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SND1 Mediated Downregulation of PTPN23 in Hepatocellular Carcinoma

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

Virginia Commonwealth University

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
μ	Micro
M	Molar
AEG-1	Astrocyte elevated gene 1
AFP	Alpha fetoprotein
AMACR	Alpha- methylacyl-coA racemase
APC	Adenomatous polyposis coli
AT1R	Angiotensin II type 1 receptor
CRC	Colorectal cancer
DMEM	Dubelco's modified eagle medium
DNA	Deoxyribonucleic acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase

FBS	Fetal Bovine Serum
HBV	Hepatitis virus B
HCV	Hepatitis virus C
HCC	Hepatocellular carcinoma
IGF	Insulin-like Growth Factor
IHC	Immunohistochemistry
IL6	Interleukin 6
H-Ras	Harvey rat sarcoma viral oncogene homolog
K-Ras	Kirsten rat sarcoma viral oncogene homolog
miR	microRNA
NFκB	Nuclear Factor kappa-light chain enhancer of activated B cells
PI3K	Phosphotidyl inositol – 3- kinase
PTEN	Phosphatidylinositol-3,4,5-triphosphate 3- phosphate
PTP	Protein tyrosine phosphatase
PTPN23	Protein tyrosine phosphate non-receptor 23
PTPR	Protein Tyrosine phosphatase receptor
PTPN	Protein Tyrosine phosphatase non receptor
OB	Oligosaccharide/ oligonucleotide binding

REMBRANDT Repository of Molecular Brain Neoplasia Data

RISC RNA induced silencing complex

RNA Ribonucleic acid

siRNA Small interfering RNA

SN Staphylococcal nuclease

SND1 Staphylococcal nuclease domain containing protein 1

Stat Signal transducer and activator of transcription

TCGA The Cancer Genome Atlas

TGF- β Transforming growth factor Beta

TNF α Tumor necrosis factor alpha

UTR Untranslated region

VEGF Vascular endothelial growth factor

ABSTRACT

SND1 MEDIATED DOWNREGULATION OF PTPN23 IN HEPATOCELLULAR CARCINOMA

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Staphyloccocal nuclease domain containing protein 1 (SND1) is identified as an oncogene in multiple cancers, including hepatocellular carcinoma (HCC). SND1 regulates gene expression at transcriptional as well as post-transcriptional level and mediates molecular pathways that culminate into carcinogenesis. SND1 is a component of RNA-induced silencing complex (RISC) and functions as a nuclease for RNAi-mediated mRNA degradation. On the other hand SND1

also binds to specific mRNAs, increasing their stability and hence expression. The aim of the present study is to identify mRNAs to which SND1 binds and modulates them either by degradation or increasing stability which might facilitate promotion of HCC by SND1. We performed RNA immunoprecipitation followed by RNA sequencing (RIP-Seq) using anti-SND1 antibody and human HCC cell line QGY-7703. More than 350 mRNAs were identified to be interacting with SND1, of which Protein tyrosine phosphatase non-receptor 23 (PTPN23) was of particular interest, since PTPN23 has been identified to be a tumor suppressor and its role in HCC has not been studied. We document that SND1 can bind to PTPN23 mRNA and induce its degradation. There is an inverse correlation between SND1 and PTPN23 levels in human HCC cell lines and PTPN23 level is downregulated in HCC. Our study thus identifies a novel mechanism by which SND1 promotes hepatocarcinogenesis and identifies PTPN23 as a potential tumor suppressor in HCC. Further studies need to be performed to explore the relationship of these two molecules in in vivo models and to develop PTPN23 overexpression as a potential therapeutic approach for HCC.
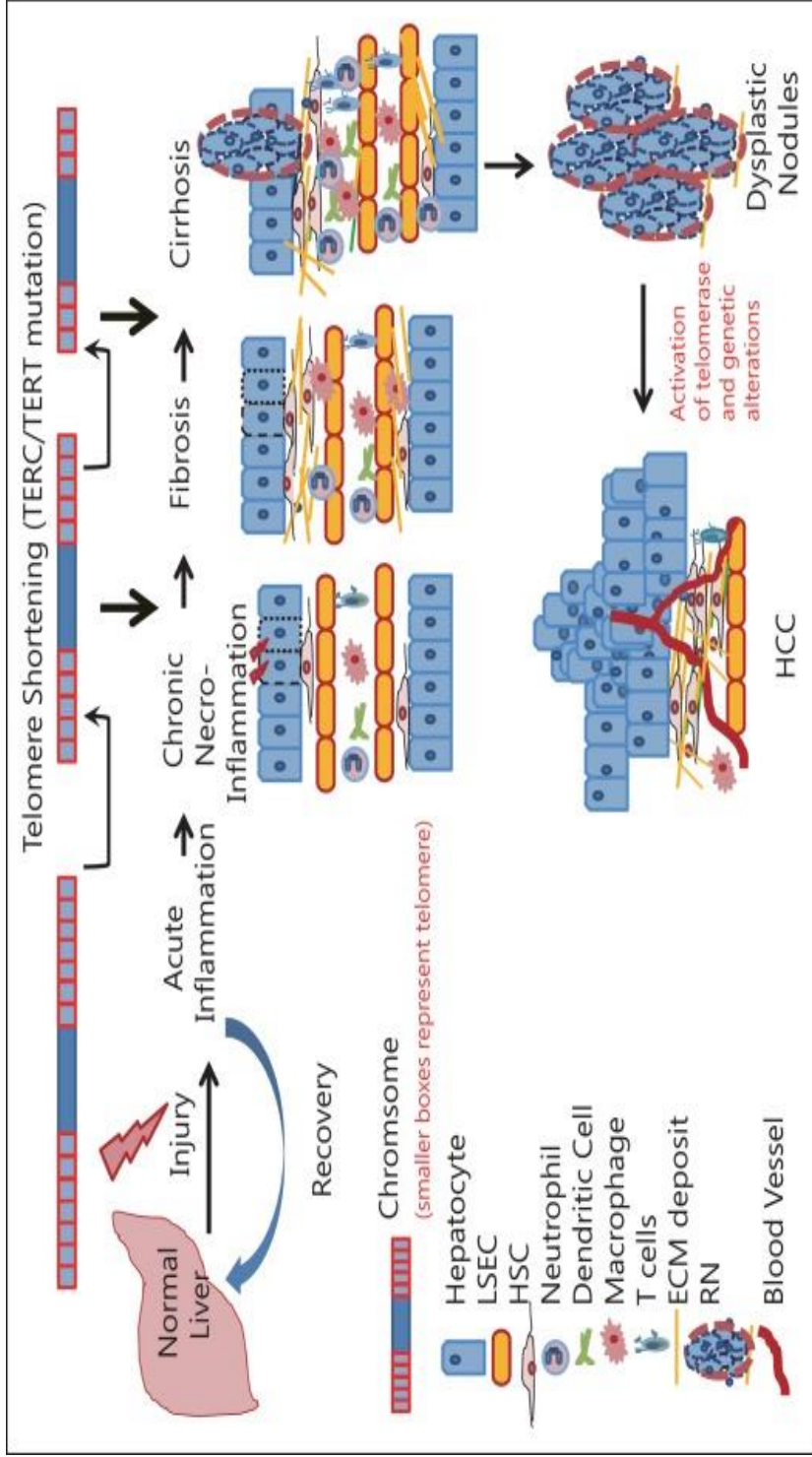
CHAPTER 1

Role of Staphylococcal Nuclease Domain Protein 1 (SND1) in cancer

Cancer is a highly aggressive disease characterized by interplay of mutations that cause cellular transformation and abnormal cell growth. Over the years, state-of-the-art studies have provided great insights into the pathogenesis of this highly complex disease. Cancer is now well defined by six hallmarks – sustained proliferative signaling, evasion of growth suppressors, invasion and metastasis, replicative immortality, angiogenesis and resistance to apoptosis (1). Change in expression patterns of any gene controlling these pathways initiate tumorigenesis that has the potential of malignancy. With increasing number of mutations being identified each day, that play a role in cancer development, molecular understanding of this disease gets more complicated. Further adding to the complexity is the effect of environmental factors in disease pathogenesis at each stage. Mutations arising in cells are mainly governed by genetic predisposition to changes in DNA structure, stability and expression, epigenetic changes and environmental factors (2). Epigenetic variations broadly include methylation pattern, microRNA expression disorders and chromatin organization (2). Diversity in cancer is not just restricted to the factors leading to cancer development, but is also observed within tumor microenvironment – broadly classified as inter-tumor heterogeneity and intra-tumor heterogeneity (2). With the advent of science, the molecular changes, which are responsible for such alterations are being identified as potential therapeutic targets.

Hepatocellular carcinoma, neoplasm arising in primary hepatocytes of liver is a highly aggressive primary liver cancer. It has a very high mortality and morbidity rate, ranking 3rd in cancer related deaths. Liver plays a primary role in metabolism and hence HCC is closely associated to metabolic pathways, especially lipid metabolism. It is well

known that majority of the HCC incidences are secondary to cirrhotic liver. A general

understanding about HCC development is aptly described in following figure (3).

Figure 1.1 Progression from Liver Injury to HCC. Molecular changes as liver injury progresses to inflammation and culminates into hepatocellular carcinoma

Ramakrishna et. al.,Liver Cancer, 2013

Inflammation has been long known to precede carcinogenesis, and is now considered one of the critical hallmarks of cancer (1). Viral infection, microbial invasion by breach in liver-gut barrier, alcohol abuse or nonalcoholic steatohepatitis (NASH) are most commonly observed molecular events that trigger inflammatory response, leading to liver cirrhosis which culminates into HCC. Necroinflammation and telomere shortening have also been reported to cause cell senescense and associated with onset of tumorigenesis, respectively (3). NF-κB- STAT3 inflammatory network is critical for progression of chronic inflammation of hepatic tissue to HCC (3). Proinflammaory molecules such as IL6 cause increased activation of STAT3 signaling, subsequently leading to cellular transformation (3). Relevance of SND1 in this network and eventual development of HCC, is discussed in detail later. Though significance of chronic inflammation with HCC development and progression has been well established by multiple studies, detailed molecular understanding of this vital association is not reported.

Staphylococcal nuclease and tudor domain containing 1 (SND1) is known to be involved in transcriptional activation, RNA splicing, editing and stability, and RNAi function (4-11). These processes are relevant for regulation of gene expression. SND1 is predicted to manifest a dynamic role modulating multiple molecular networks that control gene expression. SND1 has been proven to play a crucial role in transcriptional regulation of several genes important for tumorigenesis (6, 12-14). Structural analysis has confirmed that the protein functions include nucleic acid interaction along with protein – protein interactions (4). Several studies have illustrated the significant association of SND1 with different types of cancers, including breast, prostate, colorectal and liver cancer. It is overexpressed in these cancers and known to promote manifestation of

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aforementioned hallmarks of carcinogenesis (15). The present review provides a comprehensive description of the functional aspects of SND1 that are relevant to cancer development and progression.

SND1 protein structure

Human SND1 gene was assigned to chromosome 7q31.3 band location employing restriction fragment and fluorescence in-situ hybridization (FISH) analyses (16). Genomic gain in 7q, especially 7q31, has been demonstrated in prostate, renal and colorectal carcinoma (17-20). SND1 is overexpressed in prostate and colorectal carcinoma and genomic amplification might be an underlying mechanism for this overexpression (21-23).

Human SND1 is a 910 a.a. containing 100kDa protein with highly conserved domains, observed as low as *Caenorhabditis Elegans* in evolution. SND1 comprises of tandem repeats of four nuclease (SN) domains and a fifth domain containing fusion of Tudor and partial nuclease domains (TSN) (Fig. 1.2). Both, SN domain as well as Tudor domains have been reported to be involved in protein – protein interactions. The nuclease domains share 20% - 30% sequence homology amongst each other.(4) These domains have been well characterized and found to be structurally related to staphylococcal nuclease (SN) domains (4). These are thermo nucleases that hydrolyze DNA and RNA in a calcium dependent manner (4). However, hydrophobic cluster analysis shows that the SN domains of SND1 lack the specific amino acid residues involved in calcium dependent catalytic activity (4). SN domains are included in the oligonucleotide/ oligosaccharide binding (OB) fold superfamily, which comprises of a large number of proteins, involved in nucleic acid binding (4). OB fold proteins are critical for DNA

replication, DNA recombination, DNA repair, telomeric maintenance and cold shock response (24). OB fold domains range from 70-150 a.a in length and comprise of variable loops between conserved secondary domains (24). Other than Staphylococcal Nucleases, OB fold is also observed in bacterial enterotoxins, nucleic acid binding proteins and inorganic pyrophosphates (24). Many of the proteins in OB fold family, lack catalytic activity but carry out several other functions, such as transcriptional activation or repression, chromatin modification and DNA repair (4, 24). Tudor domains are highly conserved domains across eukaryotic species and studied in great detail employing *Drosophila* model system (25). Tudor domain containing proteins are involved in DNA interactions, specifically in epigenetic regulation, gene expression as well as snRNP, miRNA and piRNA biogenesis (25). The presence of these versatile SN and Tudor domains confer upon SND1 its diverse multifunctional properties. Below is a representation of SND1 protein structure.
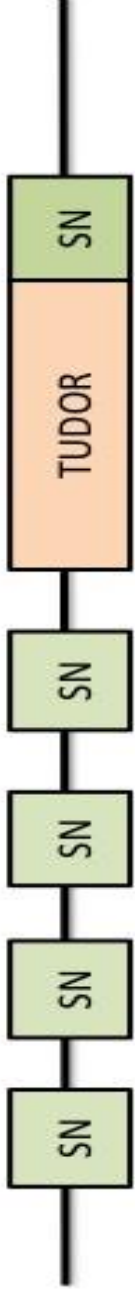
Figure 1.2 SND1 protein structure. SND1 protein structure comprises of four Staphylococcal nuclease domains and a single fusion domain, from N terminus to C-terminus. The fusion domain consists of Tudor motif and SN motif. Both, SN domain as well as Tudor domains have been reported to be involved in protein – protein interaction

Regulation of SND1 expression

The isolated 5'- flanking regulatory region of human SND1 gene spans more than 3.8 kb, including transcription start site and translation initiation codon (26). Bioinformatics analysis revealed presence of putative binding motifs for NF-κB, Sp1 and NF-Y transcription factors (26). The core promoter region does not contain TATA box, but is rich in CpG islands to form pre-initiation transcription complex (26). The lack of a TATA box, an initiator sequence for transcription is complimented by presence of CCAAT box in reverse orientation i.e. ATTGG at position -61 and -28 (26). The CCAAT box and a GC box, at -48, act as positive regulatory elements for SND1 expression (26). Inverted CCAAT sequences are often binding targets for NF-Y transcription factor (27). Luciferase assays with promoter deletion mutants show that -274 to -112, containing potential binding sites for NF-κB and Sp1 are crucial for SND1 expression (26). Interaction of NF-κB, Sp1 and NF-Y with the SND1 promoter was also detected by chromatin immnunoprecipitation (26). Mutation in GC box or CCAAT box reduced SND1 expression by 55-75%. As expected, TNFα treatment induced NF-κB mediated SND1 transcription, thereby suggesting the functional role of NF-κB in SND1 transcription regulation (26). The fact that NF-κB regulates SND1 transcription underlines the significance of this interaction in inflammation. Indeed, under pro-inflammatory conditions, there is an induction in NF-κB levels and activity, which in turn upregulates SND1 expression. Considering the supporting literature on pro-oncogenic properties of SND1, it can be speculated that Nf-κB mediated upregulation of SND1 is instrumental in progression from inflammation to carcinogenesis.

Promoter regions of human, mice and rat SND1 gene show about 80-85% sequence homology (26). Similar findings have been observed in promoter analysis of rat homologue of SND1, p102 (27). Putative binding sites for CCAAT/ enhancer binding protein (C/EBP), STATs and upstream stimulatory factor (USF) were identified (27). NF-Y binds at the CCAAT box (-370, -366) whereas the GC rich regions were identified as putative Sp1 binding sites (27). DNA elements that matched the consensus binding sequences for liver specific transcription factors, such as HNF-4 were also found (27), suggesting that SND1 might be differentially expressed in tissue specific manner.

Fashe et. al investigated cellular localization and tissue specific expression of SND1 in mice. With an exception of muscle tissues, SND1 is ubiquitously expressed in mice and the expression patterns are mostly consistent with that reported in humans (28). SND1 is strikingly upregulated in active secretory organs including pancreas, liver and mammary glands (28). Different studies have reported upregulated levels of SND1 in lipid droplets of milk secreted by mammary epithelial cells of mouse and cow (28, 29). SND1 levels were higher in exocrine pancreatic cells as compared to endocrine cells (28). SND1 is lower than in hepatocytes, however, it is more highly expressed in sinusoid endothelial cells (28). Actively proliferating cells such as crypts of Lieberkuhn and basal keratinocytes of skin and hair follicle show high SND1 protein levels compared to more differentiated or terminally differentiated cells in the same tissue (28). SND1 protein levels are much higher in spermatogonial cells than in spermatocytes and Sertoli cells (28). There is no protein detected in terminally differentiated spermatids and mature spermatozoa (28). In ovary, SND1 levels are high in follicular cells compared to that in stromal cells. There is no SND1 protein in oocytes (28). SND1 protein levels are

moderately expressed in human brain tissue, in neuronal as well as glial cells. In mice kidney, SND1 is comparatively more highly expressed in endothelial cells of Bowman's capsule than podocytes and mesangial cells (28). It is also expressed in bronchiolar epithelium, alveolar cells and pneumocytes of lung (28). Within gastrointestinal system, SND1 is expressed in ileum, deuodenum and colon. Lower SND1 levels were observed in more differentiated cells like villi and Paneth cells in ileum and absorptive cells of colon (28). Overall, SND1 is overexpressed in rapidly proliferating or precursor cells and downregulated in terminally differentiated cells. SND1 is upregulated in T-cells and co-localizes with CD3, as observed in lymphoid organs (28). Also, SND1 is not expressed in macrophages, in lymphoid organs as well as tissue residing macrophages like Kuppfer's cells in liver and alveolar macropahges in lungs (28). Further, SND1 levels in the red pulp of spleen are higher than that in white pulp (28). Differential expression of SND1 in T lymphocytes and macropahges suggests a potential role of SND1 in regulating immunity (28).

Functions of SND1: regulation of transcription

SND1, also called TudorSN or p100, was first identified as a transcriptional co-activator in an attempt to identify proteins interacting with Epstein Barr Nuclear Antigen (30). EBNA2 specifically activates transcription of genes that mediate B lymphocyte transformation (30). Along with known interacting factors such as TFIIB, TFIIH and TAF40, SND1 specifically interacts with EBNA2 acidic domain (30). This interaction is mediated by SND1 - TFIIE interaction such that SND1 acts as an adapter protein between EBNA2 and the transcriptional machinery (30). Pim kinases, found to be up regulated during Epstein Barr Virus infection, also interact with SND1 and mediate cellular

transformation by cooperating to enhance c-Myb activity (10). Pim-1 has been shown to phosphorylate SND1 and forms a stable complex, leading to an induction in c-Myb activity (10). Ectopic expression of Ras and Pim-1 also induced Myb responsive genes, since c-myb is a downstream target in this signaling cascade (10). This research study thus revealed that SND1 is a vital link in Ras and Pim-1 mediated induction of c-Myb (10). Lack of SND1 or dominant negative alleles of SND1 failed to cause an induction in c-Myb activity (10). c-Myb is linked to proliferation and differentiation, and also known to mediate cellular transformation. Hence, role of SND1 in regulating c-Myb expression via Ras or Pim-1 is significant to understand its functional impact in oncogenesis.

Another important class of transcription factors that are known to interact and cooperate with SND1 is Signal Transducers and Activator of Transcription i.e. STATs (6, 31-33). STAT proteins are sequestered to Janus kinases (JAK), which upon stimulation by cytokines like IFN-γ, undergo phosphorylation (31). Phosphorylation of STAT proteins and subsequent dimerization leads to nuclear localization where it activates transcription of target genes (31). Constitutive activation of JAK-STAT pathway has been implicated in many cancers (31). There are seven STAT proteins, which share structural and functional homology. Of these, SND1 is known to act as a transcriptional co-activator for STAT5 and STAT6. STAT5 co-immunoprecipitates with SND1, confirming the protein –protein interaction, but does not affect the phosphorylation status of STAT5 (32). Interaction with STAT5 is mediated via SN as well as Tudor domains of SND1 whereas for STAT6 – SND1 interaction involves only SN domains. SND1 acts as an adapter molecule, allowing functional bridging between CREB binding protein (CBP) and STAT6 (33). It interacts with D3-D4 domain of CBP and STAT6 transactivation

domain (TAD). Histone Acetylase Activity of CBP is stimulated by SND1, thereby allowing transcriptional activation (33).

A recent study in breast cancer model illustrated that SND1 significantly interacts with promoter regions of several genes in TGFβ signaling pathway, including Smad1-4 and TGFβ (14). We have shown that SND1 up regulation is also correlated with TGFβ signaling in HCC, as described later (34).

Post-transcriptional regulation of gene expression

Regulation of RNA-induced silencing complex (RISC) activity

Post-transcriptional regulation of gene expression can be mediated by several mechanisms including nucleocytoplasmic localization, mRNA stability, mRNA processing and translation. SND1 functions as a nuclease in RNA-induced silencing complex (RISC) that plays a significant role in modulation of gene expression at a post-transcriptional level (35). Our studies have shown that Astrocyte elevated gene-1 (AEG-1), an important oncogene, in association with SND1 and other proteins, forms a stable RISC complex (8). RISC incorporates one strand of a small interfering RNA (siRNA) or microRNA (miRNA) and uses the siRNA or miRNA as a template for recognizing complementary mRNA. Argonaute proteins are activated in RISC when a complementary mRNA is identified which then cleaves the mRNA. SND1 functions as a nuclease in RISC along with the Argonaute proteins while AEG-1 functions as a scaffold protein for proper assembly of this complex. Both SND1 and AEG-1 are overexpressed in multiple cancers and together they facilitate functions of oncogenic miRNAs (onco-miRNA).

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Indeed RISC activity in cancer cells was found to be higher than that in normal cells (8). The increased activity of onco-miRNAs leads to increased suppression of their target tumor suppressor genes. In HCC cells it was documented that overexpression of AEG-1 or SND1 resulted in decreased expression of several tumor suppressor genes that are targets of oncomiRNAs, e.g., PTEN which is a target of miR-221 and miR-21; CDKN1C (p57), target of miR-221; CDKN1A (p21), target of miR-106b; SPRY2, target of miR-21 and TGFBR2, target of miR-93 (8). The reverse finding was observed upon knockdown of SND1 or AEG-1 (8). These findings suggest that increased RISC activity conferred by SND1 and AEG-1 might contribute to the carcinogenic process (8).

In pancreatic cancer, synaptogamin-11 interacts with RISC via SND1 binding (36). It is hypothesized that this protein is the missing link between membrane trafficking and miRNAmediated gene regulation (36). Synthesis of mature mir17-92 cluster is also inhibited by SND1 protein, thereby affecting several downstream target genes (37).

Regulation of mRNA stability

SND1 interaction with mRNA transcript can be independent of the RISC. Studies show that SND1 interacts with 3'UTR of angiotensin II type 1 receptor (AT1R), a G protein coupled receptor mediating the action of angiotensin (9). Here, SND1 increases mRNA stability and translational efficiency by increasing AT1R mRNA half-life, resulting in elevated protein levels (9). A recent finding shows that SND1 and AT1R mRNA colocalize in stress granules followed by oxidative stress and SND1 is required for efficient protein – RNA aggregation (38). These findings imply that SND1 increases stability of specific mRNAs, crucial for cellular stress response (38). Using HCC cell lines, we have demonstrated that SND1-mediated increased activity of AT1R activates

TGFβ signaling cascade thereby promoting epithelial-mesenchymal transition (EMT) and increase in migration and invasion (34). Studies have also established interaction of SND1 and Dengue virus 3'UTR, leading to increased viral replication (39). It needs to be studied whether SND1 has similar role in promoting replication of Hepatitis B or C viruses (HBV or HCV), the most common cause of HCC.

Regulation of mRNA splicing

Splicing is an important post transcriptional event, involved in excluding the non-coding intronic regions of mRNA transcript and thereby allowing translation of exonic regions into a functional polypeptide (40). A large macromolecular complex, driven by several proteins is required for this processing. Splicing is tightly regulated and functionally coupled with transcription (40). Because differential regulation of genes influencing cell growth and proliferation are critical for carcinogenesis, splicing is speculated to play a vital role in establishing pathogenesis (41). Spliceosome complex comprises of five major small ribonucleoprotein – U1, U2, U4/U6 and U5 along with several small non-snRNPs (42). It has been shown that SND1 interacts with U5 component of spliceosome and other non-snRNPs (42). Immunoprecipitation studies with GST-TSN fusion protein (lacking SN domains) and GST-SN fusion protein (lacking TSN domain) demonstrated that this interaction is specifically via TSN domain (42). *In vitro*, exogenously added SND1 accelerated the kinetics of spliceosome assembly, detected in terms of a ligated mRNA product, in a dose-dependent manner.(42) However, no difference was observed in the amount of splicing products with or without SND1 (42). It was demonstrated that SND1 improved the efficiency of pre-spliceosomal Complex assembly and accelerated the formation of complex B and complex C (42).

Alternative splicing, observed in eukaryotes, allows translation of multiple polypeptides from the same gene transcript by selection of specific exons to be included in the processed mRNA (40). Recent reports have shown that deregulation of alternative splicing is associated with cancer development and progression (41). Studies have shown SND1 to play a major role in this biological process. SND1 is identified as an interacting partner for SAM68, a pro oncogenic RNA binding protein that is up regulated in prostate cancer and supports cellular proliferation (40). SAM68 is involved in alternative splicing of CD44, specifically favoring inclusion of exon v5 of this gene (40). Inclusion of variable exons (v5) in CD44 mRNA is correlated with cancer development in prior studies (40). Authors observed that SND1 is a positive regulator of the alternative splicing of CD44 via SAM68. Knocking down SND1 inhibited inclusion of upstream variable exons (v4, v5 and v7) but that of the downstream variable exons (v8-v10) and constitutive exons was not affected (40). These findings suggest that SND1 co-ordinates transcriptional and post-transcriptional events for regulation of gene expression. Future studies focused on splicing activity of SND1 might provide insights into molecular events governing malignancy.

Regulation of RNA editing

Adenosine deaminase (ADAR) proteins function in RNA A-to-I editing in which it deaminates Adenosine to Inosine which is read as guanosine on an mRNA transcript. Such processes regulate protein translation by functionally changing the mRNA sequence (11). This post-transcriptional regulation also plays a role in gene expression and miRNA processing (11). Interestingly, ADAR protein expression is tightly regulated during embryogenesis, it is highest in the oocytes and zygote and diminished in the embryo

stages.(11) A strong correlation was observed between ADAR and SND1 levels during mouse fertilization.(11) While ADAR marks hyperedited transcripts, SND1 is responsible for degradation of hyper-edited mRNA transcripts as well as miRNA precursors (11). The data suggests that SND1 and ADAR1 are functionally synchronized in A-to-I editing and eliminate most miRNA precursors, progressively from oocyte to zygote (11). However, this function is prohibited during embryogenesis. Thus SND1 plays a crucial role in early stage embryogenesis and cell differentiation (11). Analysis of a SND1 knockout mouse will provide insights into the role of SND1 in regulating gene expression during developmental stage.

SND1 and stress response

SND1 plays a key role in cellular stress response via stress granule formation (43). Several environmental stimuli can be stressful for cellular growth. In response to such stimuli, cells undergo reprogramming in gene expression that allows cell survival (43, 44). Initial studies illustrated that SND1 is a component of the cytoplasmic stress granules and SN domain is crucial for this function (43, 44). SND1 interacts with Ras GTPase Activating Protein SH3 Domain Binding Protein (G3BP) and Adenosine deaminase (ADAR1). While G3BP has been shown to be essential for assembly of stress granules(43), ADAR1 has been linked to apoptosis and stress response (44). ADAR1 levels are also induced by interferon and its deficiency causes defective haematopoesis (44). Such studies implicate ADAR1 in cell survival and immune response towards stress. SND1 was found to directly interact with ADAR1. These proteins colocalized within cytoplasmic stress granules following oxidative stress, heat shock or interferon induction (44). Knock down of SND1 prohibited assembly of smaller stress foci into

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larger granules, emphasizing its role in this cellular function (43). These studies need to be extended into in vivo models to confirm the essential role of SND1 to cope with stress responses.

Regulation of cell cycle and cell division

SND1 is shown to be an essential protein in the normal programmed cell death, a process mediated by caspases (45). SND1 is cleaved by caspase 3 during drug-induced apoptosis. A non-cleavable SND1 mutant increased cell viability and knocking down SND1 promoted drug-induced apoptosis in HeLa cells (45). Incubation with caspases completely blocked RNase activity of SND1 indicating that SND1 enzymatic activity is required for maintaining cell viability or protection from apoptosis (45).

Oncogenic functions of SND1

Role of SND1 in Hepatocellular Carcinoma (HCC)

HCC is the primary liver malignancy, characterized by a highly aggressive form of cancer. In most cases, HCC develops from a preexisting condition, such as liver cirrhosis, nonalcoholic steatohepatitis or viral hepatitis infection (3). It is the fifth most common cancer and ranks third in cancer related deaths worldwide (34). Current management options include surgical resection, tumor ablation and embolization, though there is virtually no cure for this cancer. Molecular approaches such as multikinase inhibitor Sorafenib help to moderately increase the survival of HCC patients. To curb the increasing mortality rate, there is an urgent need for identification of potential therapeutic targets that can be employed for translational applications.

Studies demonstrate multiple ways by which SND1 contributes to hepatocarcinogenesis. Immunohistochemical analysis has demonstrated that SND1 is overexpressed in a high percentage of HCC patients and SND1 levels correlate with HCC stages (8). Stable clones of human HCC cells, with either overexpression or knockdown of SND1, in nude mice xenograft studies confirmed the positive role of SND1 in regulating cell viability and growth (8). Since SND1 is also an integral component of RISC complex, we investigated if RISC activity correlated with HCC development. Increased RISC activity was observed in HCC cell lines compared to normal hepatocytes, which consequently led to increase down regulation of tumor suppressors via RNA interference (8). As pointed out earlier, SND1 interacts and co-operates with AEG-1 to form RISC along with other known RISC proteins and inhibition of SND1 activity diminished AEG-1 activity (8). SND1- AEG-1 interaction was first established in our study aimed at identifying interacting partners of known oncogene, AEG-1 (46). Further investigation established that increased SND1 protein levels trigger a cascade of molecular events that promote invasion, proliferation, migration and angiogenesis (8, 34, 47). We unraveled a linear pathway in which NF-κB activation by SND1 augments miR-221 levels (47). As a result of this interaction and increase in miR221, Angiogenin and CXCL16 protein expression was upregulated and was observed to promote angiogenesis (47). Analysis of global gene expression profiles of SND1 knock down HCC clones identified 123 genes drastically down regulated, many of which are in TGFβ signaling pathway (34). We documented that increased stabilization of AT1R mRNA by SND1 activates AT1R downstream signaling, such as activation of ERK1/2, culminating in activation of TGFβ signaling pathway. The TGFβ signaling pathway closely regulates

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EMT (34). Indeed, i*n vitro* analysis demonstrated increased invasion and migration in SND1 overexpressing clones and *viceversa* in SND1 knock down clones (34), and expression of EMT marker proteins, such as N-cadherin, Slug and Snail and Vimentin, was found to be congruent with our hypothesis (34). A tissue microarray on 50 HCC cases showed statistically significant correlation between AT1R and SND1 levels further establishing a causative relationship between SND1, AT1R and TGFβ (34).

Insulin growth factor signaling (IGF) pathways are reported to be deregulated in HCC and found to be a cause of the aggressive tumorigenesis. Insulin like growth Factor Binding protein 3, a negative regulator of IGF pathway, was reported to be significantly over expressed in SND1 knockdown HCC clones (48). Thus SND1 might contribute to HCC by inhibiting IGFBP3 and promoting IGF-1 activity (48).

SND1 promotes invasion and metastasis in breast cancer

Cancer cells possess the ability to invade circulatory system and establish tumors in tissues distant from the primary site of tumorigenesis. Angiogenesis enables them to acquire nourishment and growth factors for constitutive proliferation. Metastatic potential of a tumor is often a measure of the fatality of the cancer. Metastasis has been long known to be a major cause of disease relapse and cancer related deaths. More patients succumb to metastatic malignancy than by the primary cancer (49). An efficient prognosis and diagnostic tool has not been discovered, in spite of several attempts at analyzing differential expression patterns in tumorous tissue.

iTRAQ based proteomic analysis on breast cancer metastasis model revealed that SND1 levels are up regulated in correlation with carcinoma progression (49). This study

aimed at studying gene signatures in breast cancer metastasis model. Out of the 197 proteins differentially regulated in cancerous tissue as compared to normal tissue, only those that have not been reported in association with metastasis were shortlisted (49). This approach helped identify 10 novel proteins significantly associated with metastasis, employing Mass spectrometry and immunohistochemistry. SND1 was one of these proteins, deregulated in the breast cancer metastasis model and showed significant differential expression in normal and breast cancer tissue (49). Immunohistochemical analysis of a tissue microarray showed up regulation of SND1 in majority of the cases (49).

AEG-1, also called Metadherin, is a known oncogene, associated with most oncogenic phenotype such as metastasis, invasion, angiogenesis, chemoresistance and apoptosis (22). Studies have shown that AEG-1 is associated with poor prognosis in breast metastasis patients (22). It is involved in oncogenic signaling pathways such as ERK, NF-κB, Ras and Wnt/β-catenin pathway (22). A study aimed at exploring role of AEG-1 in breast cancer metastasis, identified SND1 as an AEG-1 interacting protein (22). Role of SND1 in metastasis was further emphasized based on the global gene expression profiling of SND1 knockdown clones. The list of gene sets globally enriched in SND1-expressing control cells *versus* SND1-KD cells was strikingly dominated by those involving genes up-regulated in some component of metastatic or oncogenic signaling (22). Genes such as ANGPTL, ID1 and EREG, known to promote specifically lung metastasis in breast cancer model, is significantly enriched in this gene set (22). Experimental lung metastasis study on nude mice with highly metastatic breast cancer cell line, with SND1 knockdown showed dramatic reduction in pulmonary metastatic

burden *in vivo* (22). Though SND1 did not promote invasion, it was found to augment chemoresistance to cells (22). SND1-KD cells demonstrated sensitivity towards chemotherapeutic drug induced apoptosis. Microarray data from SND1-KD cells showed up regulation of KiSS1 gene (22). Reporter assays later confirmed that SND1 directly down regulates the expression of KiSS1 gene that is known to suppress metastasis (22). Analysis of clinical data set of breast cancer patients with metastasis revealed that SND1 levels strongly correlated with specifically lung metastasis and metastasis free survival (22). Thus, SND1 is established to be a pro-metastasis protein. A recent finding shows significance of AEG-1-SND1 interaction in mammary tumorigenesis (50). AEG-1 knockout cells show reduced tumor initiation and sphere formation in vivo (50). This effect can be completely rescued by ectopic expression of AEG-1 in these cells (50). However, knocking down SND1 in these clones completely abolished the rescue effect of ectopic AEG-1 (50). Knocking down SND1 in AEG-1+/+ cells reduces the sphere formation in vitro and tumor formation in vivo, resembling the phenotype of AEG-1-/- cells (50). Thus SND1 is essential for pro-oncogenic manifestation of AEG-1 in breast cancer (50).

Early studies have established that SND1 cooperates with c-Myb, a differentiation and growth factor for immature hematopoetic stem cells and brings about lymphocyte transformation (10). Studies in breast cancer patients show overexpression of c-myb as well as SND1 (51). Study aimed at identifying target genes of c-Myb transcription factor revealed that SND1 promoter is one the target sites of c-Myb (51). There could be a possible positive regulatory mechanism to maintain levels of c-Myb and SND1 that

potentially maintains tumorigenesis in breast cancer. Other target genes relevant to oncogenesis identified by these authors were JUN, CXCR4 and CCNB1 (51).

SND1: an efficient diagnostic marker for Prostate cancer and Colorectal cancer

Studies focused on prostate cancer have identified SND1 as an efficient diagnostic marker (21). In a study including 174 prostrate cancer patients, SND1 levels could be correlated with histological grade of the tumor (21). SND1 protein levels were comparable to alpha-methylacyl-coA racemase (AMACR) protein levels, a currently employed marker protein for prostate cancer diagnosis (21). Though there were some cases were SND1 and AMACR protein levels differed, there was robust over expression of SND1 in tumorigenic tissue (21, 40). It was suggested that multiple protein markers, including SND1 and AMACR, should be used for better diagnosis of the disease (21). Knocking down SND1 reduces proliferation of prostate cancer cells, demonstrating the importance of SND1 in maintaining prostate cancer viability (21). SND1 also promotes prostate cancer development by positively regulating CD44 alternative splicing, allowing inclusion of variable exon v5 that is known to be pro-oncogenic (40).

SND1 is also significantly associated with colorectal cancer. A recent genome wide analysis of methylation patterns in CRC patients revealed a pool of genes that are differentially methylated in cancerous tissue in comparison to adjacent normal tissue (52). The study also included tissue from normal, healthy patients with no familial history of CRC as a control. CpG site located in SND1 gene was identified with highest discriminative accuracy, highlighting role of this protein in oncogenesis (52). Most of the hypermethylated CpG sites lied within the SND1 gene regions whereas methylation in the promoter region of SND1 was observed to be comparatively lower. SND1, along with

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other genes, was thus identified as a potential diagnostic candidate gene (52). Significant correlation is observed in nodal stage, pathological stage and co-expression of SND1 and AEG-1 in colon cancer (53). Immunohistochemical analyses of 196 colon cancer cases establish that cytoplasmic expression of AEG-1 and SND1 protein positively correlates with tumor grade and cancer progression, but negatively correlates with post-operative survival of patients (53). The study suggests potential application of these proteins as prognostic markers for colon cancer (53).

SND1 downregulates Adenomateous Polyposis Coli protein levels by post-transcriptional modification, without altering the mRNA levels of this gene, as reported in colon cancer (23). APC is a tumor suppressor in colon carcinogenesis and also associated with maintaining cell polarity and cell-cell adhesion by regulating E-cadherin localization (23). Loss of APC protein leads to stabilization and subsequent accumulation of β-catenin by preventing its proteasomal degradation with subsequent loss of contact inhibition and increased proliferation (23). These findings suggested that SND1 might be involved in early stage colon carcinogenesis. However, whether miRNAs or RISC is involved in SND1-mediated downregulation of APC protein remains to be studied.

SND1 in malignant glioma.

Malignant gliomas are the most frequent malignant brain tumor in adults (64). Current multi-modality therapies include surgery, radiation and chemotherapy, nonetheless the prognosis of malignant glioma remains extremely poor (65). The rapid growth and highly invasive nature of malignant glioma, favors its infiltration into surrounding normal brain parenchyma and facilitates recurrence after therapy (66). This aggressive disease progression necessitates further understanding of molecular mechanism involved in

glioma growth and invasion that ultimately will lead to identification of novel therapeutic targets of translational relevance. Our recent studies suggest that SND1 may provide a novel target for malignant glioma treatment (20). In this study we found higher SND1 mRNA and protein in human malignant glioma tissue as compared to normal brain. The Cancer Genome Atlas (TCGA) data showed a similar trend of high SND1 expression in human astrocytoma and glioblastoma samples as compared to normal brain. Additionally Rembrandt (Repository for Molecular Brain Neoplasia) data supports the prognostic significance of SND1 expression in which patients with intermediate levels of SND1 survived longer than patients showing elevated SND1 expression. Overexpression (OE) and knock down (KD) studies were performed to unravel the functional significance of SND1 in glioma progression. Overexpression of SND1 in immortalized primary human fetal astrocytes (IM-PHFA) (low SND1) significantly enhanced invasion and colony formation as compared to parental IM-PHFA cells. Conversely, when SND1 was knocked down in multiple glioma cell lines (high SND1), it significantly decreased invasion and colony formation, both in monolayer and in soft agar. Interestingly, SND1-KD primary glioma cells demonstrated enhanced sensitivity towards Temozolomide, an FDA approved drug used with radiation therapy as a standard of treatment for GBM patients (67). Knock down of SND1 in malignant glioma cells resulted in a flat-shaped cells, which stained positive with β-galactosidase, indicating induction of cellular senescence. Further studies documented a potential involvement of STAT-3 in SND-1-mediating glioma invasion and senescence-induced cell death. An intracranial xenograft study in nude mice using a highly invasive patient-derived malignant glioma cell line, showed a significant improved survival in SND1-KD group. This was associated with a

significant decrease in proliferation marker, activated STAT3, and angiogenesis marker and an enhanced expression of apoptotic marker. The observation that SND1 regulates several important determinants of glioma progression supports the rationale of using SND1-inhibition as a means of treating glioma patients.

Specific inhibitors of SND1

BLAST search reveals that SND1 is the only eukaryotic protein with Tudor and SN fusion domain. Hence, this quaternary fold can be employed for targeted therapeutic approaches, developing SND1 specific inhibitors. Similar protein domain is observed in *Plasmodium falciparum*, a parasite causing malarial infections in humans. Studies with *P. falciparum* SND1 have confirmed that SN domain is involved in nuclease activity whereas Tudor domain carries out the function of RNA binding (15). 3′, 5′ - Deoxythymidine bisphosphate (pdTp) is a competitive chemical inhibitor against SNases. In *P. falciparum* , it not only blocks the nuclease activity but also inhibits RNA/ protein interaction of SND1.(15) pdTp inhibited growth of both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* at a concentration of 100-200 μ M of SND1 inhibitor, suggesting that pdTp might be developed as an anti-malarial drug (15). Studies performed in our laboratory using multiple human HCC cell lines demonstrated that pdTp treatment resulted in significant reduction in call viability and colony forming potential (8). However, this effect was observed at high chemical concentrations, ranging from 100-200 μM thus rendering it ineffective for clinical applications. Further high throughput screening assays need to be performed to develop clinically relevant chemical inhibitors against SND1 enzymatic activity. Employing the C terminus fusion domain of Tudor SN might prove to be a productive effort in designing targeted molecular therapy.

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CHAPTER 2

An Introduction to Protein Tyrosine Phosphatases

Significance of kinase- and phosphatase-mediated regulation of cell functions

Kinases and phosphatases are a set of complimentary enzymes responsible for phosphorylation and dephosphorylation of proteins, respectively, associated with a multitude of human diseases (1-3). This set of reversible reactions is a common mode of post-translational modifications of eukaryotic proteins. By changing phosphorylation status of specific amino acid residues of proteins, these enzymes coordinate signaling events that determine cellular responses against external or internal stimuli. Several molecular signaling networks are tightly regulated by a balanced interplay between kinases and phosphatases. Deregulated function of these enzymes has been suggested to cause pathophysiologies ranging from autoimmune reaction to carcinogenesis (3, 4). With emerging discoveries, their role as potential drug targets becomes more evident.

Protein phosphatases have evolved from a wide spectrum of protein families, mainly categorized by the substrate amino acid residues into four classes. Of these, tyrosine phosphatases is the largest group of enzymes, encoded by more than 100 genes and subdivided into four main classes (1). Class I comprises of Dual specificity phosphatases and tyrosine phosphatases, two main groups of Protein Tyrosine phosphatases (PTPs) (1) The Dual Specificity enzymes can utilize Serine/ Threonine as well as Tyrosine residues as their catalytic substrates whereas the classical enzymes show specificity restricted to Tyrosine residue (1). Serine/ threonine phosphatases exist in vivo in a range of holoenzymes with variable regulatory or catalytic functional role (1). Physiologically, a given dual specificity phosphatase demonstrates functional specificity for either of these amino acid residue (1). The dual specificity enzyme sequence is not well conserved, demonstrates variability except for the cysteine containing catalytic domain (1). In this

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study, we focus on classical tyrosine phosphatases, described in detail below. Class II consists of a single low molecular weight phosphatase (4). Yeast cdc25 homologues make up the Class III whereas drosophila Eya homologs are categorized as Class IV PTP (4)

Initial evidence suggesting phosphatase activity was encountered while studying significance of tyrosine phosphorylation in cell growth and signaling (5). These studies were based on temperature sensitive mutants of SRC oncogene that encodes protein tyrosine kinase (5). Enzymatic activity observed to counter protein phosphorylation by kinases was the preliminary evidence of phosphatase activity (5). PTP1A and PTP1B were the first purified extracts of phosphatases, of which PTP1B was crystallized for structural analysis (5). Years of investigation later, scientists have deduced the structural details of this group of proteins. Enzymatic catalysis of these proteins has been studied in great depth.

Classical PTP structure

PTP structure has been very well characterized, identified by a signature catalytic motif $HC(X)_5R$ (1). The cysteine residue in this active site acts as a nucleophile and is essential for binding of the enzyme to target phosphate group on the substrate (1). In addition to large number of genes that encode protein tyrosine phosphatases, further diversity is contributed by mechanisms such as alternative splicing and post translational modifications (1). The complex level of diversity and the observed functional deregulation in several pathophysiologies are suggestive of the crucial role of these enzymes in cellular signaling. Based on the protein structure and cellular localization,

classical PTPs can be divided into two major categories – receptor (RPTPs) and non-receptor type of phosphatases (PTPNs).

Structures of classical tyrosine phosphatases are illustrated in figure below (1).
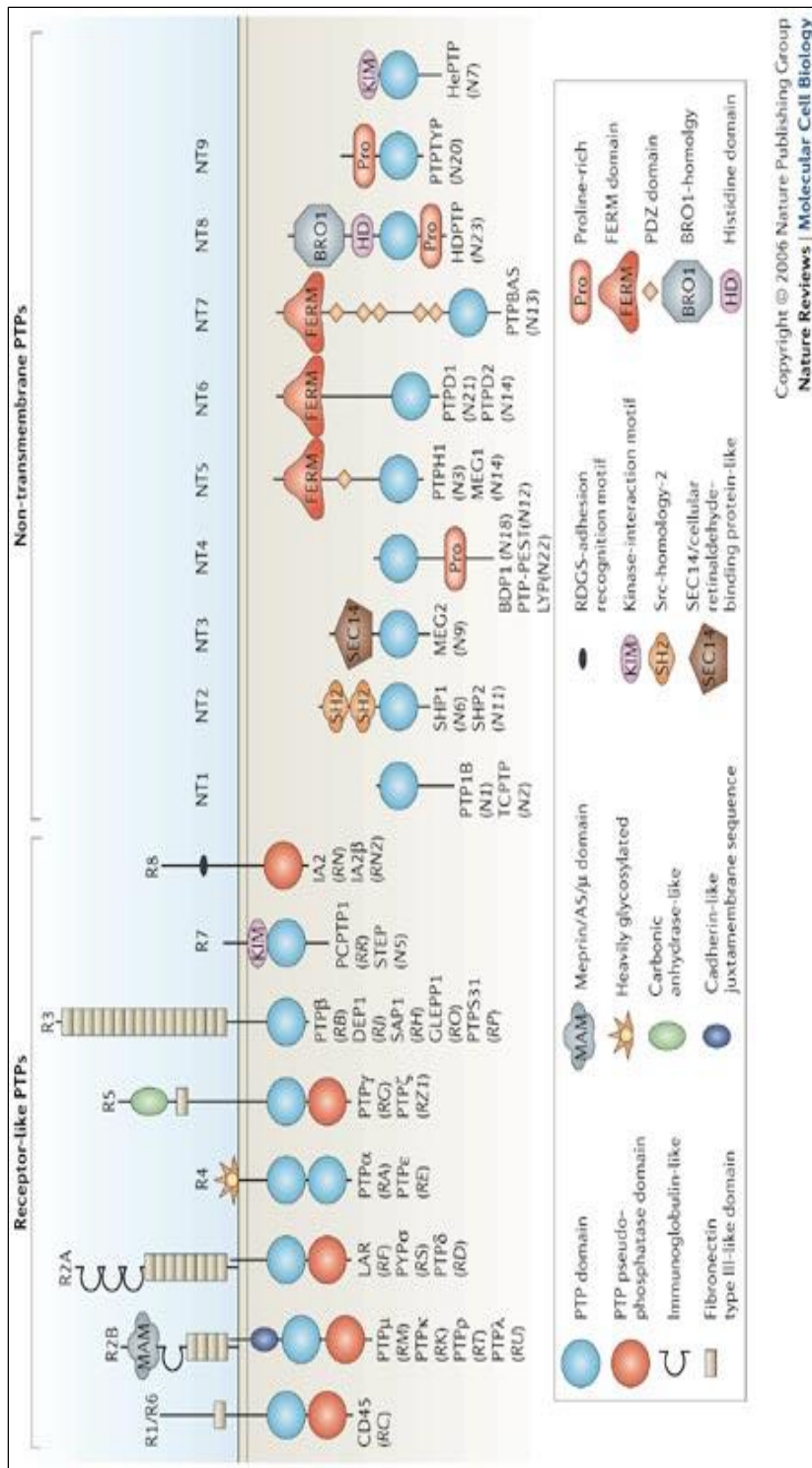
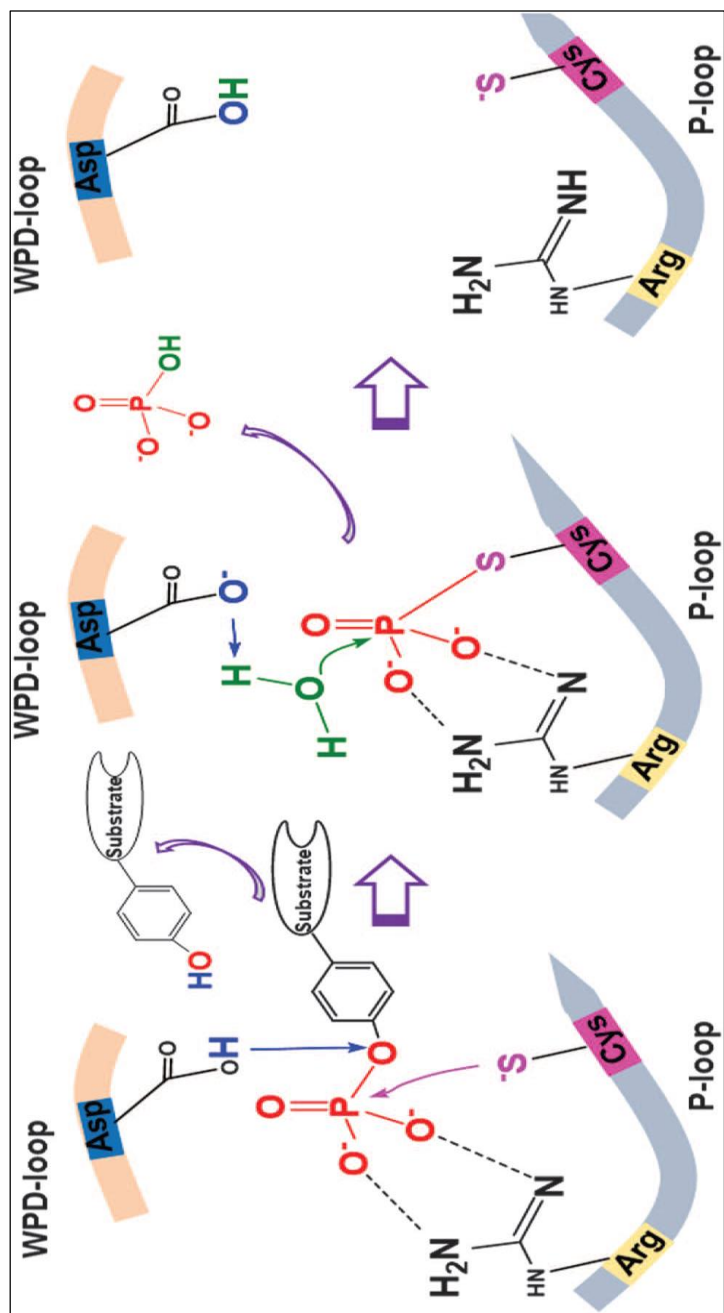*Tonks et. al; Nature Reviews Molecular Cell Biology* **7**, November 2006

Figure 2.1 Classical Tyrosine Phosphatase family and its structural diversity. Structural representation of receptor and non-receptor classical tyrosine phosphatases

Classical protein tyrosine phosphatase receptor typically comprises of a transmembrane domain that links the protein to the plasma membrane of cell. This domain is often attached to an extracellular domain, which is receptive to external signaling via specific molecules. It regulates cell signaling via substrate protein tyrosine de-phosphorylation, triggered by receptor – ligand interaction. The cytoplasmic chain consists of tandem repeats of the PTP domain (1). However, in most classical receptor type enzymes, the catalytic function is carried out by the PTP domain proximal to membrane whereas the distal domain has been proven critical for intracellular protein – protein interactions (1, 6, 7). Several of the enzymes belonging to this class have been associated with cell- cell adhesion(8) and cell- matrix interactions (1). Dimerization of the protein tyrosine phosphatases receptor followed by ligand interaction has been implicated in regulation of enzyme activity (1, 9).

Protein tyrosine phosphatase non receptor type comprises of regulatory units flanking the enzymatic domain (1). These flanking regions are responsible for substrate specificity and subcellular localization of the active protein thereby assisting in catalytic de-phosphorylation (1). SHP1, PTPN23 (HD-PTP) and PTP1B are some of the most well characterized non-receptor PTPs. For example, SH2 domain in SHPs directs the protein towards target substrate tyrosine sites for phosphorylation (10). Similarly, FERM domains target the PTPs to interface between plasma membrane and cytoskeleton (11) and BRO1 domain targets proteins to endosomes (12). Apart from stringent subcellular targeting, these PTPs show high sequence identity and non-redundant functional specificity (5).

PTP function and regulation of enzymatic activity

PTPs are known to negatively regulate tyrosine phosphorylation, thereby terminating the signaling cascade. This enzymatic activity is essential to balance phosphorylation dependent molecular signaling, in order to maintain cellular physiology. With the advances in science, significance of these proteins has been emphasized in regulation of cellular metabolism and cell cycle. General catalytic reaction of PTPs is aptly described in figure 2.2. Cysteine residue in the catalytic domain functions as a nucleophile and the attacks the phosphate group on substrate tyrosine residue to break the phosphorus–oxygen bond. The aspartate in the WPD loop acts as a generate acid and donates a proton to the dephosphorylated tyrosine (4). This generates an unstable phosphocysteine intermediate and the dephosphorylated substrate is released (4, 13). The phosphocysteine intermediate is cleaved via hydrolysis by the aspartate residue and results in the release of free phosphate (4).

Figure 2.2. Enzymatic catalysis by classical tyrosine phosphatases. The cysteine (Cys) residue in catalytic domain acts as a nucleophile and attacks the phosphate group on substrate, whereas the Aspartate (Asp) residue behaves as a proton donor to mediate dephosphorylation of the target protein.

Zhao et al, Oncogene (2014)

Reactive oxygen species have been implicated in altering tyrosine phosphatase mediated signaling pathways, under normal physiology as well as in cancer cells (1, 14). The nucleophilic nature of cysteine residue within the active domain renders it susceptible to reversible oxidation by ROS thereby resulting in inhibition of the enzymatic action (15). Oxidation of nucleophile, cysteine brings about conformational changes that are non-conducive for substrate binding (15). Several ligand molecules are known to trigger reversible oxidation of receptor tyrosine phosphatase. For example, insulin, epidermal growth factor (EGF) and TNFα stimulate oxidation of PTP1B, PTEN and MKPs, respectively (1). Ligand interaction mediating dimerization of the receptors is also a proposed mechanism for regulation of receptor PTP activity. Crystallization studies have elucidated that such dimerization of RPTPs result in reciprocal occlusion of active sites, thereby inhibiting the enzymatic activity (9). However, only few ligands have been identified so far, in this context. For example, RPTPζ activity following binding of pleiotrophin is shown to result in increased phosphorylation of β- catenin, a known substrate of RPTKα (1, 9, 16).

According to current understanding of tyrosine phosphorylation mediated signaling, an external physiological stimuli triggers inhibition of the tyrosine phosphatases, thereby allowing amplification of the tyrosine phosphate mediated signaling (1). Loss of stimuli results in activation of the phosphatase and subsequent termination of the signaling pathways (1). Inhibition of phosphatase activity is complimentary to kinase activity that promotes tyrosine phosphorylation, and downstream signaling. Hence, an optimum balance between phosphatase and kinase activity is crucial for maintenance of normal physiology.

Identification of HD-PTP or PTPN23

First study that identified PTPN23 as HD-PTP (His domain containing PTP) was aimed at identifying phosphatases that play critical role in hypertrophy in cardiomyocytes. Among the 16 target phosphatases, HD-PTP was a novel discovery. Though the protein was observed to be ubiquitously expressed, distinctly high levels were detected in rat neonatal cardiomyocytes (17). PTPN23 is localized within cytoplasm and protein localization was found to be independent of the PTP domain (17, 18). Preliminary mRNA sequence analysis established the presence of a BRO1 domain, a yeast signaling protein, on the N terminus of translated protein (17). The identified HD-PTP cDNA sequence showed a variant from the generic catalytic site sequence of most PTP's identified to date. Specifically, serine residue was observed to be replaced by an alanine residue (17). However, the overall sequence was observed to be in synchrony with that of the phosphatases. This study also demonstrated that PTPN23 inhibits Ha-Ras mediated cellular transformation. When PTPN23 was overexpressed in Ha-Ras expressing cells, foci formation was significantly reduced by 33% (17). This was the first evidence implying a tumor suppressive role of PTPN23.

PTPN23 genomic cDNA sequence was first isolated in an attempt to characterize human genomic sequences that promoted anchorage independent growth of cells. These sequences were derived from B – cell lymphoma (18). Southern hybridization with human genomic DNA probe identified nine repeat regions (18). Each of these sequences was then employed for Northern blot analysis for identification of corresponding mRNA sequence (18). A common 6kb sequence was identified using three different probes (18). These probes were than utilized for screening human cDNA library obtained from tumor

cell line (18). Analyzing the identified mRNA sequence confirmed that the protein encoded by this gene belonged to the PTP superfamily, later identified to be PTPN23 (18). PTPN23 gene was localized on short arm of chromosome 3, specifically 3p.21.3 (18). This region is observed to be frequently deleted in many cancers including lung, breast, renal, urogenital and bladder carcinoma (19, 20), further implying PTPN23 as a tumor suppressor gene. Alterations within the sequence were mostly restricted to the substrate-binding domain, indicating altered substrate specificity.(18)

PTPN23 structure and expression pattern

Initial bioinformatics analysis revealed that PTPN23 protein is proline rich (17%) and lacks transmembrane domain as well as a unique localization peptide (17). The N terminus of PTPN23 also shows 45% similarity with BRO1 domain (17, 18). Literature confirms that BRO1 domain is homologous with a yeast signaling protein, involved in mitogen activated protein kinase signaling cascade. Detailed analysis also revealed presence of two SH-3 binding domains towards the C terminal end of BRO1 domain (17). This protein sequence was also found to be significantly similar to mouse AIP1, an apoptosis regulatory protein. The C terminus of PTPN23 carried an amino acid sequence similar to a known 'PEST' protein destabilizing sequence, indicative of a very short half-life of this protein (17, 18). Thus the PTPN23 protein structure can be explained as follows: a functionally uncharacterized BRO1-like domain (BRO), a histidine-rich domain (HIS), a classical protein tyrosine phosphatase domain (PTP) and a proteolytic degradation targeting sequence (PEST motif). Below is representation of PTPN23 structure (21).
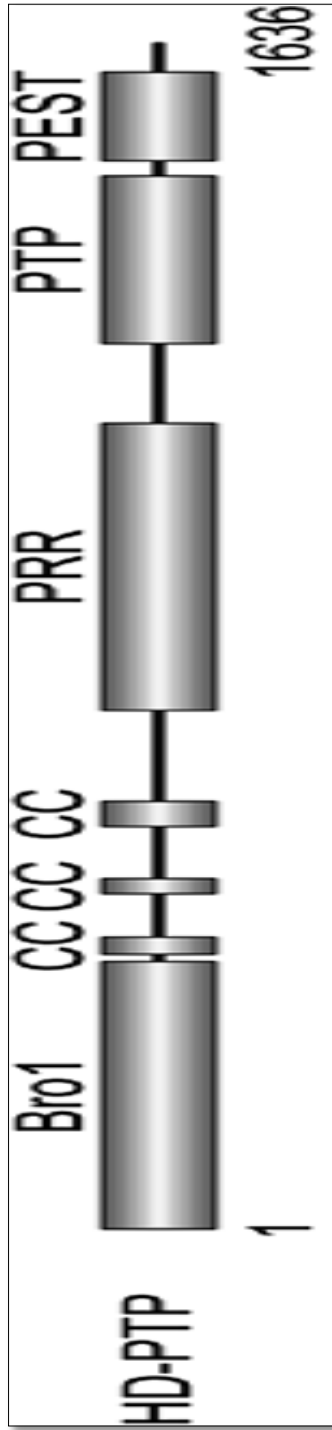
Figure 2.3 PTPN23 protein stricture. PTPN23 comprises of four important domains, from N terminus to C- terminus - a yeast signaling domain, BRO1, Histidine and proline rich, a catalytic phosphatase domain and PEST domain

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Though in vitro biochemical assays indicate a lack of phosphatase activity, an in vivo loss of PTPN23 mouse models, provides insight into the crucial role of this protein in mammalian development. PTPN23 gene was disrupted by insertion of β-galactosidase-neomycin- phosphotransferase II (β-geo) DNA sequence (22). Such insertion resulted in loss of wild-type PTPN23. This protein was found to be essential for mouse embryological development and loss of 3′ region of PTPN23 was embryonically lethal at E8.5 (22). Expression analysis of WT mouse showed distinct expression of PTPN23 in neurological tissues of an E9.5 embryo, specifically within neuroepithelial lining of midbrain, hindbrain, inner side of optic and ottic vescicle and ventricular area of forebrain (22). At E12.5, PTPN23 expression is more pronounced encompassing more organs including tongue, heart, gut, common bile duct, precursor stomach, lung, bronchia and vertebral column (22). With advancing embryonic age, PTPN23 expression was observed to be more cell-specific, ubiquitous but variable (22). Extended expression analysis in adult tissues revealed similar pattern, conserved in adulthood as well, although loss of expression was observed within spleen, heart and muscle tissue (22). Expression of PTPN23 in adult mammalian skeletal tissue is controversial, with two independent studies showing distinctly high to low levels of mRNA, respectively (17, 22, 23). Another study affirms the fact that particularly high levels of PTPN23 are observed in brain and kidneys of fetal tissue (17, 23). Negligible to very low levels of PTPN23 transcript was detected in liver tissue (17, 23).

In vitro biochemical studies reported that PTPN23 lacks catalytic phosphatase activity, and the specific amino acid substitution is implicated to be the causative factor (17, 24). Although, later studies have identified Src Kinase to be a direct substrate of the

phosphatase activity of PTPN23 (25). At the same time, role of PTPN23 in endosomal cargo sorting and epidermal growth factor receptor (EGFR) transport to lysosomes is well documented, apart from its effect in several oncologically relevant cellular functions. It was observed that RNAi mediated knockdown of PTPN23 caused disruption in recycling EGFR to lysosomal machinery and accumulation of ubiquitinylated proteins on endosomal compartments (26).

Mutations in PTP genes are associated with a variety of diseases

Reversible phosphorylation of tyrosine residues of proteins has been studied as a popular signaling mechanism under physiological conditions. Phosphatases regulate levels of phosphorylation and their mode of action is well conserved in eukaryotes, highlighting their significance in normal development and physiology. Research has demonstrated role of phosphatases in a range of molecular pathogenesis, including developmental disorders, autoimmune reactions and cancer.(2, 4) Deregulation of phosphatase function in association with human diseases has been studied in great details.

Loss-of-function as well as gain-of-function mutations in PTP genes have been reported in reference to human diseases. Several single nucleotide polymorphisms in PTP genes are shown to be associated with diseases, though underlying molecular mechanisms are not studied yet. A single nucleotide insertion within the 3′UTR of PTPN1 leads to increased mRNA stability and subsequent overexpression of PTPB1 protein (27). PTPB1 dephosphorylates and inactivates insulin receptor, thereby increasing insulin resistance, a trait commonly observed in type 2 diabetes mellitus patients (28). Studies in knockout mice models have demonstrated that loss of PTPB1 protein confers resistance to high fat diet induced diabetes and obesity (28) A study has also shown association between a

polymorphism in PTPN1 intron 6 and body fat distribution and diabetes mellitus type 2 (29). PTPN2 is important in immune regulation and has been identified as a susceptibility locus in inflammatory bowel disease as well as autoimmune reactions.(2, 4) SNPs in PTPN2 are associated with autoimmune disorders such as Rheumatoid arthritis (30, 31) whereas loss of PTPN2 is also observed in a subset of leukemia cases therefore implicating its role as a tumor suppressor (32). SNPs causing increased expression of STEP (Striatal enriched protein tyrosine phosphatase), encoded by PTPN5 and expressed in a brain specific manner, has been associated with increased susceptibility to cognitive disorders such as hereditary schizophrenia(33) SNP causing reduced PTPN12 expression is embryonically lethal, so is a gain of function mutation in PTPN11. Similarly, SNP resulting in partial loss of function of PTPN12 is associated with increased risk of developing breast cancer (34). A range of mutation in PTPN11 are linked to several diseases, from autosomal dominant dysmorphic Noonan syndrome to leukemia and autoinflammatory Lupus erythematosus (35, 36). SNPs in PTPN13 are studied in association with familial hepatocellular carcinoma, lung carcinoma and colorectal cancer (37, 38). A SNP in PTPN22 gene (p.R620W) resulting in amino acid substitution (arginine to tryptophan) is associated with increased risk of autoimmune disorders such as Graves' disease and Rheumatoid arthritis (39). Broadly, non-receptor PTPs are implicated in inflammatory disorders, psychiatric disorders, metabolic disorders such as type 2 diabetes mellitus and cancer predisposition.

In case of receptor PTP, mutations0 have been linked to a similar array of disorders. For example, mutations in PTPRA, PTPRC and PTPRD are associated with Schizophrenia, Severe Combined Immunodeficiency and renal cancer, respectively.(2, 4) These

comprehensive analyses underline the role of tyrosine phosphatases in maintenance of cellular functions, its association with human pathologies as well as its significance in developing therapeutics.

Role of PTPs in cancer

Due to their integral role in cellular signaling, PTPs are strongly suggested to be involved in cancer development and progression. Early attempts to study role of PTPs in cancer were focused on colorectal cancer. Research identified deregulation of PTPRT, PTPRF, PTPRG, PTPN3, PTPN13 and PTPN14 in approximately 26% of the CRC cases (40). A more recent study has identified PTPRT, PTPRC, PTPRD and PTPRM as tumor suppressors in head and neck squamous cell carcinomas (41). Detailed information about PTP mutations in human cancer is now available via high throughput sequencing analyses. Protein tyrosine phosphatase receptor T (PTPRT) manifests missense mutation in multiple cancers such as colon, bladder, endometrium, esophagus, head and neck squamous carcinoma, lung and stomach (40, 41). Mutations resulting in truncated protein product are also widely observed in human tumors, suggesting a putative tumor suppression function of this protein (40). Studies on PTPRT knock out mice have shown early development of carcinogen induced colorectal tumors (42). Loss of PTPRT due to promoter DNA methylation is also reported in gastric and colon cancers. STAT3 dephosphorylation is proposed to be the underlying signaling mechanism, mediating tumor suppressive function of PTPR (42).

Genetic deletions and promoter DNA methylation of PTPRD gene, resulting in loss of phosphatase activity has been observed in multiple cancers, indicating tumor suppressive function. Mutations in this gene have also been observed in glioblastoma multiforme,

neuroblastoma, head and neck, lung and colon cancers (43, 44) PTPRD knock out mice manifest enhanced gliomagenesis whereas overexpression of this protein in colon cancer cells inhibited migration, implying its role in metastasis (44).

PTPRK, known to promote cell adhesion, migration and proliferation via EGFR signaling,(45, 46) is frequently mutated in breast, cervical, colon, endometrial and skin cancers as well as glioma and lymphoma. It is also known to dephosphorylate Her2, subsequently inhibiting growth of breast cancer cells. Almost 26% of angiosarcoma cases manifest nonsense or frame-shift mutations in PTPRB gene suggestive of its role as tumor suppressor (47). Knock out of PTPRB is embryonically lethal, emphasizing the importance of this protein in angiogenesis and development (48).

PTPRJ has been implicated in breast, colon, pancreatic and thyroid cancer (49-51). Intra-(52)injections of PTPRJ conjugated adeno-associated virus suppresses growth of pancreatic cancer cell xenografts (53). PTPRM inhibits proliferation, invasion and migration in breast, prostrate and brain tumor (54). Proteolytically cleaved products of this protein have been observed in tumor samples (55). These fragments are found to translocate to nucleus, where they are hypothesized to mediate cancer specific functions (55). Exact molecular mechanisms underlying this hypothesis have not been elucidated.

Among non-receptor PTPs, PTPN11 is a rare PTP to function as an oncogene in juvenile myelomonocytic leukemia, acute myeloid leukemia . Germ line gain of function mutation in PTPN11 was first observed in Noonan syndrome cases (52). Contrarily, PTPN11 is also demonstrated to act as a tumor suppressor in hepatocellular carcinoma by a recent study. Liver specific knockout of PTPN11 in mouse model resulted in increased susceptibility to carcinogen-induced HCC (56). PTPN11 is also suggested to be a tumor

suppressor in metachrondomatosis (57). Loss of function of PTPN11 have also been implicated in other diseases such as Leopard syndrome (58). At cellular level, it has been shown to regulate several critical signaling pathways including Jak/Stat, PI3K/AKT, NFκB and Ras-ERK MAPK.

PTP1B, encoded by PTPN1 is overexpressed in ~72% of breast cancer cases (58). Protein levels of PTP1B are correlated with Erbb2 expression and known to activate c-Src and Ras signaling (58). PTPN1 is also implicated in tumor suppression, according to genome wide sequencing. It is mutated in ~20% Hodgkin's lymphoma and primary mediastinal B cell lymphoma cases (59). Most of the identified mutations are loss of function, and few of them are non-sense or missense mutation, parallel with the tumor suppressive function (59). PTPN1 dephosphorylates insulin receptor and JAK2, thereby negatively regulating JAK-STAT and PI3K/AKT signaling (28) PTPN1 knockout mice demonstrate delayed Erbb2 induced breast cancer and decreased lung metastasis (60).

Mutations leading to loss of function of PTPN13 are identified in endometrial, lung, liver, breast and colorectal cancers (40, 61). In colorectal cancer, PTPN13 is identified as a substrate of mir200, known to promote EMT. It is also known to interact with Bcr-Abl, such that Bcr-Abl expression in myeloid progenitor cells up regulated PTPN13 protein levels and induce apoptosis (62). PTPN13 is known to regulate Adenomatous Polyposis Coli/ β-catenin pathway and PI3K/AKT signaling. Insulin receptor 1 and Ephrin B1 are identified PTPN13 substrates whereas phosphorylation of ERK and Her2/Erbb2 is inversely correlated with PTPN13 expression (63). Negative regulation of PI3K/AKT pathway via PTPN13 leads to apoptosis in breast cancer cells (64). Epigenetic silencing

of PTPN13 is observed in non-Hodgkin's and Hodgkin's lymphoma, breast, gastric and hepatocellular carcinoma (61).

PTPN14 is known to regulate β-catenin pathway(65), promoting cell adhesion, migration and cell growth. It was initially identified as a tumor suppressor in breast cancer, where it was observed to promote anchorage independent proliferation of mammary cells (65). Currently, it is established that PTPN14 mutations are associated with breast, colon and endometrium cancers. Point mutation at Y128 residue of p130Cas, a direct substrate of PTPN14 promotes migration, adhesion and tumorigenesis in colon (66). Dephosphorylation of this tyrosine residue results in slower growth of colon cancer xenograft tumors, reduced migration and colony formation as well as impaired anchorage independent growth (66). It is also observed to mediate epithelial-mesenchymal transition via TGFβ pathway regulation.

Significance of non-receptor PTPs in hepatic physiology and function

With increasing evidence emphasizing role of phosphatases and kinases in cellular functions, there have been some rigorous studies on elucidating role of PTPs in liver physiology. Liver is a critical link between metabolism and diseases and deregulation of hepatic functions can result in multifarious effects on normal physiology. Hence, molecular signaling pathways in liver are very tightly regulated and most of these mechanisms are dependent on tyrosine phosphorylation.

PTP1B, a non-receptor type PTP, is studied as a negative regulator of insulin receptor signaling and leptin signaling (67). Phosphorylation of IR is necessary to activate multiple downstream signaling molecules, such as AKT, mTORC and PI3K via

phosphorylation of IR substrates (67). PTP1B dephosphorylates IR and thereby inhibits the subsequent cellular signal transduction (67). Leptin, secreted by white adipose tissue, is expressed in proportion to body fat mass. It is a key signaling molecule to communicate with hypothalamus region of brain, in order to regulate fat metabolism (67). Interaction between leptin and its receptor is known to activate JAK2, thereby affecting the JAK-STAT signaling network. Dephosphorylation of JAK2 leads to immediate termination of Leptin signaling. PTP1B has been established to directly mediate dephosphorylation of JAK2 (67). Hence it is hypothesized that inhibition of leptin signaling, via PTP1B can be a potential mechanism to promote obesity. This hypothesis is consistent with the fact that global knock out of PTP1B in mice results in lower adiposity in spite of a high fat diet (67). Observations were concurrent with increased brown fat and lower leptin levels in serum. Improved glucose tolerance and lipid profile was observed in liver specific knock out mice (67). Lack of PTP1B activity also led to attenuation of endoplasmic reticulum stress (ER), a known cause of obesity (67). There is convincing evidence that PTP1B is a potential therapeutic target for diabetes as well as obesity. An independent study revealed that lack of PTP1B also inhibits insulin resistance and increase in body fat associated with aging (68). Non-significant increase in hepatic glucose production was observed in aged, PTP1B knock out mice, as opposed to that in wild type age-matched controls (68). Loss of PTP1B also prohibited hyperinsulinemia and enlargement of islets indicative of absence of age-related insulin resistance (68). Accumulation of white adipose tissue is linked to inflammation that can potentially progress into carcinogenesis. PTP1B knock mice not only demonstrated reduced adiposity, but also maintained normal tissue specific as well as serum levels of pro-

inflammatory molecules such as TNFα, indicating absence of inflammation (68). However, there was a marked increase in the levels of inflammatory molecules in age matched wild type mice, indicating inflammation in hepatic tissue of wild type mice (68). Elevated levels of adipose tissue are correlated with steatosis and inflammation in liver. These conditions give rise to steatohepatitis, culminating into cirrhosis and possibly, carcinogenesis. Loss of PTP1B also protected the hepatic tissue against obesity induced steatosis and inflammation by blocking activity of specific kinases (68). Hepatic insulin signaling was observed to be maintained at normal levels, with advent of age in PTP1B null mice (68). Under normal physiological functioning, hepatic insulin signaling declines with age. Insulin resistance, deregulated insulin signaling in liver, increased accumulation of white adipose tissue, elevated levels of pro-inflammatory molecules were all congruent with the observed elevation in PTP1B levels of aged mice, as compared to young ones (68). This was also affirmed by differential expression levels of negative regulators of insulin signaling pathway, in PTP1B null mice versus age-matched normal wild type mice (68). Researchers have also established that PTP1B levels are down regulated in most of the HCC cases. Lower PTP1B levels also correlated with overall survival rate of the patients (69). Lower PTP1B levels were thus proved to be associated with poor prognosis and survival prediction of patients (69). Tumor initiating cells are shown to have lower proliferative potential with higher PTP1B levels (69). PTP1B levels are also inversely proportional to β-catenin nuclear levels (69). Since Wnt/β-catenin is an important pathway mediating expansion of cancer stem cells, lower PTP1B levels cause increased tumorigenesis (69). Role of PTP1B in hepatocyte proliferation has been well studied. Lack of PTP1B activity results in increased EGFR-

and HGFR-mediated tyrosine phosphorylation, thereby activating Akt and ERK signaling pathways (70). Increased proliferation of PTP1B deficient hepatocytes and subsequent delay in termination of liver regeneration is observed in PTP1B null mice (70). This phenomenon was confirmed by correlation of multiple marker protein levels such as TGFβ and cyclin D (70). Thus, PTP1B is a strong candidate for developing molecular therapeutics in obesity, diabetes and cancer management.

A tissue microarray study on 42 HBV or HCV associated HCC samples, followed by gene expression assay identified PTPN4A3, also called phosphatase of regenerating liver-3 (PRL3) in correlation with poorly differentiated tumors, poor prognosis and survival chance and higher rate of HCC recurrence.(71) Twelve out of 13 hepatoma cells lines demonstrated lower PRL3 levels, confirming its potential role as a tumor suppressor (71).

Another non-receptor tyrosine phosphatase linked to obesity and cancer is PTPN6 (72). Heterozygote knockout mice of this gene manifest resistance to high fat diet induced hepatic stress and insulin resistance (72). Heterozygote PTPN6 liver shows normal insulin signaling and insulin sensitivity with high fat diet, as opposed to that observed in wild-type mice (72). Steatohepatitis was also augmented in heterozygote PTPN6 mice, with comparable cholesterol levels (72). Loss of PTPN6 results in increased activity of SREBP1 (sterol-regulated element binding protein 1) and other lipogenic enzymes such as fatty acid synthase (72). Expression of CD36 and FABP5 (Fatty acid binding protein) increases in PTPN6 knockout mice, and subsequently results in increased hepatic uptake of fatty acids (72). PTPN6 heterozygote knockouts are also protected against hepatic inflammation, an important molecular stage allowing transition from steatosis to carcinogenesis in liver (72). This phenomenon was supplemented by no significant

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increase in inflammatory cytokines IL-3 and IL-6 after 16 weeks of high fat diet (72). Serum ALT and AST levels were also detected to be within normal range, thus indicating regular liver function (72). They also demonstrate significantly lower levels of hepatic gluconeogenesis as compared to the wild-type counterparts (72). PPARγ was overexpressed in primary hepatocytes of PTPN6 knock out mice (72). Global expression analysis revealed that loss of PTPN6 significantly impacts lipid metabolism, in accordance with the *in vivo* observations (72).

Shp2, an intracellular tyrosine phosphatase with Src homology-2 domain containing protein is encoded by PTPN11. Gain of function mutation in this gene has been observed in leukemia, establishing this protein as rare PTP oncogene (56). However, recently, PTPN11 has been implicated as a tumor suppressor in hepatocellular carcinoma (56). Reduced expression of this protein is also observed in 11.5% of HCC cases (56). Liver specific knockout of this gene caused increased DEN induced carcinogenesis in mice (56). This was supplemented by necrosis and infiltration of hepatic tissue with inflammatory cells, indicated by histological analysis (56). Inflammatory cytokines such as IL-6 and TNFα were also overexpressed in PTPN11 knock out mice hepatocytes (56). Splenomegaly and aggravated immunological response to LPS was observed in knock out mice compared to the wild type mice (56). Spontaneous hepatocellular adenoma development as a result of sustained STAT3 signaling was observed in knock out mice (56).

PTPN12 has also been studied as a tumor suppressor in hepatocellular carcinoma. Protein levels of PTPN12 are inversely correlated to tumorigenesis (73). Decreased PTPN12 levels also correlated with reduced patient survival rate and serum AFP levels (73).

Clinopathologic analysis established that PTPN12 levels were significant for prognosis in patients of HCC stage I/II and stage III/IV (73). To further emphasize the role of PTPN12, researchers proved that decrease in PTPN12 protein levels is concurrent with increased risk of recurrence and hence, reduced chance of disease free survival (73).

PTPN13 is recently studied as a mir200 target in hepatic tissue. Loss of PTPN13 results in augmentation of hepatic fibrosis, a key driver of hepatic carcinogenesis (74). These molecular events are promoted via deregulation of Src signaling cascade in hepatic fibroblasts (74). PTPN13 protein levels are also down regulated in hepatocellular carcinoma cell lines, transformed liver fibroblast cell line and 45.8% of HCC tumor tissues, as compared to normal hepatocytes or corresponding non-tumorous tissue (74). Accordingly, overexpression of PTPN13 is congruent what increased proliferation. Reduced expression is a consequence of promoter DNA methylation (74). Genetic alteration in PTPN13 gene is significantly associated with multiplex familial risk of hepatocellular carcinoma, strengthening its role as a tumor suppressor gene (74).

Role of PTPN 23 in cancer

PTPN23 gene is located at chromosomal position 3p21.3 and contains 25 exons as established in the preliminary studies (18). This region is highly susceptible to loss of heterozygosity mutations in cancer cells (18-20). Multiple mutations within the genes have been identified, though no single mutations are correlated with a particular type of cancer or tumor stage so far.

Significance of PTPN23 in neoplasm and cellular transformation was first studied in cardiomyocytes where it was observed to promote hypertrophy of cardiac tissue (17).

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This group of researchers were first to identify PTPN23 as a potential tumor suppressor. They observed that Ha-ras mediated foci formation was reduced by more than 3 fold if cells were transfected with PTPN23 (17). This observation required presence of the catalytic domain of PTPN23, since the mutant DNA vector, lacking this domain failed to inhibit cellular transformation (17). Although *in vitro* tyrosine phosphatase biochemical assays established that PTPN23 lacked enzymatic activity (17). However, later studies have identified Src- kinase and Focal adhesion kinase as direct targets dephosphorylated by PTPN23. Different studies have demonstrated that cellular transfection with PTPN23 DNA promotes anchorage independent cell growth and Ha-Ras mediated transformation (17).

PTPN23 is also known to inhibit endothelial migration (75). Angiogenesis, an important hallmark of cancer progression involves migration of endothelial cells from existing vasculature, into tumorous tissue to form new blood vessels. These endothelial vessels are essential to maintain high metabolism of cancerous cells. Molecular signaling cascades leading to endothelial migration as well as angiogenesis are tightly regulated by coordinated activity of kinases and phosphatases (76). Role of protein tyrosine phosphatases is emphasized by the fact that PTP inhibitors cause neovascularization *in vitro* as well as *in vivo* (76). This fact was consistent with the observation that knock down of PTPN23 protein levels in human umbilical vein endothelial cells promoted migration (75). Cellular phenotype was observed to change from rounded, epithelial to more taper-ended mesenchymal phenotype, with presence of lamellipodia and reorganization of actin cytoskeleton (75). Further, this study also established that Focal Adhesion kinase (FAK) interacts with PTPN23, as indicated by immunoprecipitation

analysis. FAK is crucial for formation of focal adhesions and is known to manifest phosphorylation dependent kinase activity (75). Endothelial migration is complimented by a dynamic, cyclic change in the cytoskeleton mediated by focal adhesion molecules and stress fibers. PTPN23 knock down was correlated with increased phosphorylation of FAK and specific localization of this protein, along the plasma membrane to allow formation of focal adhesions (75). Lack of PTPN23 activity produces results similar to those observed post 24-hour FGF treatment in these cells. Cells with high PTPN23 protein levels also showed cytoplasmic localization of FAK (75). Bioinformatics analysis demonstrates structural homology between PTPN23 and Alix/ AIP1, known to interact with FAK (75). Interestingly, PTPN23 also interacts with Src kinase (25). Src is a non-receptor kinase, working in conjugation with FAK to modulate turnover of focal adhesions. An independent study elucidated that PTPN23 regulates phosphorylation of Src and negatively regulates kinase activity (25). Immunoprecipitation studies confirmed PTPN23- Src interaction. Alix1, also known to interact with Src shares the biding domain sequence with PTPN23 (25). Researchers have also demonstrated that augment in migration of endothelial cells, upon inhibition of PTPN23 is mediated via Src kinase activity (25). PP2, inhibitor of Src kinase, reverses the scattered, migratory phenotype. Thus, PTPN23 inhibits Src kinase activity as well as FAK kinase activity, both of which are essential for membrane localization of FAK and subsequent formation of focal adhesions. Down regulation of PTPN23 reverses the molecular cascade, allows for FAK localization and thereby augments cellular motility and migration.

Magnesium is a known chemo attractant for endothelial cells, promoting migration. Treatment of Human vein endothelial cells (HUVEC) with increasing concentrations of

magnesium is correlated with reduced migration as well as down regulation of PTPN23 protein levels (77). These results reaffirm the significance role of PTPN23 in endothelial migration and angiogenesis.

Similar effects in cellular migration have been observed in bladder carcinoma. PTPN23 is shown to be inversely correlated with cell motility and migration. The research group confirmed that epidermal growth factor (EGF) not only enhanced interaction between PTPN23 and Src, but also observed mislocalization of Src within the cytosol in presence of PTPN23 (78). Src was found to be a key regulator of PTPN23 tyrosine phosphorylation, which subsequently controlled FAK phosphorylation status and its localization at focal contacts (78). A more recent finding has shown that loss of PTPN23 function bladder carcinoma is attributed to calcium dependent calpain degradation (79). The authors have shown that PTPN23 protein levels are in inverse correlation with those of calcium.

Another interesting observation in context of PTPN23-EGFR interaction was made by *Doyotte et. al*. This study demonstrated that loss of PTPN23 inhibits delivery of cell surface signaling molecule EGFR to lysosomal machinery (26). Significance of constitutive EGFR signaling is well described in literature. This observation can lead to the hypothesis that loss of PTPN23 results in deregulation of intra-cellular protein trafficking and EGFR signaling (25). Subsequently, due to lack of lysosomal degradation of EGFR, there is an increase in EGFR mediated cell signaling, promoting carcinogensis (49).

PTPN23 is studied to interact with Grb2 and GrpL proteins (80). These are adapter proteins belonging to the Grb2 family. It has been established that Grb2 family of

proteins co-ordinate a multitude of downstream signaling pathways that are relevant in carcinogenesis, from cell adhesion to invasion and metastasis (81). PTPN23 was found to be interacting with the adapter proteins via His domain, deduced by yeast two hybrid screening employing human colon cDNA (80). This finding further strengthens the evidence, implicating role of PTPN23 in cancer.

A wide scale loss-of-function screening of PTPs in breast cancer cell lines, employing RNAi-mediated down regulation, identified PTPN23 as a key regulator of cellular motility and invasion (13). Specifically, down regulation of PTPN23 leads to epithelial-mesenchymal transition of cellular phenotype (13). It is concurrent with increased expression of mesenchymal marker proteins and internalization of E-cadherin (13). These molecular events are supplemented by marked reduction in cell migration. The study also affirmed that Src kinase activity was essential to manifest downstream effect of PTPN23 depletion (13). In addition, authors identified β-catenin and E-cadherin as novel substrates of PTPN23 (13). Authors proposed that loss of PTNP23 in breast cancer cases might lead to increase Src kinase activity, subsequently causing increased phosphorylation of β-catenin/E-cadherin complex (13). Phosphorylation status of this protein complex is crucial in promoting proliferation, growth and invasive properties of breast cancer cells.

Though chromosomal deletion is a common mode of loss of PTPN23, a recent finding has suggested potential role of microRNAs and post-transcriptional RNA regulation in regulating PTPN23 protein levels. Studies in testicular cancer model have demonstrated that PTPN23 protein levels are negatively correlated with mir142, suggesting PTPN23 as putative microRNA target (13, 82). Further investigation revealed that PTPN23 functions

as a tumor suppressor in testicular cancer, inhibits colony formation on soft agar as well as tumorigenesis in xenografts (82).

In this study, we identified and analyzed SND1-mediated inhibition of PTPN23 expression in hepatocellular carcinoma.

Molecular therapeutic approach

With the advances in research and realization of molecular complexities of human disorders such as cancer, emphasis is being laid on developing molecular medicine that shows high specificity and increased efficacy. Molecules with unique structural feature make it possible to develop specific chemical inhibitors. This approach is especially critical for oncogenes, inhibiting their expression and downstream signaling. At the same time, tumor specific over expression of proteins that inhibit carcinogenesis allows therapeutic application of tumor suppressors via gene therapy. Viral constructs identifying and targeting strictly cancer cells are available and efforts are made in employing them for successful clinical trials with several known tumor suppressor genes. PTPN23, with a strong impact on cellular physiology could potentially be one such tumor suppressor that could be successfully employed in cancer management. Strategic chromosomal location, often deleted in human cancers, and literature underlining its significance in more than one cancer hallmarks, makes this protein a very promising candidate for gene therapy.

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CHAPTER 3

Identification and Understanding Role of PTPN23 in Hepatocellular Carcinoma

INTRODUCTION

SND1 regulates gene expression both at transcriptional and post-transcriptional levels. As a component of RISC, SND1 mediates RNAi-mediated mRNA degradation (1). However, SND1 has also been shown to bind to 3′-UTR of specific mRNAs, increase their stability and hence gene expression. SND1 binds to AT1R mRNA and increases AT1R mRNA stability (2) and we have shown that activation of AT1R-downstream signaling by SND1 also plays an important role in the activation of TGFβ signaling and EMT (3). We hypothesized that in addition to its RNAi function, SND1 might bind to additional mRNAs and modulate their half-life, either by increasing stability or directly degrading these mRNA. To identify these mRNAs, we chose to perform RNA immunoprecipitation study using anti-SND1 antibody employing a HCC cell line expressing high basal level of SND1. RNA sample was amplified and subjected to sequencing for identification of genes. From a milieu of genes, we chose nine genes that show 10 fold amplification in SND1 IP as compared to the control IgG IP. After initial expression screening, we focused on PTPN23 because of its role as a tumor suppressor (4). We hypothesized that SND1 might bind PTPN23 and degrade it and this downregulation of PTPN23 by SND1 might contribute to tumor promoting function of SND1. We also explored the potential role of PTPN23 as a tumor suppressor in HCC since PTPN23 has not been studied in HCC before. We designed our experiments to interrogate these questions.

MATERIALS AND METHODS

1. Cell culture

QGY-7703, Hep3B, Huh 7 and HepG3 human HCC cell lines were cultured in 10% FBS containing DMEM cell culture media (Life Technologies #12491-015, #11095-072), at 37°C in CO_2 incubators, under aseptic conditions. Hep3B clones stably overexpressing SND1 (H-17) and QGY clones with siRNA mediated stable knock down of SND1 (QGYsh2, QGYsh24) were also cultured under similar conditions (1). Transient transfection of QGY-7703 was carried out with pcDNA3.1-2Flag-PTPN23 construct. For experimental control, QGY-7703 were also transfected with empty vector pcDNA3.1, under similar condition. For colony formation assay, 2000 cells were plated in 6-cm dishes and colonies > 50 cells were counted after 2 weeks

2. RNA- immunoprecipitation and Sequencing

QGY-7703 was employed for immunoprecipitation, using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore EMD #17-700). Rabbit anti-SND1 (Sigma) was used as primary antibody. Extracted RNA sample was amplified and sequenced to identify target genes. Illumina TruSeq RNA sample preparation kit was used for preparing RNASeq library, which was sequenced on Illumina HiSeq2000 platform. RNA-Seq libraries were pooled together to aim about 25-40M read passed filtered reads per sample. All sequencing reads were aligned with their reference genome (UCSC human genome hg19) using TopHat2 and the Bam files from alignment were processed using HTSeq-count to obtain the counts per gene in all samples. The counts were read into R software using DESeq package and plot

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distributions were analyzed using Reads Per Kilobase Million (RPKM) values. Data was filtered based on low count or low RPKM value (<40 percentile). Pairwise tests were performed between each group using the functions in DESeq. Genes showing absolute fold-change of >2, False Discovery Rate (FDR) of <0.1 and p-value of <0.01 were selected. Gene cluster analysis was performed using bioinformatics tools for gene ontology such as KEGG.

3. Tissue microarray

Human HCC tissue microarray from Imgenex (IMH360) was used. For immunostaining, Rabbit anti-PTPN23 (Sigma Aldrich #HPA016845) was used at a concentration of 1:100.

4. Immunohistochemistry

Tissue sections of human HCC tumors and corresponding non-neoplastic tissue sections were obtained from Liver Tissue Cell Distribution System (LTCDS). Rabbit anti-PTPN23 (Sigma Aldrich #HPA016845) was used at a concentration of 1:100 as primary antibody. Standard protocol for immunohistochemical staining was followed.

5. Isolation of total RNA, cDNA synthesis, Reverse Transcriptase PCR and qRT-PCR

Total RNA was extracted from HCC cell lines or mouse tissues using the miRNAeasy Mini Kit (QIAGEN). cDNA preparation was done using cDNA Synthesis Kit (Applied Biosystems). Real-time PCR (RT-PCR) was performed using an ABI ViiA7 fast real-time PCR system and specific TaqMan gene expression assays according to the manufacturer's protocol. For RT-PCR, gene specific primers, spanning 300-400

base pair sequence were obtained from Nucleic acid Research Facility (NARF) at Virginia Commonwealth University. Standard PCR protocol was used.

6. Western blots

Cell lines described above, were lysed in 1.5% DDM lysis buffer. Protein sample was loaded at a concentration of 50μg/μl on 8% SDS-PAGE gel. Rabbit anti-SND1 (Santacruz Biotechnology Inc. #sc-67128), rabbit anti- PTPN23 (ProteinTech #10472-1-AP) and mouse anti- GAPDH (Santacruz Biotechnology Inc. #sc-166545) were used as primary antibodies at 1:1000 concentration, in 5% blocking buffer. Anti-Rabbit and anti- mouse were used as secondary antibodies, used at 1: 2000 and 1:5000 concentrations, respectively in 5% blocking buffer.

7. Synthesizing radio-labelled 3′ UTR-PTPN23 mRNA probe

3′ UTR sequence of PTPN23 was amplified by PCR and cloned into pGEMT-easy vector (according to manufacturer's protocol) such that target sequence was transcribed by T7 promoter (Primer sequence: Fwd; 5' acaggttttgcctacctggtc 3' and Rev; 5' acgggccacagaacagggt 3'). Plasmid was linearized using Sca1 restriction endonuclease, at 37°C. As a non-specific control, IGFBP-7 pGEMT-easy plasmid clone was linearized in a similar way. Linearized plasmids were used for *in vitro* transcription with T7 Maxiscript kit (Life Technologies # AM1312). In the presence of radioactive UTP labeled with ^{32}-P (Perkin Elmer) radiolabelled transcription products were gel purified on 4% non-denaturing gel and eluted in DEPC water at 37°C.

8. Binding assay

SND1 protein was synthesized *in vitro*, employing T7 translation kit (Promega # L1171). Protein sample was diluted 1:2 in Buffer D (20 mM HEPES, pH 7.0, 0.1 M NaCl, 3 mM MgCl2, 0.4 mM EDTA, 1 mM DTT, 20% glycerol.). SND1 extract was incubated with radiolabelled 3'UTR-PTPN23 mRNA, in presence of 10X Binding buffer (0.1 M HEPES, pH 7.0, 1 M NaCl.). Non-specific radiolabelled probe was used as control reaction. Reaction was carried out at 4°C for 2 hours.

9. Electrophoretic Mobility Shift Assay (EMSA)

Binding reaction mixtures were analyzed on a 4% non-denaturing gel. Gel was run at 200V for 1-2 hours, at 4°C. Gels were dessicated onto Whatmann's filter paper and analyzed by autoradiography.

RESULTS

1. Identification of target mRNAs interacting with SND1

With strong evidence suggesting SND1 functions as a global regulator of gene expression, the aim of this study was to identify mRNAs under direct regulation of SND1. This was aimed to understand molecular changes brought about by SND1 by modulating gene expression, specific to cancer. For this purpose, we chose to perform RNA immunoprecipitation sequencing (RIPSeq). RNA immunoprecipitation employing anti-SND1 revealed several target mRNAs to be significantly interacting with SND1 protein. Out of the total mRNAs sequenced, those with a Read per KiloBase Million (RPKM) value below 45th percentile were filtered out. RPKM value was calculated taking into consideration reads per gene and read length. Of the remaining mRNAs, only those showing significant enrichment in SND1 IP samples (S1-S5) in comparison to the control IgG IP (C1-C3) were considered for further analysis. Out of the total mRNAs sequenced, 370 were found to be significantly enriched in SND1 IP samples, implying putative association of SND1 protein to the respective mRNA transcripts. It was speculated that SND1 regulates expression of these genes at post-transcriptional level. Databases such as Gene Ontology Consortium were employed to identify biological processes that could be potentially regulated by SND1, depending on the mRNAs identified to interact with this protein. Critical processes implicated to be under direct SND1 mediated regulation include RNA processing and splicing, mRNA metabolism, cell junction organization and assembly, angiogenesis and vasculature development (Figure 3.1, 3.2). Bioinformatic tools such as KEGG were used for gene clustering and annotation study. Critical

pathways identified by KEGG analysis included ECM- receptor interactions, focal adhesion and pathways in cancer (Figure 3.1, 3.2). This data analysis helped us infer that SND1 affects molecular pathways, via regulation of global gene expression. It regulates expression of a milieu of genes, transcriptionally as well as post-transcriptionally and affects a variety of cellular processes altered in cancer. Based on the fold change value, we shortlisted nine genes (Table.1) showing at least a 10 fold enrichment in SND1 IP as compared to the control IgG IP. These genes were further analyzed to understand SND1 mediated regulation of the respective transcripts. For this purpose, mRNA levels of the nine genes were analysed in QGY-7703 cells and SND1 knock down clone in QGY (QGYsh24), employing Reverse Transcriptase PCR. Out of the nine genes, three genes (PCSK9, PTPN23, PNPLA7) negatively correlated and three genes (KCNJ, CELSR2, COL4A2) were in positive correlation with SND1 protein levels, whereas three genes (BCAM, AGRN, LAMB3) showed no change in mRNA expression (Figure 3.3). Since PTPN23 mRNA levels are upregulated in SND1 knock down clones, it was speculated that SND1 negatively regulates PTPN23 expression. With relevant literature, role of PTPN23 was further studied in detail with respect to HCC.
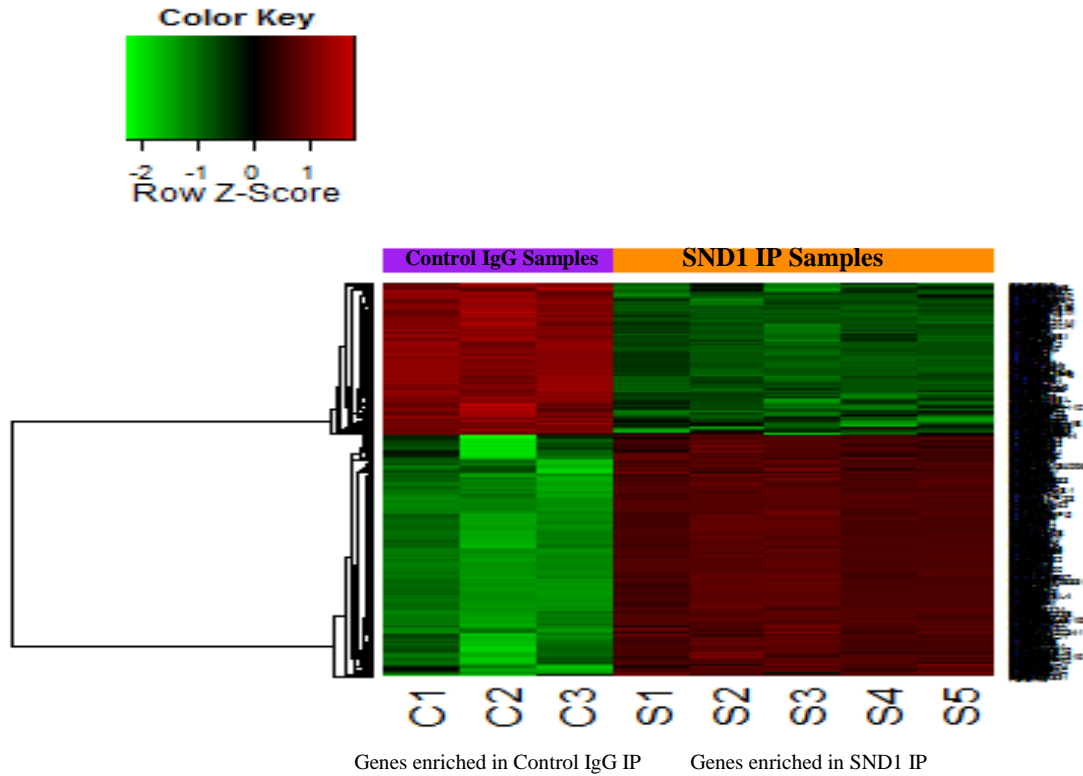
Figure 3.1. Heat Map of RIPSeq data. Heat map indicating the mRNAs enriched in SND1 IP samples (S1- S5) as well as the control IgG samples (C1- C3). A total of 370 mRNAs were significantly enriched in SND1 IP samples in comparison to the IgG IP sample. These mRNAs were then filtered based on fold change to shortlist few mRNAs significantly interacting with SND1.

Annotation Cluster 14	Enrichment Score: 2.03	G		Count	P_Value	Benjamini
GOTERM_BP_ALL	RNA splicing	RT		19	2.3E-3	8.8E-2
GOTERM_BP_ALL	RNA processing	RT		28	7.7E-3	1.9E-1
GOTERM_BP_ALL	RNA metabolic process	RT		41	1.5E-2	2.8E-1
GOTERM_BP_ALL	mRNA metabolic process	RT		20	1.6E-2	2.9E-1
GOTERM_BP_ALL	mRNA processing	RT		18	1.7E-2	2.9E-1
Annotation Cluster 15	Enrichment Score: 1.94	G		Count	P_Value	Benjamini
GOTERM_BP_ALL	cell junction organization	RT		7	7.0E-3	1.8E-1
GOTERM_BP_ALL	cell junction assembly	RT		6	7.3E-3	1.8E-1
GOTERM_BP_ALL	cell-substrate junction assembly	RT		4	3.0E-2	4.2E-1
Annotation Cluster 16	Enrichment Score: 1.84	G		Count	P_Value	Benjamini
GOTERM_BP_ALL	blood vessel development	RT		16	7.1E-3	1.8E-1
GOTERM_BP_ALL	vasculature development	RT		16	8.8E-3	2.0E-1
GOTERM_BP_ALL	anatomical structure formation involved in morphogenesis	RT		19	2.0E-2	3.2E-1
GOTERM_BP_ALL	angiogenesis	RT		10	3.4E-2	4.5E-1

Annotation Cluster 1	Enrichment Score: 6.82	G		Count	P_Value	Benjamini
KEGG_PATHWAY	ECM-receptor interaction	RT		23	5.3E-14	6.0E-12
KEGG_PATHWAY	Focal adhesion	RT		28	1.3E-9	7.3E-8
REACTOME_PATHWAY	REACT_13552:Integrin cell surface interactions	RT		15	2.5E-6	1.3E-4
KEGG_PATHWAY	Small cell lung cancer	RT		12	1.7E-4	6.3E-3
KEGG_PATHWAY	Pathways in cancer	RT		23	2.7E-3	4.4E-2

Figure 3.2 Biological Processes and pathways potentially regulated by SND1. mRNAs enriched in SND1 IP samples were found to be significantly involved in above mentioned biological processes, as studied using Gene Ontology Consortium database. KEGG analysis was used to identify major pathways in which the enriched 370 mRNAs were playing an important role. The listed pathways are potentially regulated by SND1, as indicated by KEGG analysis of sequencing data.

Gene Id	Control mean	SND1 IP mean	Fold Enrichment	P- value	Protein Function
BCAM	84.99	1426.40	16.78	1.73E-65	Cell adhesion
PCSK9	2.13	34.94	16.39	1.14E-09	Cholesterol homeostasis
AGRN	695.054	8559.479	12.314	2.21E-31	Cell – ECM interaction
KCNJ12	5.0861	58.448	11.491	9.43E-14	Senescence
PNPLA7	2.274	24.514	10.777	1.17E-05	Lipid metabolism
CELSR2	252.998	2604.322	10.293	1.60E-69	Circulating Lipid Levels
COL4A2	211.599	2141.286	10.119	6.18E-38	Metastasis
LAMB3	446.552	4520.898	10.124	1.30E-108	Cell adhesion
PTPN23	313.245	3144.123	10.037	1.56E-114	Migration/ Invasion

Table 1. A shortlist of nine mRNAs regulated by SND1. A list of nine mRNAs were shortlisted on the basis of fold change (>10 value) and significance level, for preliminary *in vitro* screening. Identified mRNAs were speculated to be under SND1 mediated post-transcriptional regulation.
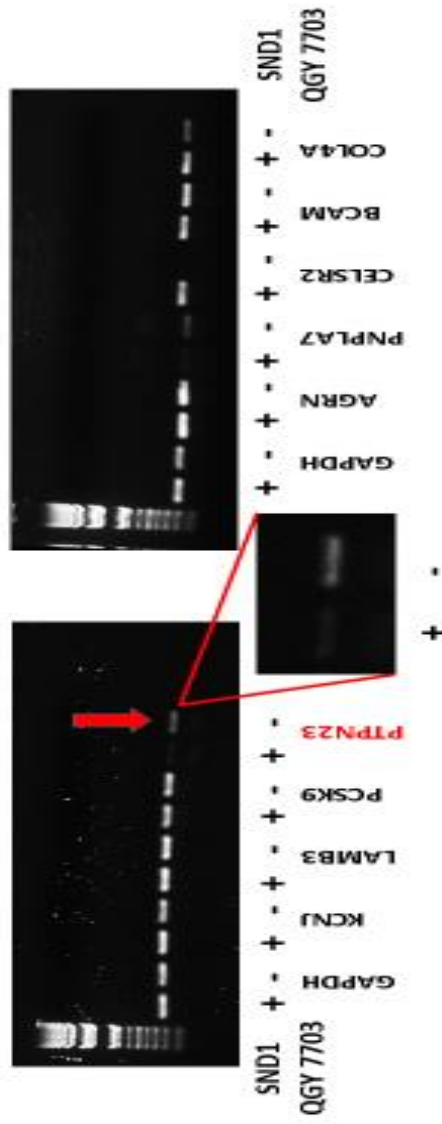
Figure 3.3. SND1 differentially regulates each of the nine mRNAs. Reverse Transcriptase PCR analysis was performed on RNA sample obtained from QGY-7703 cells and SND1 knockdown clones of QGY-7703 (QGYsh24) Primers spanning 300-400 bp region of each of the nine shortlisted genes were synthesized and used for PCR. LAMB3, AGRN and BCAM showed equal mRNA levels in QGY cells and knock down clones whereas KCNJ, CELSR2 and COL4A2 were downregulated in SND1 knockdown clones. PCSK9, PTPN23 and PNPLA7 mRNA levels were upregulated in SND1 knockdown clones.

2. PTPN23 is down regulated in HCC

With preliminary studies indicating downregulation of PTPN23 expression in HCC and thus suggesting a tumor suppressive function of this protein, it was important to observe PTPN23 expression pattern in HCC. Immunohistochemical analysis of PTPN23 expression was performed on a tissue microarray (Imgenex) of 38 hepatocellular carcinoma, 2 cholangiocarcinoma, 10 metastatic hepatocellular carcinoma and 9 corresponding non-neoplastic liver tissue samples. Approximately 40% of these cases demonstrated down regulation of PTPN23 levels (Figure 3.4). Immunohistochemical staining was also performed on human HCC sections with adjacent non-neoplastic tissue. Loss of PTPN23 expression was specific to neoplastic, highly proliferative tissue. PTPN23 expression was also corelated with SND1 protein levels in HCC cell lines, at mRNA as well as protein level. SND1 knockdown clones in QGY-7703 cell line showed higher PTPN23 mRNA as well as protein expression, in comparison to parental cell line (Figure 3.5a). SND1 knock down correlated with up to 3 fold increase in PTPN23 transcript level in comparison with parental QGY-7703 cells (Figure 3.5a) SND1 over-expressing clone (H17) in Hep3B cell line did not show significant decrease at mRNA level, though a marked reduction in PTPN23 protein was observed (Figure 3.5a-b). Overall, PTPN23 mRNA levels were observed to be lower in HCC cell lines Huh-7, HepG3 and QGY-7703 in comparison to normal hepatocytes (Figure 3.5a). Thus, SND1 protein levels negatively correlated with PTPN23 expression in HCC, at transcriptional as well as post-transcriptional level.
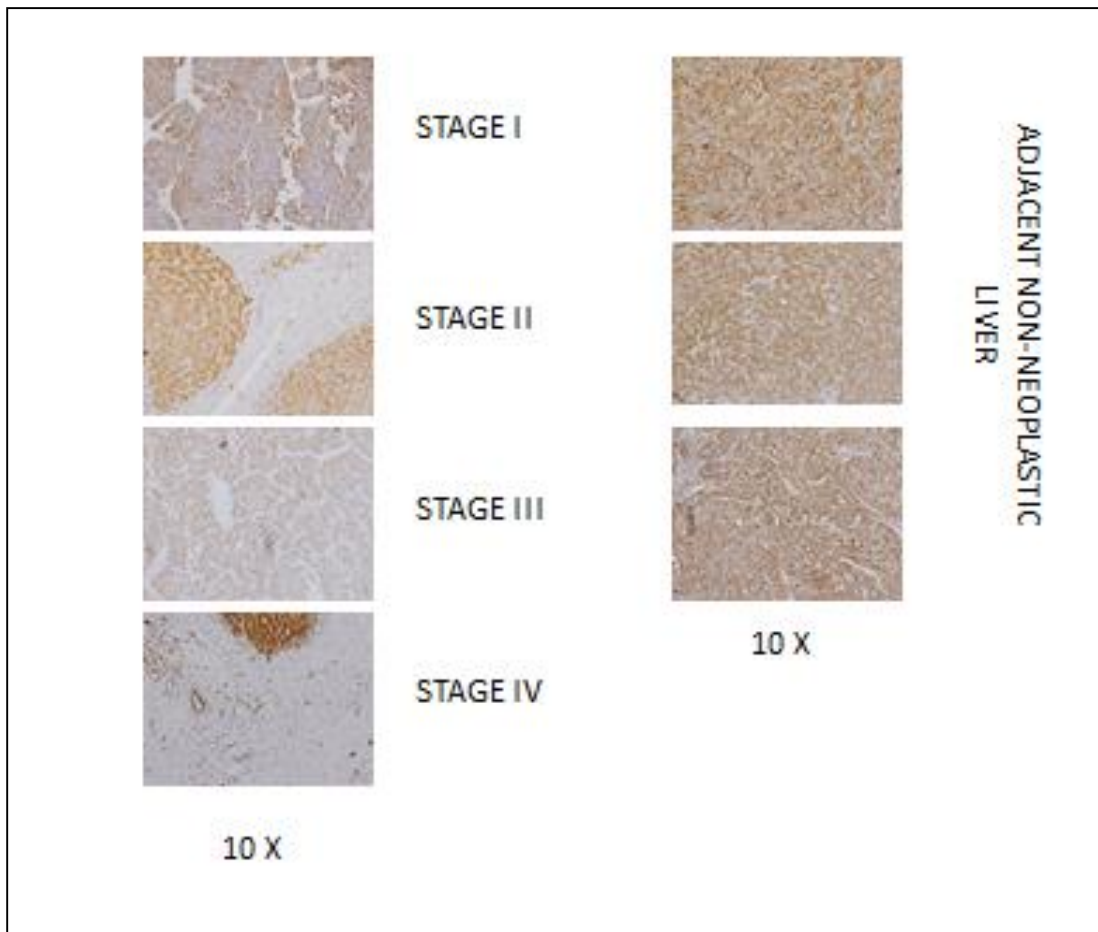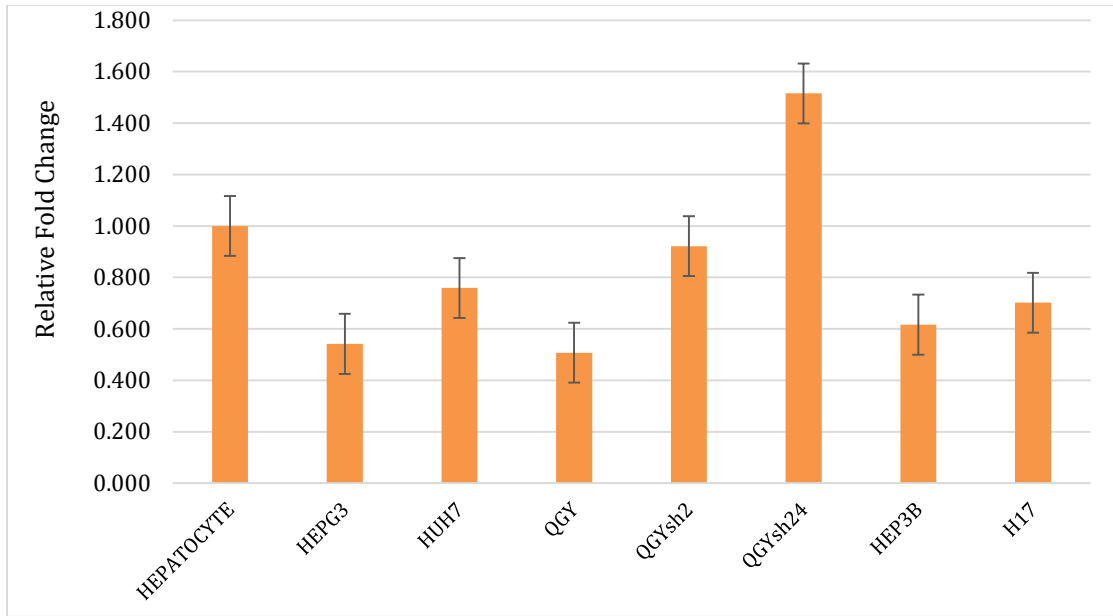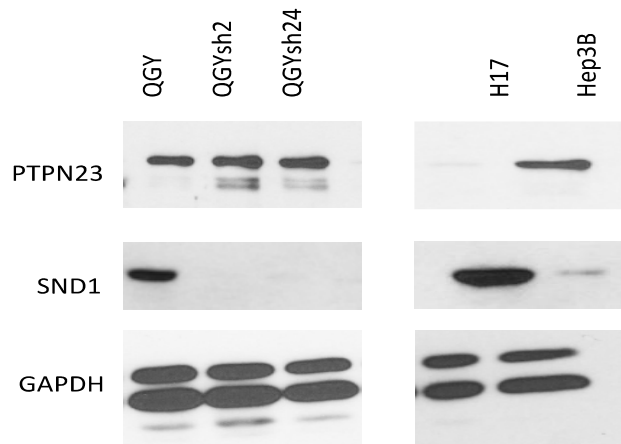
Figure 3.4 HCC tissue microarray. Immunohistochemical staining of a HCC tissue microarray was performed. Loss of PTPN23 protein expression was observed specifically in HCC tissue sections and compared to normal non-neoplastic tissue sections

(a)

(b)

Figure 3.5 SND1 protein levels negatively correlate with PTPN23 expression (a): Quantitative Real Time PCR analysis showed up to 2-fold decrease in PTPN23 transcript levels in human HCC cell lines (HepG3, Huh7, QGY, Hep3B) in comparison to normal human hepatocytes. SND1 knockdown clones (QGYsh2, QGYsh24) in QGY-7703 cells showed up to 3-fold increase in PTPN23 transcript levels in comparison to parental QGY-7703 cells. Not a significant difference is observed in PTPN23 transcript levels in SND1 over expressing clones (H17) and parental Hep3B cells.

Figure 3.5 (b): Western Blot analysis showed upregulation of PTPN23 protein expression in SND1 knockdown clones (QGYsh2, QGYsh24). PTPN23 protein levels were significantly reduced in SND1 overexpressing clone (H17) in comparison to parental Hep3B cell line.

2. PTPN23 inhibits cell proliferation in HCC cells

Since tyrosine phosphorylation dependent signaling is heavily implicated in cell proliferation and growth, it was hypothesized that PTPN23 regulates this cellular function. For this purpose, we performed colony formation assay in cells overexpressing PTPN23. QGY-7703 cells were transfected with pcDNA3.1-Flag-PTPN23 construct. Cellular proliferation as measured by Colony formation assay showed more than 90% decrease in the number of colonies formed by QGY-7703 overexpressing PTPN23, as compared to control QGY-7703 cells (Figure 3.6). Thus it can be inferred that over expression of PTPN23 in cancer cells might inhibit tumor growth *in vivo*, in HCC.
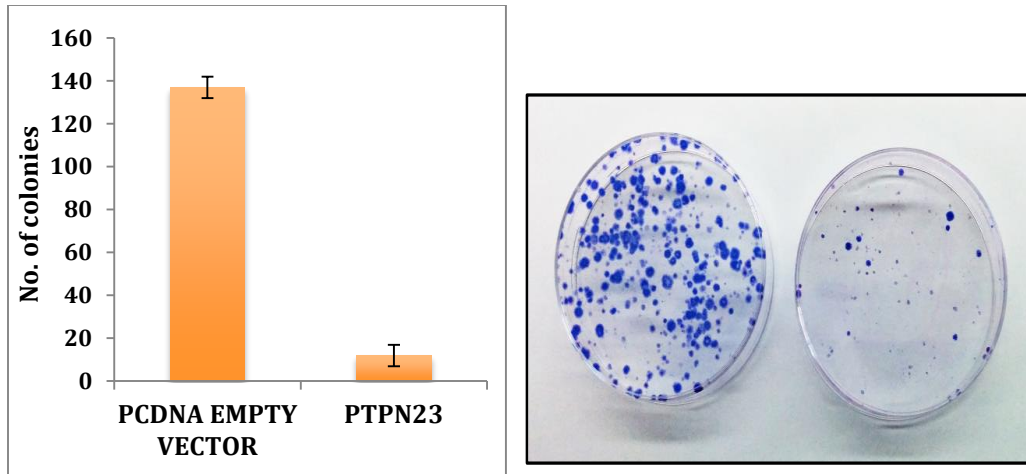
Figure 3.6 Overexpression of PTPN23 inhibits cellular proliferation in HCC. QGY-7703 cells were transiently transfected with PTPN23 pcDNA3.1 construct and empty vector pcDNA3.1 as control. Colony formation assay was performed with transiently transfected cells. PTPN23 overexpressing cells showed more than 90% reduction in colony formation.

3. SND1 protein binds to PTPN23 mRNA

Since RIPSeq does not affirm direct protein-RNA interaction and relevant literature indicating this interaction as a potential regulatory mechanism, it was necessary to observe SND1 and PTPN23 mRNA interaction *in vitro*. We performed an *in vitro* binding assay with SND1 protein and 32-^{P} labelled 3'UTR of PTPN23 mRNA, the results of which were analyzed using EMSA. This was aimed to help us understand how SND1 suppresses PTPN23 expression in HCC. Using T7 MaxiScript kit, radiolabelled 3′UTR PTPN23 mRNA was synthesized *in vitro*. The RNA probe was gel purified and utilized for subsequent binding assay. SND1 protein was also synthesized *in vitro* with T7 translation kit. Binding reaction was carried out at 4°C for 2 hours and the reaction mixture was analyzed by electrophoretic mobility shift assay. A shift in band position was observed when SND1 was incubated with 3′UTR PTPN23 (Figure 3.7). Furthermore, when reaction mixture was incubated at 37°C, no corresponding band was observed, indicating absence of radiolabelled mRNA, possibly due to SND1 mediated degradation (Data not shown). For further confirming specificity of SND1 protein binding to the 3′UTR mRNA sequence, binding reactions were subjected to RNase treatment at 37°C for 15 minutes and the samples were analyzed by EMSA. IGFBP7 transcript was not observed, whereas a clear band of 3′UTR transcript was seen by autoradiography (Data not shown). It was inferred that SND1 binds to the 3′UTR and therefore offers protection against RNase degradation. Thus, it can be hypothesized that SND1 binds to PTPN23 transcript at 3′UTR and possibly degrades it under physiological conditions, causing down regulation of protein.
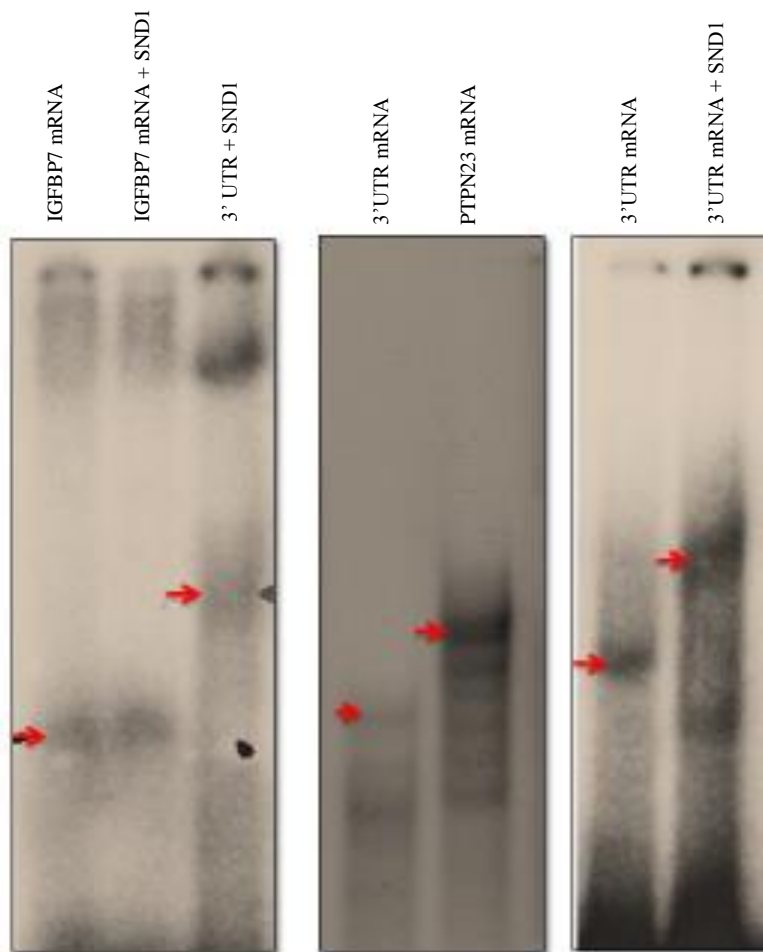
Figure 3.7. SND1 binds to 3'UTR of PTPN23 mRNA. 32-^{P} radiolabeled mRNA probes spanning 3'UTR of PTPN23 and IGFBP7 full length transcript (as positive control) were incubated with in vitro translated SND1 protein, at 4°C. Electrophoretic Mobility Shift Assay demonstrated a supershift in band position when 3'UTR of PTPN23 is incubated with SND1 protein, indicating a protein – mRNA interaction.

CHAPTER 4

Discussion and Future perspectives

DISCUSSION

Staphylococcal Nuclease Domain containing protein 1 (SND1) is crucial mediator of molecular events that culminate into carcinogenesis. Role of SND1 has been studied in multiple cancers as an oncogene, promoting proliferation, invasion, angiogenesis and EMT (5-9). In reference to hepatocellular carcinoma, we have previously demonstrated that SND1 promotes tumorigenesis using both *in vitro* assays as well as nude mice xenograft models (1, 3, 10). It was also established that functional role of SND1 in RNA induced silencing complex (RISC) is one of the critical mechanisms via which this protein regulates global gene expression (1). Specifically, we observed that SND1 down regulates expression of tumor suppressor genes such PTEN via RISC activity employing miR-221(1). There are several reports elucidating role of SND1 in regulation of gene expression at transcriptional and post-transcriptional level (2, 11, 12). Since SND1 shows RNA binding as well as differentially regulates expression of multiple genes employing a diverse array of functions, we were interested in identifying target mRNAs that are modulated by SND1 interaction, at a post-transcriptional level. The rationale was to understand SND1 mediated changes in gene expression and subsequent molecular pathways, in reference to cancer and inflammation. We employed RIPSeq approach for this aim and observed a vast number of mRNAs potentially interacting with SND1. In accordance to our hypothesis, RIP-Seq data identified multiple SND1 interacting mRNAs, mostly affecting RNA processing and splicing, mRNA metabolism, cell junction assembly and morphology, cell cycle and division, replication, vasculature development and angiogensis. The relevance of these pathways in tumor development, cell cycle and

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signaling further confirms the notion that SND1 mediates dynamic changes at molecular level, affecting cellular physiology. Such changes in global molecular signaling are key to development of cancer and hepatocellular carcinoma, in particular. It should be noted that the mRNAs identified in RIPSeq data are speculated to potentially interact with SND1 protein. However, the effect of post-transcriptional regulation by SND1 on each of them is independent and needs to be studied individually, *in vitro*.

We selected nine genes with a fold change value >10 and functional relevance for further *in vitro* analysis. Since SND1 is known to specifically down regulate or upregulate gene expression, it was necessary to understand SND1 mediated regulation of these nine genes. Expression pattern of these mRNAs were analyzed in correlation to SND1 protein levels, by Reverse Transcriptase PCR using QGY-7703 and SND1 knock down clone (QGYsh24). As expected, all nine mRNAs were found differentially correlate with SND1 protein levels. Three out of nine genes were up regulated whereas three were down regulated in SND1 knock down clones. Remaining three genes showed no change in expression in correlation to SND1 levels. This observation affirms the theory that SND1 modulates gene expression by more than one mechanism. There have been reports indicating increased mRNA translation conferred by stabilization of the transcript by SND1 protein. We observed that PNPLA, PCSK9 and PTPN23 are up regulated when SND1 is knocked down in QGY-7703 cells. Down regulation of CELSR, COL4A2 and KCNJ was correlated with low SND1 protein levels in knock down clones. This varied observation can be attributed to nuclease activity or increased mRNA stabilization and translation by

SND1. PNPLA7 is a phospholipase whereas PCSK9 is known to plays a crucial role in cholesterol homeostasis. It degrades LDL receptor expression, thus reducing cellular intake of LDL. SND1 mediated down regulation of PCSK9 is thus, in agreement with increased lipid uptake of hepatocytes, a condition characterized as steatosis that initiates inflammation and leads to carcinogensis. PCSK9 is currently being pursued as a potential molecular target for familial hypercholesterolaemia (13). CELSR2 is identified in a genome- wide meta-analysis study, to be significantly associated with increased risk for coronary artery disease and influencing circulating lipid levels (14). These findings suggest that SND1 might be a regulator of cholesterol metabolism. COL4A2 expression is altered and significantly associated with metastasis in laryngeal cancer (15). In epithelial ovarian cancer cells, COL4A2 upregulation mediates anoikis resistence (16). Interestingly, three genes BCAM, AGRN and LAMB3 did not show a difference in expression levels upon SND1 knock down. Detailed mechanistic analysis will help understand the role of SND1 in regulating the identified genes in the context of their functional pathways. It is also with relevant literature supporting its role as tumor suppressor, was the highlight of this study (4, 17, 18). PTPN23 is so far implicated as a potential tumor suppressor in breast cancer, where it regulates invasion (18) and testicular cancer, where it's expression is lost as result of miR142-3p mediated degradation (17). Preliminary investigation in HCC tumor samples by immunohistochemical approach reveal that PTPN23 expression is lost in HCC liver sections in comparison to non-neoplastic normal liver sections. Further analysis proved that SND1 protein levels negatively correlate with PTPN23 expression, at a transcriptional as well as post-transcriptional

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level. According to the results observed, one of the possibilities is that SND1 represses transcription of PTPN23 as a co-repressor protein. Hence, in SND1 knock down clones (QGYsh2, QGYsh24) we observe a 3 fold induction in PTPN23 transcript levels. An independent mode of regulation would be inhibition of translation of the PTPN23 mRNA. This possibility would explain the lack of difference in PTPN23 mRNA levels in SND1 overexpressing clones (H17) but a significant reduction in protein levels of the same clone. Although it is very critical to confirm the qRT-PCR analysis of PTPN23 mRNA levels in SND1 overexpressing clone (H17) and rule out any discrepancy. Especially since in subsequent experiment we find compelling evidence that SND1 might be degrading PTPN23 transcript, and this could also be a potential underlying mechanism of downregulation of PTPN23 protein in SND1 overexpressing (H17) cells.

Loss of 3p21.3 chromosomal location, housing PTPN23 gene has been associated with multiple cancers (19-21). Tissue microarray data, analyzing PTPN23 expression in normal and neoplastic liver tissue sections from HCC patients, helped in strengthening the tumor suppressive role of PTPN23. A marked decrease in PTPN23 protein was observed in tumor tissue as compared to adjacent normal tissue. Although, there was no correlation between PTPN23 protein expression and progression of HCC stages and metastasis.

Accordingly, overexpression of PTPN23 protein in a highly aggressive HCC cell line (QGY-7703) resulted in severe decrease in cellular proliferation. However, these results need to be confirmed in clones with inducible expression of PTPN23 and eventually in an *in vivo* model. Forced overexpression of PTPN23 causes severe cell

death (data not shown), emphasizing the necessity to establish inducible clones, which can be employed for long term *in vitro* studies. The exact mechanism, underlying reduction in proliferation remains to be studied. It will be interesting to understand whether this observation is result of increase in apoptosis or decrease in replicative potential of cells. Such studies shall be instrumental in understanding the molecular pathways under regulation of phosphatase activity of PTPN23.

In this study, we were successful in establishing the SND1- PTPN23 interaction *in vitro.* According to the observation by EMSA, in vitro translated SND1 protein binds to 3'UTR region of PTPN23 mRNA at 4°C, under conducive conditions. This binding interaction between SND1 and 3′ UTR of PTPN23 transcript was observed to be a specific event and presumed to lead to mRNA degradation. This assumption was based on the observation that the binding reaction mixture comprising of SND1 and 3′ UTR-PTPN23 transcript, incubated at 37°C, for 2 hours, showed no band in EMSA analysis (data not shown).

Studies so far have deduced that this phosphatase interacts with critical kinases such as Src and Focal Adhesion kinase (FAK) to inhibit cellular migration (22, 23). Initial studies have confirmed decrease in Ha-Ras mediated cellular transformation and proliferation, upon over expression of PTPN23 (4). There is also strong evidence implicating role of PTPN23 in endothelial migration regulating angiogenesis, migration of cancer cell lines as well as inhibition of invasion in breast cancer patients (24). These studies, emphasizing role of PTPN23 in manifesting hallmarks of cancer, indicate that reviving PTPN23 expression in cancer cells specifically, should

ameliorate disease condition. PTPN23 expression is expected to manifest as a protective mechanism against tumorigenesis in *in vivo* mice model.

Employing SND1 as a therapeutic target is at a risk of affecting global molecular networks, with increased chances of unfavourable downstream effects. However, it seems interesting to target SND1 specifically within tumor cells, and subsequent reduction in carcinogenesis. With a unique Tudor-SN fusion protein domain, specificity and high efficacy drug targeting is not a far reaching goal. Loss of SND1, an anti-apoptotic protein is expected to induce wide scale cellular death. It should also allow up regulation of tumor suppressors, which subsequently induces cell death. If targeted restrictively to cancer cells, SND1 is a very promising candidate in molecular drug development.

Alternatively, specific relevant downstream targets of SND1 would be potential molecular therapy candidate genes. Due to high significance studies proving role of PTPN23 as a tumor suppressor gene, testing its therapeutic potential on liver specific SND1 transgenic model or a xenograft study with adeno-associated viral construct of PTPN23 seems promising.

According to current findings, SND1 and PTPN23 are crucial for HCC development as well as progression. Pursuing detailed studies on understanding function of PTPN23 in cancer, specifically HCC, employing *in vitro* studies with stable clones and eventually an *in vivo* model, would be an interesting future direction.

CONCLUSION AND FUTURE DIRECTION

SND1 is known to orchestrate a series of changes that affect global gene expression and brings about cellular transformation. It regulates gene expression employing multiple unique mechanisms that function at transcriptional as well as post-transcriptional level. With increasing relevance of studies on SND1 in the context of carcinogenesis it is important to elucidate the molecular mechanisms underlying SND1 activity. Studies so far suggest it is a promising molecule for clinical investigation and targeted therapeutic management of cancer. Considering the pleiotrophic functions of SND1, one important question that has not been addressed is that how important is SND1 for maintaining normal physiological function including growth and development. An SND1 knockout mouse, conditional and global, will not only provide comprehensive insights into the physiological functions of SND1 but also will be an ideal model to interrogate the role of SND1 in immortalization, transformation, metastasis and overall cancer development and progression. Conversely, organ-specific SND1 transgenic mouse will also provide useful insight into the oncogenic function of overexpressed SND1. Is SND1 overexpression alone sufficient for transforming normal cells into cancer cells? Can SND1 function as a driver for tumorigenesis or is it a promoter following initial mutagenic events? Which particular aspects of SND1 function are most relevant to confer its oncogenic properties? Future studies need to be focused toward these angles.

PTPN23 is a potential discovery that can be pursued for gene therapeutics. Inducing PTPN23 expression in cancer cell specific manner is expected to inhibit tumorigenesis by reducing cell proliferation, migration and increasing apoptosis.

Whether these *in vitro* findings remain true under *in vivo* conditions needs to be elucidated by over expression of PTPN23 in a HCC mice model. Though embryological expression patterns of PTPN23 have been elucidated, its role in maintenance of normal physiology is not known. Hence, a global knock out or liver specific knock out of this gene will illustrate its role in maintaining normal physiology as well as its tumor suppressor functions. Very little is known so far about this protein in normal hepatic physiology, and it would be an imminent question to be answered before employing this protein in molecular medicine. First and foremost, we need to understand the signaling pathways that are under direct regulation of PTPN23. For this purpose, it is necessary to identify the phosphorylated tyrosine substrates of the phosphatase activity of this protein.

Another important discovery in this study is PCSK9, a known regulator of plasma LDL levels. PCSK9 is known to bind to LDL receptors, mediate their degradation by endocytosis thereby causing hypocatabolism of cholesterol in liver and increased levels of circulating LDL. Decreased PCSK9 levels correlate with increase in LDLR activity and subsequent increase in uptake of LDL in human hepatocytes, such as HepG2 and Huh7 (25, 26). Such an increased uptake of LDL within hepatocytes leads to hepatosteatosis, which is expected to initiate inflammatory response that progresses to HCC. Currently, PCSK9 is also being employed for therapeutic targeting for developing therapies against familial hypercholesterolaemia (13). Since steatosis induced cirrhosis is one of the major conditions causing HCC development, it would be interesting to study SND1 mediated down regulation of PCSK9 and subsequent impact on hepatic physiology.

There are several other mRNAs identified in RIPSeq analysis, which are implicated in lipid metabolism and cholesterol homeostasis, affirming that SND1 might be closely regulating these biological processes.

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