Savagner P. (2009) Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. Am J Pathol **174** pp. 1588-1593

Kopan R, Nye JS, Weintraub H. (1994) The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development **120** pp. 2385-2596.

Kopstein L, Christofori G. (2006) Metastasis: cell autonomous mechanisms versus contributions by the tumor microenvironment. Cell Mol Life Sci **63** pp. 449–468

Krebs, LT., Shutter, JR., Tanigaki, K., Honjo, T., Stark, KL. and Gridley, T. (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev **18** pp. 2469 -2473

Lacombe M-L, Milon L, Munier A, Mehus J, Lambeth D. (2000) The human Nm23/nucleoside diphosphate kinases. J Bioenerg Biomembr **32** pp. 247–258

Laffin B, Wellberg E, Kwak HI, Burghardt RC, Metz RP, Gustafson T, Schedin P, Porter WW. (2008) Loss of singleminded-2s in the mouse mammary gland induces an epithelial-mesenchymal transition associated with up-regulation of slug and matrix metalloprotease 2. Mol Cell Biol **28** pp. 1936-46.

Lagna G, Carnevali F, Marchioni M, Hemmati-Brivanlou A. (1999) Negative regulation of axis formation and Wnt signaling in Xenopus embryos by the Fbox/WD40 protein beta TrCP. Mech. Dev **80** pp. 101–106.

Lai EC. (2004) Notch signaling: control of cell communication and cell fate. Development **131** pp. 965-973.

Lee JM, Dedhar S, Kalluri R, Thompson EW. (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol **172** pp. 973-981.

Lewis, J. (1998) Notch signalling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol **9** pp. 583-589.

Li CM, Guo M, Borczuk A, Powell CA, Wei M, Thaker HM, Friedman R, Klein U, Tycko B. (2002) Gene expression in Wilms' tumor mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition. Am J Pathol **160** pp. 2181-2190.

Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X. (2002) Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell **108** pp. 837-847.

Lohi H, Ianzano L, Zhao XC, Chan EM, Turnbull J, Scherer SW, Ackerley CA, Minassian BA. (2005) Novel glycogen synthase kinase 3 and ubiquitination pathways in progressive myoclonus epilepsy. Hum. Mol. Genet **14** pp. 2727–2736.

Lu KH, Patterson AP, Wang L, Marquez RT, Atkinson EN, Baggerly KA, Ramoth LR, Rosen DG, Liu J, Hellstrom I, Smith D, Hartmann L, Fishman D, Berchuck A, Schmandt R, Whitaker R, Gershenson DM, Mills GB, Bast RC Jr. (2004) Selection of potential markers for epithelial ovarian cancer with gene expression arrays and recursive descent partition analysis. Clin Cancer Res **10** pp. 3291-3300.

Maccioni RB, Munoz JP, Barbeito L. (2001) The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch Med Res **32** pp. 367–381.

Matsuda S, Katsumata R, Okuda T, Yamamoto T, Miyazaki K, Senga T, Machida K, Thant AA, Nakatsugawa S, Hamaguchi M. (2000) Molecular cloning and characterization of human MAWD, a novel protein containing WD-40 repeats frequently overexpressed in breast cancer. Cancer Res **60** pp. 13-17.

Matsukawa J, Matsuzawa A, Takeda K, Ichijo H. (2004) The ASK1-MAP kinase cascades in mammalian stress response. J Biochem **136** pp. 261-265.

Mimeault M, Batra SK. (2007) Interplay of distinct growth factors during epithelial mesenchymal transition of cancer progenitor cells and molecular targeting as novel cancer therapies. Ann Oncol **18** pp. 1605-1619

Minella AC, Welcker M, Clurman BE. (2005) Ras activity regulates cyclin E degradation by the Fbw7 pathway. Proc Natl Acad Sci U S A **102** pp. 9649-9654.

Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM, Leach SD. (2003) Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell **3** pp. 565-576.

Mora A, Komander D, van Aalten DM, Alessi DR. (2004) PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol **15** pp. 161-70.

Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY. (2004) The ankyrin repeat as molecular architecture for protein recognition. Protein Sci **13** pp. 1435-1448

Moustakas A, Heldin CH. (2007) Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci **98** pp. 1512-1520.

Nadella KS, Jones GN, Trimboli A, Stratakis CA, Leone G, Kirschner LS. (2008) Targeted deletion of Prkar1a reveals a role for protein kinase A in mesenchymal-to-epithelial transition. Cancer Res **68** pp. 2671-7.

Nelson WJ, Nusse R. (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science **303** pp. 1483-7.

Noseda M, Fu Y, Niessen K, Wong F, Chang L, McLean G, Karsan A. (2006) Smooth Muscle alpha-actin is a direct target of Notch/CSL. Circ Res **98** pp. 1468-1470.

Orsulic S, Huber O, Aberle H, Arnold S, Kemler R. (1999) E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. J Cell Sci **112** pp. 1237-1245.

Perez-Moreno MA, Locascio A, Rodrigo I, Dhondt G, Portillo F, Nieto MA, Cano A. (2001) A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. J Biol Chem **276** pp. 27424-27433.

Popov N, Wanzel M, Madiredjo M, Zhang D, Beijersbergen R, Bernards R, Moll R, Elledge SJ, Eilers M. (2007) The ubiquitin-specific protease USP28 is required for MYC stability. Nat Cell Biol **9** pp. 765-774

Qiu L, Joazeiro C, Fang N, Wang HY, Elly C, Altman Y, Fang D, Hunter T, Liu YC. (2000) Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. J Biol Chem **275** pp. 35734-35737.

Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP. (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. Embo J **20** pp. 3427-36.

Rao MK, Pham J, Imam JS, MacLean JA, Murali D, Furuta Y, Sinha-Hikim AP, Wilkinson MF. (2006) Tissue-specific RNAi reveals that WT1 expression in nurse cells controls germ cell survival and spermatogenesis. Genes Dev **20** pp. 147-152.

Reynolds TC, Smith SD, Sklar J. (1987) Analysis of DNA surrounding the breakpoints of chromosomal translocations involving the beta T cell receptor gene in human lymphoblastic neoplasms. Cell **50** pp. 107-117.

Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman,I. (2006) Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature **444** pp. 1083-1087

Roehl H, Bosenberg M, Blelloch R, Kimble J. (1996) Roles of the RAM and ANK domains in signaling by the C. elegans GLP-1 receptor. EMBO J **15** pp. 7002-7012

Ronchini C. and AJ. Capobianco (2001) Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). Mol Cell Biol **21** pp. 5925-5934.

Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. (1996) Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science **272** pp. 1023–1026.

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P. (1997) Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science **275** pp. 1790-1802.

Salahshor S, Woodgett JR. (2005) The links between Axin and carcinogenesis. J Clin Pathol **58** pp. 225–236.

Salghetti SE, Caudy AA, Chenoweth JG, Tansey WP. (2001) Regulation of transcriptional activation domain function by ubiquitin. Science **293** pp. 1651-1653.

Sasai Y, Kageyama R, Tagawa Y, Shigemoto R, Nakanishi S. (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev **6** pp. 2620-2634.

Savagner P, Yamada KM, Thiery JP. (1997) The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J Cell Biol **137** pp. 1403-1419.

Seong HA, Jung H, Ha H. (2007) NM23-H1 tumor suppressor physically interacts with serine-threonine kinase receptor-associated protein, a transforming growth factor-beta (TGF-beta) receptor-interacting protein, and negatively regulates TGF-beta signaling. J Biol Chem **282** pp. 12075-96.

Seong HA, Jung H, Choi HS, Kim KT, Ha H. (2005) Regulation of transforming growth factor-beta signaling and PDK1 kinase activity by physical interaction between PDK1 and serine-threonine kinase receptor-associated protein. J Biol Chem **280** pp. 42897-42908.

Sharma VM, Draheim KM, Kelliher MA. (2007) The Notch1/c-Myc pathway in T cell leukemia. Cell Cycle **6** pp. 927-930.

Sheng W, Wang G, La Pierre DP, Wen J, Deng Z, Wong CK, Lee DY, Yang BB. (2006) Versican mediates mesenchymal-epithelial transition. Mol Biol Cell **17** pp. 2009-2020.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A **96** pp. 5522-5527.

Stambolic V, Ruel L & Woodgett JR. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signaling in intact cells. Curr Biol **6** pp. 1664-1668.

Steeg PS, Horak CE, Miller KD. (2008) Clinical-translational approaches to the Nm23-H1 metastasis suppressor. Clin Cancer Res **14** pp. 5006-5012.

Sutherland C, Leighton IA, Cohen P. (1993) Inactivation of glycogen synthase kinase-3 beta by phosphorylation: New kinase connections in insulin and growth factor signalling. Biochem J **296** pp. 15–19

Sundqvist A, Bengoechea-Alonso, Ye X, Lukiyanchuk V, Jin J, Harper J, Ericsson J. (2003) Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF. Cell Metabolism **1** pp. 379-391

Takahashi K, Adachi K, Yoshizaki K, Kunimoto S, Kalaria RN, Watanabe A. (2010) Mutations in NOTCH3 cause the formation and retention of aggregates in the endoplasmic reticulum, leading to impaired cell proliferation. Hum Mol Genet **19** pp. 79-89.

Takashima A, Murayama M, Murayama O, Kohno T, Honda T, Yasutake K, Nihonmatsu N, Mercken M, Yamaguchi H, Sugihara S, Wolozin B. (1998) Presenilin 1 associates with glycogen synthase kinase-3beta and its substrate tau. Proc Natl Acad Sci U S A **95** pp. 9637-9641.

Talora C, Campese AF, Bellavia D, Pascucci M, Checquolo S, Groppioni M, Frati L, von Boehmer H, Gulino A, Screpanti I. (2003) Pre-TCR-triggered ERK signalling-dependent downregulation of E2A activity in Notch3-induced T-cell lymphoma. EMBO Rep **4** pp. 1067-1072

Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, Furukawa T, Honjo T. (1995) Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). Curr Biol **5** pp. 1416-1423

ter Haar E, Coll JT, Austen DA, Hsiao HM, Swenson L, Jain J. (2001) Structure of GSK3beta reveals a primed phosphorylation mechanism Nat Struct Biol **8** pp. 593–596.

Tretyakova I, Zolotukhin AS, Tan W, Bear J, Propst F, Ruthel G, Felber BK. (2005) Nuclear export factor family protein participates in cytoplasmic mRNA trafficking. J Biol Chem **280** pp. 31981-31990

Uren A, Toretsky JA. (2005) Ewing's sarcoma oncoprotein EWS-FLI1: the perfect target without a therapeutic agent. Future Oncol **1** pp. 521-528.

Vadlamudi U, Espinoza HM, Ganga M, Martin DM, Liu X, Engelhardt JF, Amendt BA. (2005) PITX2, beta-catenin and LEF-1 interact to synergistically regulate the LEF-1 promoter. J Cell Sci **118** pp. 1129-1137.

Vanderburg CR, Hay ED. (1996) E-cadherin transforms embryonic corneal fibroblasts to stratified epithelium with desmosomes. Acta Anat (Basel) **157** pp. 87-104.

Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G. (2001) Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech Dev **108** pp. 161-164.

Wang QM, Fiol CJ, DePaoli-Roach AA and Roach PJ. (1994) Glycogen synthase kinase-3β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. J Biol Chem **269** pp. 14566-14574.

Wei W, Jin J, Schlisio S, Harper JW, Kaelin Jr WG. (2005) The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. Cancer Cell **8** pp. 25–33.

Weinmaster G, Kintner C. (2003) Modulation of notch signaling during somitogenesis. Annu Rev Cell Dev Biol **19** pp. 367-395.

Welcker M, Orian A, Jin J, Grim JE, Harper JW, Eisenman RN, Clurman BE. (2004) The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proc Natl Acad Sci U S A **101** pp. 9085-90.

Wells A, Yates C, Shepard CR. (2008) E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis **25** pp. 621-628

Weng AP, Ferrando AA, Lee W, Morris JP 4th, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC. (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science **306** pp. 269-71.

Wharton KA, Johansen KM, Xu T, Artavanis-Tsakonas S. (1985) Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell **43** pp. 567-81.

Winston J.T., Strack P., Beer-Romero P., Chu C.Y., Elledge S.J., Harper J.W.
(1999) The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. Genes Dev **13** pp. 270– 283.

Woodgett JR. (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO **9** pp. 2431-2438.

Wu G, Lyapina S, Das I, Li J, Gurney M, Pauley A, Chui I, Deshaies RJ, Kitajewski J. (2001) SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Mol Cell Biol **21** pp. 7403-7415.

Xu C, Kim NG, Gumbiner BM. (2009) Regulation of protein stability by GSK3 mediated phosphorylation. Cell Cycle **8** pp. 4032-4039.

Yamaguchi N, Oyama T, Ito E, Satoh H, Azuma S, Hayashi M, Shimizu K, Honma R, Yanagisawa Y, Nishikawa A, Kawamura M, Imai J, Ohwada S, Tatsuta K, Inoue J, Semba K, Watanabe S. (2008) NOTCH3 signaling pathway plays crucial roles in the proliferation of ErbB2-negative human breast cancer cells. Cancer Res **68** pp. 1881-1888.

Yamamoto H, Kishida S, Kishida M, Ikeda S, Takada S, Kikuchi A. (1999) Phosphorylation of Axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. J Biol Chem **274** pp. 10681–10684.

Yada M, Hatakeyama S, Kamura T, Nishiyama M, Tsunematsu R, Imaki H, Ishida N, Okumura F, Nakayama K, Nakayama KI. (2004) Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. Embo J **23** pp. 2116-2125.

Yeasmin S, Nakayama K, Rahman MT, Rahman M, Ishikawa M, Iida K, Otsuki Y, Kobayashi H, Nakayama S, Miyazaki K. (2010) Expression of nuclear Notch3 in cervical squamous cell carcinomas and its association with adverse clinical outcomes. Gynecol Oncol **117** pp. 409-416.

Zeng Q, Li S, Chepeha DB, Giordano TJ, Li J, Zhang H, Polverini PJ, Nor J, Kitajewski J, Wang CY. (2005) Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell **8** pp. 13-23

Zhang W, Yang J, Liu Y, Chen X, Yu T, Jia J, Liu C. (2009) PR55 alpha, a regulatory subunit of PP2A, specifically regulates PP2A-mediated beta-catenin dephosphorylation. J Biol Chem **284** pp. 22649-22656.

Zhong TP, Rosenberg M, Mohideen MA, Weinstein B, Fishman MC. (2000) gridlock, an HLH gene required for assembly of the aorta in zebrafish. Science **287** pp. 1820-1824.

Zumbrunn J, Kinoshita K, Hyman AA, Nathke IS. (2001) Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. Curr Biol **11** pp. 44–49.