Analyses of role of SG2NAs in cell cycle progression

Thesis submitted to School of Life Sciences, JNU for the partial fulfillment of the degree of M. Phil

2012

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CERTIFICATE

The research work embodied in this thesis entitled **"Analyses of role of SG2NAs in cell cycle progression"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. This work is original and has not been submitted so far, in part or full, for the award of any degree or diploma of any university.

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July 2012

To my family....

Contents

Acknowledgements

I would like to express my gratitude to all who have helped me in making this work of mine a reality.

First I would like to thank Almighty for blessing me with such a loving, caring & extremely beautiful family.

I owe my debt of gratitude to my supervisor Prof. Shyamal K. Goswami for providing me an opportunity to explore the world of science. Thank you Sir for your constant support & motivation. I admire your patient, caring & understanding nature.

I thank the current Dean, SLS, Prof. N.B. Sarin & previous Dean Prof. R. Madhubala for providing me all the necessary infrastructure & facilities in SLS. I also extend my gratitude to Prof K.C. Upadhayay for his valuable advice at the time when I joined the SLS, the time when I needed it the most.

It is truly an impossible task to pen down my feelings towards my friends & labmates, OK..... let me try..... I am really happy to be a part of lab#220. I express my gratitude towards all the labmates Anitadi, Indranidi, Ekta ma'am, Pooja di, Goutam sir, Santosh sir, Sangeeta ma'am, Jahangir sir, Nikhat, Buddhi, Neetu & Sagrika. My heartfelt thanks to "my Goutam Sir", frankly I do not know how should I write, I am not exaggerating, but truly 51% of this thesis belongs to you. With so many silly questions & stupid answers you have really bare me two years. Thanks for shouting & yelling at me, because it shows that you care, although care (shouting) was a bit more but that makes me feel important. Thanks.....

Now, one of the sweetest girls I have ever met Pooja di, ever ready to help every one. Thanks is a very small word for your loving & affectionate nature, You make me feel as if we are family. Indrani di , my first mentor in lab. I have learnt a lot from you ma'am. Anita di, another sweet girl, thanks for advising me at the times of need & of course for sending me the papers which are not accessible in JNU. We all miss both of you a lot. Thanks to Ekta ma'am for her help & support. Santosh Sir, to be there every time I need him with & without bike & for teaching us how to manage everything in a lab. Jahangir Sir & Buddhi for being my snacks time partner & helping me in my labwork. My friend & lab mate Nikhat, we shared same kind of feelings when we joined the lab, we have prepared many liters of MiliQ together. Sagrika & Arshdeep for being my cute

friends & for all the invaluable advice to become a decent person. One day I will follow it.

I also appreciate the warmth & care received from my friends Soni, Munazzah, Baby Santosh, Neha rats, Shweta, Asish, Nitesh, Meghna, Tapan, Somesh, Amod & especially Mrigya & Manjari.... Thanks for being there for me always....., the birthday celebrations, the lunch time, the moonlight tracking..... Every moment we shared together is extremely beautiful. Really I can't have more beautiful picture of life in JNU without all you guys.

I am gratified to whole CIF, AIRF staff especially Mishra sir, Meena sir, Joginder sir, Plabon sir & Ashok sir and all the office staff especially Shini ma'am, Kirti ma'am and Meenu ma'am. From the very first day Meenu ma'am has been very supportive, she use to scold us but as a person who cares.

Fellowship provided by CSIR is highly acknowledged.

Last but most importantly I would say..... Papa ji, Mummy & my Somu I love you a lot. You are "my lifeline".

Shweta

Abbreviations

AP-1	Activator Protein 1
APA	Alternative Polyadenylation
APC/C	Anaphase-promoting complex/cyclosome
AS	Alternative Splicing
bp	Base pairs
BSA	Bovine Serum Albumin
CaM	Calmodulin
CCM	Cerebral Cavernous malformation
CDK	Cyclin-Dependent Kinase
CKA	Connector of Kinase to AP-1
DDB1	DNA damage binding protein 1
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
DNMTs	DNA methyltransferases
EDTA	Ethylene diamine tetra acetic acid
eIFs	Eukaryotic initiation factors
ER	Endoplasmic Reticulum
ERK	Extracellular Regulated kinase
ERα	Estrogen receptor α
FBS	Fetal Bovine Serum
GFP	Green fluorescent protein
IGF2R	Insulin-like growth factor 2 receptor
IPTG	Isopropyl-β-D-1-thiogalactopyranoside

Abbreviations

JNK	c-Jun N-terminal Kinase
kb	Kilobase
kDa	kilo Dalton
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
Min	Minutes
mRNA	Messenger RNA
ml	Milliliter
NDPK	Nucleotide diphosphate kinase
ORC	Origin Recognition Complex
ORFs	Open reading frames
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
PP2A	Protein Phosphatase type 2A
PSD	Post synaptic density
PTB	Polypyrimidine tract-binding protein
RNA	Ribonucleic acids
RT PCR	Reverse Transcription PCR
SCF	Skp, Cullin, and F-box
SH	Src homology
SS	Splice site
SSU	Small subunit

TAB	Transforming growth factor-β activated protein kinase (TAK1)-binding protein
TAE	Tris, Acetate, EDTA buffer
TLR	Toll-like receptor
UTR	Untranslated region
µl	Microliter
µg	Microgram
µM	Micromolar

Introduction

A). Scaffold proteins:

Cells exhibit an array of specific responses towards various stimuli which are mediated by a wide range of signalling networks. These networks comprise of disparate as well as similar pathways including numerous ligands, receptors, mediators, effectors etc. which are assembled into specific complexes. The same molecule, depending upon intra & extracellular environment, may convey divergent downstream messages. Although the shared usage of molecules exists but the signalling networks are accurately regulated. Scaffold proteins play a major role in precise control of the events in signalling cascade and usually contain multiple modular interaction domains or motifs (Buday and Tompa, 2010; Zeke et al., 2009).

A.1. Mechanisms in scaffold signalling.

Scaffold employs a variety of mechanisms to enforce modification of the signalling components.

(a) Scaffolds tether enzymes close in space and enhance effective local concentrations. Tethering mechanism represents one of the most important mechanism regulating signalling and is associated with various merits and demerits as described: lowering of the entropy of signalling interactions by co localization of enzyme & substrate; restriction of the conformational freedom of interacting partners to enhance the rate of signal transfer; titration of enzymes & substrates in different complexes, away from each other at higher concentrations (Dickens M et al., 1997, Zeke et al., 2009).

(b) They can mediate assembly of signalling complexes in a combinatorial manner by participating in signalling through different pathways using distinct scaffold proteins.

(c) The function of full signalling modules can be dynamically modulated by regulating the accessibility of the scaffold without involving individual components (Zeke et al., 2009).

(d) Some scaffolds can also modify the conformation of enzymes binding to them, or in turn the conformation of the scaffold can also be modified (Smock RG, 2009)

A.2. Scaffolds in signalling.

Scaffolds can influence signalling events in a spatio-temporal & dose dependent manner. Increasing number of evidences demonstrates that the scaffold protein can be the direct

target of regulation: some pathways can be turned on or off by modification of scaffold proteins. Their phosphorylation may leads to inhibitory effects, e.g., phosphorylation of Ste5, a scaffold protein of yeast by cyclin dependent kinase 1 (cdk1) blocks the association of Ste1 with plasma membrane resulting in the attenuation of mating signals (Strickfaden SC et al., 2007). In a signalling pathway, scaffold proteins can coordinate complex feedback loops as exemplified by KSR scaffold in mammals regulating MAPK pathway (Rajakulendran et al., 2009).

Another distinct feature of scaffold proteins is that they can facilitate the formation of new pathways. Pathogens use scaffold like proteins to rewire signalling response of the hosts. (Selyunin AS et al., 2011). For example, in the course of infection by HIV, a host protein APOBEC3G (a cytidine deaminase which interferes with the replication of virus), is destroyed by Vif scaffold protein by ubiquitinylation (Yu X et al., 2003). Although scaffold proteins share more or less common functions, the heterogeneity in structure & sequence suggests that they appeared independently during evolution. Some of the WD repeat proteins are good examples of scaffolds.

B). <u>WD-40 repeats family:</u>

The family of WD-40 repeat containing proteins comprises enormous proteins with an array of structural organisation & functions found in some prokaryotes & all eukaryotes (Li D et al. 2001). They are also reported in some bacteria e.g, *Thermomonospora curvata* and *Cyanobacterium synechocystis* (Janda et al., 1996; Stirnimann et al., 2010). They are characterized by WD domains which mediate protein-protein or protein-DNA interactions. By acting as scaffolds, WD-40 domain mediates numerous molecular recognition events. Most WD-40 domain proteins possess additional domains with catalytic or other functional activities such as ubiquitylation and protein degradation (FBOX, RING, SOCS), microtubule dynamics (LisH), phospholipid binding (FYVE) and vesicle coating (CLH) (Stirnimann et al., 2010).

B.1. Architecture of WD-40 repeat domain.

WD40 domain containing proteins are characterized by several copies of 44–60 amino acid residues that typically contains the GH dipeptide 11–24 residues from its N-terminus and a tryptophan-aspartic acid (WD) pair at the C-terminus (Li and Roberts, 2001).

Although, these two signature dipeptides are not conserved, the contribution to these repeats is made by several other amino acids including aspartic acid 6 residues upstream to WD repeat (Neer EJ, 1994; Smith TF et al., 1999 & Smith TF, 2008).

Structurally, the domains containing WD-40 repeats belong to the larger class of proteins having the beta propeller fold. They are highly dynamic in nature and each WD repeat sequence form propeller blade of four antiparallel β strands. Three β strand of one blade form a sheet with outer strand of next blade, termed as velcro closure which stabilize the β propeller folding by hydrophobic interaction between β-sheet. Velcro closure is not essential for WD-40 domain β propeller assembly but for its stabilization. The conserved residue in β sheet form strong H-bond network and stabilize the WD-40 repeat fold. Besides, intrachain hydrophobic networks also contribute to the maintenance of the propeller structure (Smith, 2008). At least seven WD-repeats are required to make the most stable structure (Murzin, 1992).

B.2. Diverse functions of WD-repeat proteins.

WD-40 domains represent one of the ten most abundant domain types across eukaryotic proteomes and ideally suited to be hubs in cellular interaction networks (Stirnimann et al., 2010). Several WD proteins form homo or heterodimers & comprise other domains resulting in enhanced number of binding partners & therefore overall functions, e.g., a ubiquitin ligase, β transducin repeat containing protein (β-TrCP1) has both WD repeat & F box domains in addition to a RING domain. The degradation of some regulatory proteins such as p53 requires (β-TrCP1) (Vinas-Castells R et al., 2010; Xia Y et al., 2009). WD repeat also effects its subcellular localisation, thereby enabling the protein to play a role in transcription & circadian rhythm (Ohsaki K et al., 2008; Seo E et al., 2009; Kimbrel EA et al., 2009). WD-40 domains play versatile roles in mediating critical cellular functions (Table 1) including signal transduction, cell cycle control, vesicular trafficking, cytoskeletal assembly, development, apoptosis and also in many diseases. The WD-repeat-β propeller protein Gβ and the alpha-helical, isoprenylated polypeptide Gγ are considered to be obligate dimerization partners, required for the functional coupling of Gα subunits to receptors (Sondek and Siderovski, 2001). ORC-associated (ORCA), a WD-40 repeat domain protein facilitates association of Origin recognition complex (ORC) to chromatin, thereby playing a critical role in DNA replication initiation and cell cycle progression (Shen et al., 2010). RACK1 with seven tandem WD-40 domains is scaffolding protein

Table 1# representing summarized functions of WD repeat proteins.

FUNCTION	EXAMPLE OF GENE
G protein signal transduction	**Gbb/FYVE**
RNA processing & splicing	SOF1, PRP4,PRP19
Translation initiation complex formation	**TFIID**
Chromatin assembly, histone acetylation	CAF1/ RpaP/MSI1
Vesicular & Golgi trafficking	**NORI1/WRD1& SEC1**
Regulation of cell cycle progression	CDC40/POP1/POP2
Cell division & chromatin separation	**HIR1**

involved in signalling and development process. It also has role in innate immunity (Ren Q et al., 2011). Fbw7 is highly expressed in the nervous system and controls neural stem cell differentiation and apoptosis via Notch and c-Jun (Hoeck et al., 2010).

C. Striatin family:

The striatin family constitutes a group of three highly homologous and structurally related metazoan proteins belonging to the WD-40 repeat superfamily of scaffolding proteins (Castets et al., 2000). They have been detected in species as diverse as filamentous fungi to mammals but absent in plants and budding yeast (Benoist et al., 2006). The three mammalian members of this family are Striatin, SG2NA and Zinedin (Gaillard et al., 2006).

C.1. Sub cellular localization.

The prototype member of the family is striatin (780 aa), originally purified from rat brain synaptosomes and later found in abundance in the striatum, hence named "Striatin" (Benoist et al., 2006). It is largely expressed in the central nervous system in the somato-dendritic compartment of neurons, especially in dendritic spines (Castets et al., 1996). SG2NA (also called striatin 3), the second member of the striatin family, was originally identified as a nuclear protein. Immuno-cytochemistry and subcellular cell fractionation studies carried out so far reveal that SG2NA is exclusively a cytosolic and membrane-bound protein (Baillat et al., 2002; Gaillard et al., 2006). Zinedin, (also called Striatin 4), the third member of the striatin family, is a cytoplasmic protein specifically expressed in the central and peripheral nervous systems (Castets et al., 2000; Benoist et al., 2008).

C.2. Structural features of striatin family.

All members of striatin family are derived from same ancestral gene (Castets et al., 2000). They have identical structure, i.e., similar number of exons (except Zinedin which has 17 exons instead of 18). They share same protein-protein interaction domains from N to C termini, viz., caveolin binding domain, coiled coil domain, calcium-calmodulin binding domain & WD repeats (Fig 1). These are found exclusively in striatin family and thus serve as signature for this family (Benoist et al., 2006).

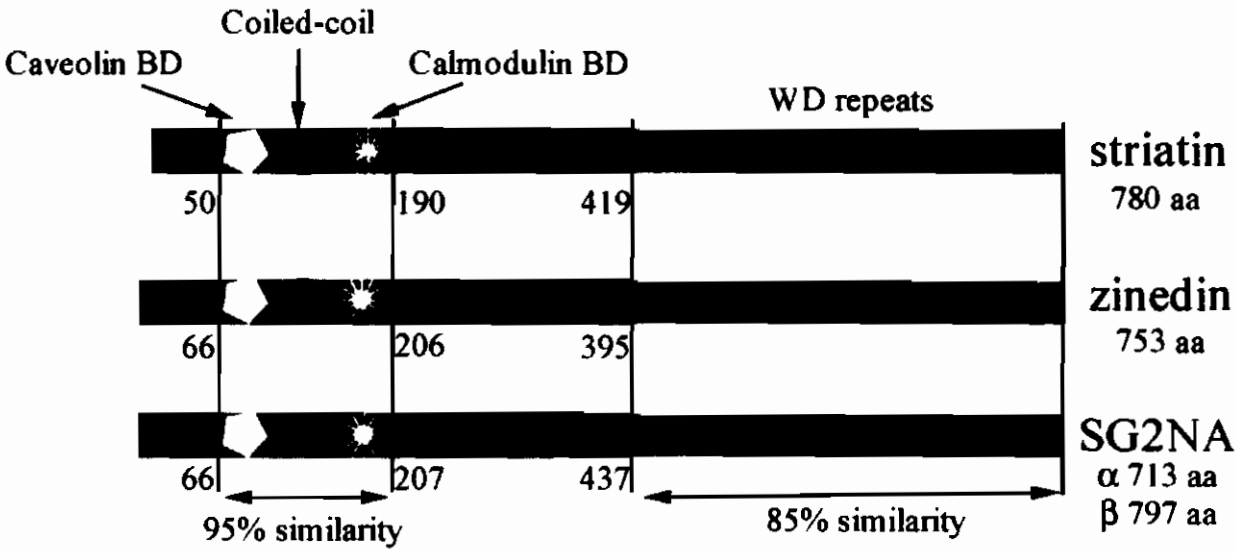


Figure 1: Domain structure of the mammalian striatin family members. Striatin, zinedin and SG2NA a and b are composed of several protein–protein interaction domains, from the N to the C terminus: caveolin-binding domain (BD, yellow), coiled-coil domain (pink), calmodulin-binding domain (green) with 95% similarity between the family members and WD-repeats (cyan) with 85% similarity between the members. (aa: amino acids).

Adapted form Beonist et al. 2006

- **Caveolin binding domain**: Caveolae are domains of plasma membrane specialised in clathrin independent endocytosis & cholesterol homeostasis. All the members of striatin family are known to incorporate a consenses sequences LHFIQHEWARF which corresponds to caveolin binding sequence αxαxxxxα, where α denotes aromatic amino acid & x any amino acid, often α is replaced by leucin or isoleucin. (Couet et al., 1997; Carman et al., 1999). Pull down experiments demonstrated that they interact directly with caveolin, which is inhibited in the presences of calcium (Gaillard et al., 2001).
- **Coiled coil domain:** It is another domain conserved in the striatin family. It is present at the amino terminus and it is highly conserved (94% homology amongst members) (Castets et al., 2000). Proteins, especially those with scaffolding functions, oligomerize via α helical coiled coil structure (Lupas, 1996; Kammerer, 1997; Yu, 2002). This oligomerization is necessary to facilitate the routing of striatin family proteins in the rat synaptosomes (Gaillard et al., 2006).

- **Calcium- calmodulin binding domain:** The calcium CaM binding domain is located in between the residues 149 to 166 of Striatin family and show calcium-dependent CaM binding activity (Castets et al., 2000; Moreno et al., 2000). Presence of Ca^{2+}–CaM-binding domain in members of the striatin family allow them to act as Ca^{2+} sensors in the protein complexes, enabling them to localize in different sub membranous micro-domain in response to Ca^{2+} fluctuations (Benoist et al., 2006).

- **WD40 repeat domain:** It consists of ~40 minimally conserved amino acids with a pair of glycine-histidine (GH) at the amino terminus and a pair of tryptophan-aspartic acid (WD) residues at the carboxy terminus (Castets et al., 1996; Castets et al., 2000). Striatin has eight WD repeats at its carboxy terminus (Castets et al., 1996). The WD-repeat domains thus enable these proteins to establish multiple protein-protein interactions to fulfil various functions.

C.3. Functions of the striatin family.

Numerous functions have been assigned to the members of the striatn family (as shown in Fig 2). Some of which are described below:

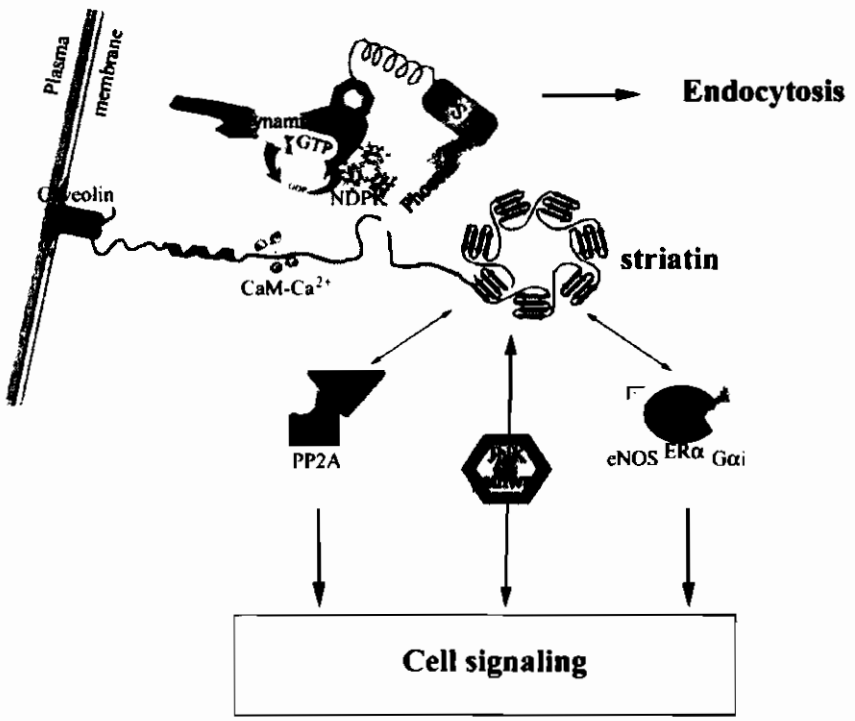


Figure 2 : Synthetic model of striatin function in dendritic spines. Most of the binding partners and interacting pathway of striatin are represented. Mammalian striatin family members interact with caveolin, Ca2+–CaM complex, PP2A A and C subunits, ERa, and phocein. In D.melanogaster, striatin plays a role in the JNK pathways. All these interaction are in accordance with involvement of striatin family members in cell signaling and endocytosis.

Adapted from Beonist et al. 2006.

Membrane trafficking: Yeast two hybrid assay using full length Striatin as bait revealed phocein protein as their interacting partner. Phocein is a ubiquitously expressed, highly conserved intracellular protein comprising 225 amino acids, It is homologous to the sigma subunit of clathrin adaptor protein. It interacts with nucleoside diphosphate kinase (NDPK) and Eps15 (Baillat et al., 2002). NDPK interacts with C terminal proline rich domain of Dynamin I, which plays an important role in endocytosis (Baillat et al., 2002; Takei et al., 2005). Eps15 has also been implicated for endocytic pathway. This suggests that phocein-Striatin serve an important role in membrane trafficking in general and membrane budding in particular.

Localisation of estrogen receptor: SG2NA has been shown to promote the localization of estrogen receptor to plasma membrane (Lu et al., 2004). In endothelial cells, SG2NA recruits the ER–eNOS signalling complex to the membrane. Estrogen regulates physiological processes in reproductive as well as non-reproductive tissues including cardiovascular system, brain and bones. Estrogen receptors can exert genomic and non-genomic effects by functioning as transcription factor regulating gene expression and acting as a signalling protein respectively. The caveolin binding, coiled coil and calcium CaM binding domain of Striatin in the N terminus interact with the DNA binding domain of estrogen receptors (Benoist et al., 2006).

The new class of B regulatory subunit of PP2A: SG2NA has been shown to be an integral part of Protein Phosphatase 2A (PP2A, Moreno et al., 2000). PP2A is a serine/threonine phosphatase present in all eukaryotes and aid in cell cycle regulation, viral transformation and neuronal signalling (Janssens and Goris, 2001; Lechward et al., 2001; Berndt, 2003; Van Hoof and Goris, 2003). It is a heterotrimeric protein comprising of three different subunits (A, B and C) serving as structural, regulatory & catalytic functions (Berndt, 2003). B subunit forms a complex with A/C dimer regulating its activity as well as specificity. Binding of different B subunits to the holoenzyme and methylation of catalytic subunit of PP2A is the major point of its regulation. In the absence of B subunit, Striatin family binds to PP2A (Moreno et al., 2000) and inhibits its activity resulting in increased phosphorylation of striatin family members. It is thus suggested that striatin family members serve as the substrate for PP2A by interacting with them and possibly confining them to a specific location (Benoist et al., 2006).

Developmental role: Striatin family members serve an indispensable role in development of motor neurons. Decrease in striatin results in impairment of dendritic outgrowth (Bertoli et al.,1999). Calcium is essential for maturation of neurons and Striatin possibly aids to dendritic arborisation via calcium signalling (Jourdain et al., 2003). During the development of *Drosophila*, various kinases viz., Misshapen (Msn), Slipper, Hemipterous (Hep) and Basket (Bsk) are activated by the JNK pathway which results in the activation of D-Jun. A transcriptional complex, Activator protein 1 (AP-1) is formed by the association of Jun with Fos, that regulate the expression of a number of downstream genes (Martin and Wood, 2002). CKA, the single *Drosophila* homologue the mammalian Striatin and SG2NA (56% homology) interacts with HEP, BSK, JUN and FOS aiding in organising the kinases and facilitating the phosphorylation-activation of AP-1 (Chen et al., 2002). Therefore, Drosophila Striatin acts as a scaffolding protein connecting various components of JNK signalling during development.

Role in cell cycle, cell dynamics and diseases: Members of striatin family associate with various other proteins, including Mst3, Mst4, and GCK-III (Germinal Center Kinase III) involved in control of cell cycle, cell migration, cell polarity (Fidalgo M et al., 2010. Cornils H et al., 2011). Striatin complexes have been implicated in various diseases like cerebral cavernous malformation, angioma wherein CCM3 is mutated. Lower level of Striatin due to small deletion in the 3' untranslated region of Striatin complexes have been linked to several clinical conditions in canine model of arrhythmogenic right ventricular cardiomyopathy (Meurs KM et al., 2010).

D. Insights into SG2NA and its isoforms: a member of the striatin family.

SG2NA (also called striatin 3), is a member of the striatin sub-family of WD-40 repeat proteins (Benoist et al., 2006; Castets et al., 1996). It was originally identified as a nuclear autoantigen in cancer patient (bladder cancer followed by adenocarcinoma of lungs) whose expression is augmented during S to G2 phase of cell cycle, hence named SG2NA (Landberg and Tan, 1994; Muro et al., 1995). Later studies established its structural and functional relatedness to striatin (bearing 80% homology) (Castets et al., 2000). Human SG2NA comprises of 713 aa containing six tandem WD-40 repeats at its C-terminus (Castets et al., 2000; Muro et al., 1995). Now, it is well established that SG2NA is

exclusively a cytosolic and membrane-bound protein (Castets et al., 2000; Gaillard et al., 2006).

Our laboratory has established that SG2NA exists in multiple isoforms arising out of alternative splicing (Table 2) involving exons 7-9. Splicing variation of SG2NA is also found in chick and human, suggestive of an evolutionarily conserved trait. There are at least six alternatively spliced isoforms of SG2NA (35, 38, 78, 82/52, 83 and 87 kDas) which differ in the exons organization. These isoforms are variably expressed in various tissues and their expression profile changes with state of differentiation. (Sanghamitra et al., 2008). Apart from being present in the nucleus, cytoplasm and nucleus; they are also present in various cellular organelles differentially (Chauhan P, 2012; Ph.D thesis). More recent study also suggests that members of the striatin family emerged from a common ancestor in lower eukaryotes wherein the emergence of SG2NA preceded that of others (Tanti GK, 2012; manuscript in preparation). Apart from alternative splicing, SG2NA transcripts are also characterized by differential polyadenylation, mRNA editing (by the deletion of one adenosine at (1571 position) in the 82 kDa transcript resulting in formation of premature stop codon at 473 instead of 750 in the ORF) and the generation of non-coding reverse transcripts arising out of its 3'-UTR region (unpublished data). Our laboratory has also shown the existence of various sites of post translational modifications of SG2NAs. Posttranslational modifications regulate activity, localization and interaction with other proteins, thereby influencing almost every aspect of cell function (Table 3). Besides, SG2NA expression is also modulated by MAPKinases, Ca^{2+} signalling and stress conditions. Taken together, such extensive variation in transcripts strongly suggests a potentially important but yet unknown function for SG2NA.

D.1. Mechanisms controlling the expression of gene.

ALTERNATIVE SPLICING:

Alternative splicing represents one of the most extensively used mechanisms, which accounts for the genomic diversity and tissue specificity of higher eukaryotic organisms. Alternative splicing signifies the basis for the discrepancy between the estimated ~24,000 protein-coding genes in the human genome and ~100,000 different proteins that are postulated to be synthesized in various tissues (Chen and Manley, 2009; Keren et al., 2010). Recent studies based on RNA sequencing revealed that ~90% genes in *Homo*

Table 2# representing the exon structure and possible of stop codon in mSG2NA isoforms.

MOLECULAR WEIGHT	EXON STRUCTURE OF CORRESPONDING TRANSCRIPT	POSITION OF STOP CODON FROM THE BEGINNING OF ORF
87kD	Exon 1-18 present	2388-2390
83 kD	Only exon 8 is missing	2277-2279
82kD	Only exon 9 is missing	2247-2249
78kD	Both exon 8 & 9 are missing	2136-2138
38kD	Complete exon 7 & a part of 7/8 intron is present; both exon 8 & 9 are missing.	1041-1043
35kD	First four nucleotides of exon 7 present, part of 7/8 intron & exon 8-18 are present.	903-905

Table 3 # representing possible sites of post translational modifications of SG2NAs as summarised below (adopted from Tanti G, M. Phil thesis)

POSITION	DESCRIPTION
153-159; 168-174; 97-403	CK1 phosphorylation site.
204-210; 254-260	CK2 phosphorylation site.
319-326; 392-399	GSK3 phosphorylation recognition site.
165-171; 194-200; 660-666	PIKK phosphorylation site.
319-325	PKA phosphorylation site.
444-450	Phosphorylated by polo like kinases.
321-324; 388-391; 396-399; 419-422; 516-519	Glycosaminoglycan site.
26-31; 211-216; 367-372; 392-397	Motif for N- glycolation.
125-128	Motif recognised for modification by SUMO.

sapiens, ~60% in *Drosophila melanogaster* and ~25% of *Caenorhabditis elegans* undergo alternative splicing. Mechanistically, mRNA splicing is the process in which introns are removed from primary transcripts and the exons that encode amino acid sequences are joined together to generate mature protein-coding mRNAs (Fedor, 2008). According to the mechanisms involved, alternate splicing (AS) events are classified into four main categories. The first type is exon skipping where cassette exon is spliced out of the transcript together with its flanking introns. The second and third types are alternative 3′ splice site (3′SS) and 5′ splice site (5′SS) selection which occur when two or more splice sites are recognised at one end of an exon. In the fourth type of AS events, an intron is retained in mature mRNA. Other less frequent events that give rise to alternative transcript variants include mutually exclusive exons, alternative promoter usage and alternative polyadenylation (Keren et al., 2010). The plethora of splicing events are regulated by a balance between positive & negative regulators: positive regulators comprises SR proteins (being rich in serine & arginine) while negative regulators are hnRNPs (heterogeneous nuclear ribonucleoproteins, Lin and Fu, 2007).

CELL CYCLE REGULATION:

Reproduction is one of the most fundamental characteristics of living system. An ordered series of event through which cells undergo during division is termed as cell cycle. The typical mammalian cell cycle consists of four sequential phases viz., G1, S, G2 and M, each of which should be completed errorless before the next begins (Malumbres and Barbacid, 2006; Williams and Stoeber, 2012). Cell cycle regulation is a result of highly organised and complex orchestration of various proteins in the cell. Any discrepancy in it can result in cell death or onset of various diseases. In cancer cells, tight control of cell cycle regulation fail, causing aberrant proliferation of cells (Harbour and Dean, 2000; Bonn Bong et al., 2012; Marlow et al., 2012). So, it is imperative that cell division is a tightly controlled process. External signals & internal cues determine the progression of cell through cell cycle. The precision of control of cell cycle is mainly attributed to combination of cyclin dependent kinases (Cdks) and their interacting partners, the cyclins (Malumbres and Barbacid, 2006; Viallard et al., 2001; Williams and Stoeber, 2012). In cell cycle progression, various levels of control impinge upon Cdks, the small Serine/Threonine kinases. This control involves regulated expression and disruption of cyclins and inhibitory proteins that associate with CDKs or CDK/cyclin complexes;

activating and inhibiting phosphorylation- dephosphorylation of the CDKs. Although the concentration of cyclin dependent kinases remains constant throughout the cell cycle, the abundance of cyclins determines the activation or inactivation of CDKs. Ubiquitin ligase complexes execute the degradation of proteins by covalent attachment of ubiquitin chains. Two poly-ubiquitinating E3 complexes predominantly affecting the cell cycle are SCF (Skp1, Cullin & Fbox) and Anaphase Promoting Complex, also called as cyclosome (APC/C). While the SCF remains functional throughout the progression of cell cycle, APC/C becomes active during prometaphase & remains active till G1 phase (Leuken et al., 2008). Transfer of ubiquitin to their respective substrate is promoted by RING ligase, SCF & APC/C as they serve as scaffold protein which bring together all the molecules in close proximity. Multiple evidences have supported the notion that for accurate cell cycle progression mitotic phosphatases are required. Over 90% of total Ser/Thr phosphatase activity in the cells is accounted by protein phosphatase 1 (PP1) and protein phophatase type 2A (PP2A) (Trinkle-Mulcahy et al., 2006; De Wulf et al., 2009; Barr et al., 2011). As stated earlier, numerous regulatory subunits enable the phosphatase PP2A to regulate diverse functions, like control of cellular dynamics during cell cycle. For example B55α regulatory subunit controls the postmitotic assembly of nuclear envelope & Golgi bodies (Schmitz MH et al., 2010). A study using a library of siRNAs revealed that spindle formation, kinetochore functions and progression into cytokinesis is administrated by the regulatory subunit (Chen F et al., 2007; Archambault V et al., 2007).

Misshapen-like kinase 1 (MINK1) is a member of the germinal center kinase (GCK). It is essential for cytoskeletal organisation & oncogene induced senescence. Using mass spectroscopic analysis, it has been shown that MINK1 is a component of STRIPAK (striatin interacting phosphatases & kinases). STRIPAK comprises striatins, catalytic and structural subunits of PP2A, and other accessory proteins (Goudreault et al., 2009; Gordon et al., 2011; Kean et al., 2011). STRN4, a regulatory subunit of PP2A, is shown to directly associate with MINK1, induction of multi nucleated state and inhibition of abscission occur when STRN4 is knocked down (Hyodo T et al., 2012).

STRESS RESPONSES:

Stress response is an evolutionary conserved mechanism that enables cells to reciprocate to adverse metabolic and environmental conditions. Multiple mechanisms have been evolved to surmount the genotoxic and non-genotoxic effects (Fig 3). At the molecular level, stress results in the induction of specific subset of cellular proteins, viz., heat shock proteins, glucose related proteins (Grps) under heat and glucose starvation respectively. Several lines of evidences suggest that genotoxic stress results in the arrest of cells in G1/S & G2/ M checkpoints, enabling them to repair the damage prior to entry to next phase of the cycle (Morgan & Kastan, 1997; Schwartz & Rotter, 1998). A number of stress responses in context to cell cycle involves:

Phosphorylation of eIF2: Three kinases activated under stress can induce the phosphorylation of eIF2. Heme regulated inhibitor of translation (HRI) is activated in response to heme deprivation, heat shock and by heavy metals. PKR is activated in response to double stranded RNA, formed following viral infection. GCN2 in stimulated by nutrient deprivation. All these results in phosphorylation of eIF2, which in turn makes eIF2B unable to exchange nucleotides.

Phosphorylation of eIF4E: While growth factors induce ERK1, p38 is a stress responsive kinase (Dhanasekaran and Reddy, 1998). MAPK-mediated phosphorylation of eIF4E enhances its ability to bind to the `cap' increasing the rate of translation initiation (Raught and Gingras, 1999). p38 promotes phosphorylation of eIF4E inhibitory proteins E4-BPs, preventing its interaction with eIF4G and consequently with the 43S complex (Waskiewicz et al., 1999; Clemens and Bommer, 1999; Raught and Gingras, 1999). Similarly proteolytic cleavage of eIF4G during stress-induced apoptosis results in protein synthesis inhibition, via caspases (Marissen & Lloyd, 1998; Clemens et al., 1998).

Activation of kinases and DNA-integrity checkpoints: Under UV/γ irradiation, X rays, oxidative stress and other cytotoxic and genotoxic agents, cells activate stress kinase and DNA-integrity checkpoint pathways. Both pathways mediate the cellular response to stress by regulating the transcription factors that control stress-response genes. Moreover, both pathways function in a number of common processes, including development, cell differentiation and apoptosis. It is thus suggested that the stress kinase and DNA-integrity checkpoint pathways perform complementary roles in the stress response. MAPKK–p38γ cascade is required for a γ-radiation induced G2 arrest. Ionizing radiation activates MKK6 and p38, resulting in cell-cycle arrest in G2 phase (Wang X et al., 2000). ATM-deficient

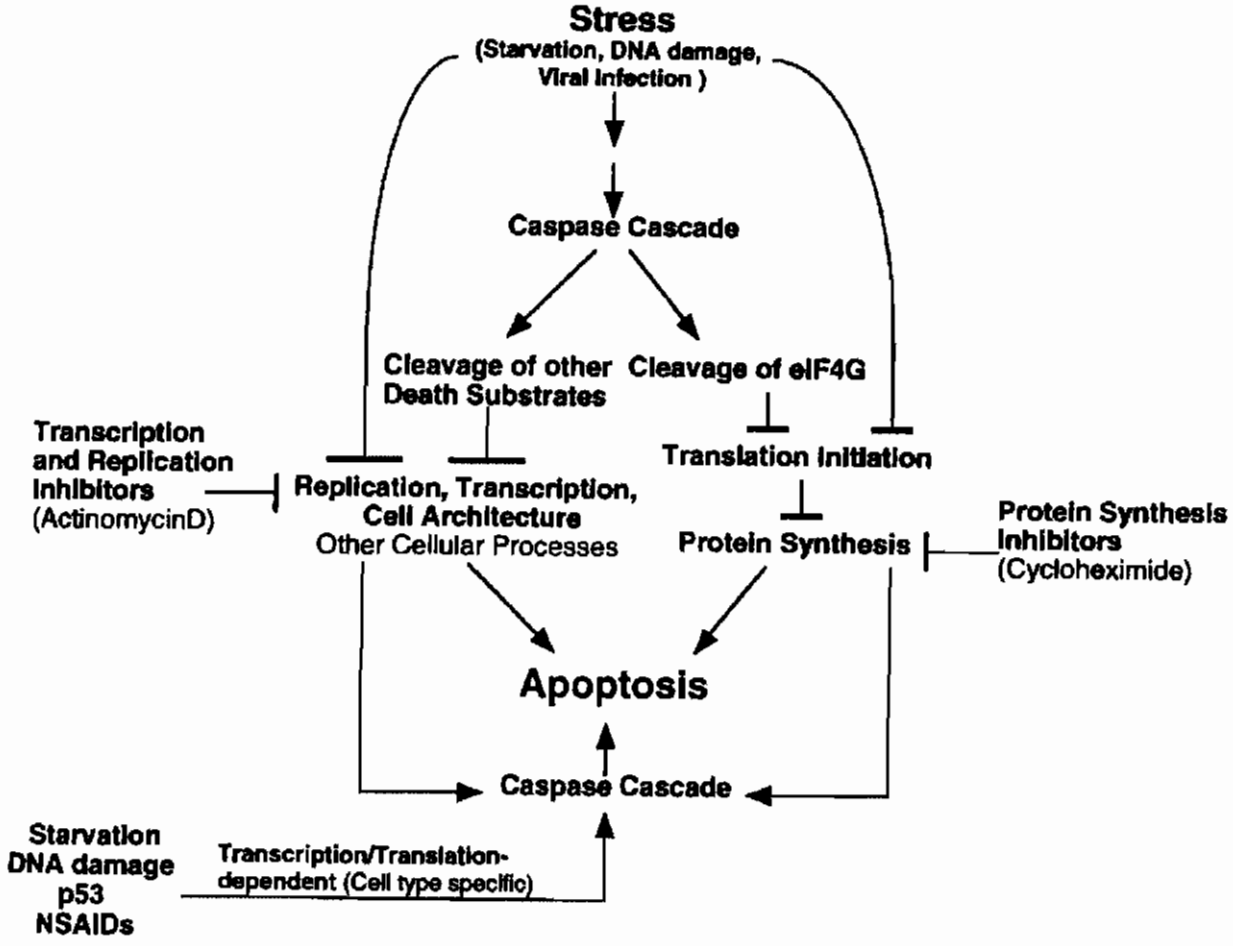


Figure 3 :Stress-induced apoptosis may involve activation of caspase cascade; caspases can cleave cellular proteins that control basic cellular processes including replication, transcription and building of cellular architecture.

Adapted from Meek et al 2003

cells are unusually sensitive to a variety of oxidants, including nitric oxide, superoxide and hydrogen peroxide (Green MH et al., 1997).

In budding yeast under hypertonic stress; Hog1, together with the kinase Swe1 regulates entry into G2. In human glioblastoma cells T98G, upon depletion of JNK1 or JNK2 cells are arrested in S phase (Potapova O et al., 2000).

Thus, numerous evidences suggest that stress can affect cell cycle. Similarly there are number of examples which show that during cell cycle progression, various proteins have varied functions depending on their subcellular localisation.

SUBCELLULAR LOCALISATION:

Compartmentalisation of cells in various organelles ultimately results in division of labour as well as organised and efficient working. As the intracellular pH, enzyme system etc. vary from compartment to compartment, the function of molecules may also vary according to the location. mRNA localization to subcellular compartments provides a means for the regulatory aspects of gene expression providing temporal and spatial control. It is intertwined with the translational and mRNA turnover machinery of the cell to achieve the goal of local protein production (Czaplinski and Singer, 2006; Martin and Ephrussi, 2009). The cellular ''address'' of transcripts is encoded by cis-acting elements in the RNA (most frequently in the 3'UTR), often known as ''localization elements'' or ''zipcodes''(Martin and Ephrussi, 2009). Distinct localization elements mediate distinct steps in localization and can have a complex secondary or tertiary structure, as in the Bicoid localization element, or they can be short, defined nucleotide sequences, sometimes in repeated elements (such as in the case of the Xenopus localized transcript Vg1) (Mignone et al., 2002; Martin and Ephrussi, 2009). Localization elements are recognized on the basis of mRNA sequence and/or secondary structure by trans-acting proteins such as Zipcode binding proteins, exon-junction complex, locasomes, Staufen, and fragile X-mental retardation protein (FMRP) etc (Holt and Bullock, 2009; Martin and Ephrussi, 2009). Localisation can alter the function of proteins as exemplified: following genotoxic stress, the transcription factor ATF2 moves to mitochondria where it impairs the HK1/VDAC complex and mitochondrial membrane integrity; sensitizing cells to apoptosis. However, under high level of expression of PKCε retains ATF2 to the nucleus preventing apotosis. Thus ATF2 can switch between its oncogenic transcriptional function and tumor suppressor function (inducing mitochondrial apoptosis, Meek DW et al., 2003).

Akt associates with MDM2 in the cytoplasm and once activated, it phosphorylates MDM2 and promotes its entry into the nucleus. The phosphorylated MDM2 associates with p300, stimulates the degradation of p53, and blocks its inhibition by ARF (Meek DW et al., 2003).

Aims and Objectives

Striatin family members comprising Striatin, SG2NA and Zinedin, play key roles in vesicular trafficking, cell cycle, signaling, and differentiation. In early studies, SG2NA was shown to be involved in cell cycle regulation while Striatin in signal transduction & vesicular trafficking in dendritic neurons (Benoist et al., 2006; Muro et al., 1995). In a recent study, Zinedin (also known as Striatin 4) has been shown to be involved in cytokinesis (Hyodo et al., 2012). Such disparate functions by the members of the family suggest context specific scaffolding activities.

We, in our laboratory have demonstrated that SG2NA has multiple splice variants that are preferentially but not exclusively located in organelles like mitochondria, endoplasmic reticulum, golgi body; as well as in plasma membrane and cytosol. Most of these isoforms (except 82 kDa) are modulated during the progression of cell cycle and under quiescent condition. Also, in both growing and quiescent cells, isoforms of SG2NA transiently localize to the nucleus. We thus hypothesize that SG2NA variants might have similar but distinctive roles in cell growth and stress responses. To explore that hypothesis, we propose to study its role in cell cycle progression by:

1. Developing cell lines (NIH3T3) stably expressing recombinant (myc tagged) SG2NAs.
2. Identifying its interacting partners in the nucleus.

Materials & Methods

Materials

Cell Culture: Dulbecco's Modified Eagle's Medium (DMEM), Penicillin-Streptomycin solution, Amphotericin B and Sodium bicarbonate were purchased from Sigma-Aldrich, USA. Fetal Bovine Serum was from Biowest, France. Trypsin-EDTA solution was purchased from Invitrogen Life Technologies, USA. Tissue culture plastic ware was from Corning International, USA and Greiner, Germany. The filter unit for medium filtration was purchased from Nalgene International, Denmark and filter membranes (0.22µm pore size) were from Millipore. Tissue culture grade water was prepared in the laboratory by using Quartz distillation system (Bhanu Scientific, Bangalore, India). DMEM was prepared in tissue culture grade water and sterilized by filtration through 0.22µm filter. Phosphate buffered saline (PBS), pH 7.2- 7.4, was prepared in tissue culture grade water and autoclaved.

Mouse embryonic fibroblast cell line (NIH3T3) were procured from National Centre for Cell Sciences, Pune, India (originally from ATCC, USA). Hela cells were kind gift from

Biochemical and Molecular Biology Reagents: All biochemicals were procured from Sigma-Aldrich, USA and Qualigens, India unless mentioned otherwise. All restriction enzymes and DNA modifying enzymes were procured from Fermentas, Inc. USA and New England Biolabs, USA. Bacterial growth media (LB and Agar) was purchased from HiMedia. DNA ladder (1 kb and 100 bp) were obtained from MBI Fermentas, Inc. USA. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Amresco. Gel extraction kit and Plasmid Midi Kit were from QIAGEN, Germany. Cell viability was measured using MTT assay from Sigma-Aldrich (USA).RNA Isolation reagent was from Ambion, Inc USA. Reverse Transcription kit was from Epicentre Biotechnologies. Madison, Wisconsin. Escort (IV) Transfection Reagent was purchased from Sigma, USA. Power SYBR® Green Master Mix was procured from Applied Biosystems, Inc., USA. Glutathione-Beads were from GE healthcare biosciences Uppsala. All plastic wares were from Tarsons, Germany.

Plasmid Constructs: pcDNA3.1(neo)-SG2NA (35,52,78 and 87kda) variants , GST-tagged SG2NA variants used in this study were available in the lab (Tanti G, M.Phil thesis., 2009).GFP-SG2NA i.e., pEGFP-C3 variants stable cell lines (35,52,78 and 87kda)

used were also available in the lab (Chauhan P, PhD thesis., 2012) pGFP-V-RS vector (from which puromycin gene was released) was purchased from Origene.

Antibodies: Mouse monoclonal antibody for SG2NA (cat no. 05-1115) was from Millipore, USA. SP1 rabbit polyclonal antibody: cat no. sc-14027; goat polyclonal VDAC-1 (N-18) antibody: sc-8828 was from Santacruz, ; goat ploYclonal GRP-78 (N-20) sc-1050 antibody; mouse monoclonal antibody for β actin :cat no.A2228 were purchased from Sigma Aldrich, USA.

Oligonucleotide primers were synthesized from Sigma Aldrich, India.
The sequences were as follows:

Puro f: 5' GGTTCACGTAGTGTGGTGTGGAAAGTCCCCAGGC 3'

Puro r: 5' CATTTCGAATCAGGCACCGGGCTTGCG 3'

Puro forward 5GAGCTGCAAGAACTCTTCCTC 3'

Puro reverse 5'GCTCGTAGAAGGGGAGGTTGC 3'.

Methods

Generation of puromycin kill curve and stable cell lines

In order to generate stable cell lines expressing the gene of interest, it was important to determine the minimum amount of puromycin required to kill non-transfected cells. This was done by generating a puromycin kill curve in HeLa cells. The cells were plated in a 48-well plate and incubated overnight. The next day, the growth media was replaced with the media containing the dilutions of the antibiotic ranging from 0μg/ml to 2μg/ml, into the appropriate wells. Every day, freshly prepared selective media was added and cells were monitored. The percentage of surviving cells was calculated by MTT assay. HeLa cells were transfected with puro pcDNA-SG2NA constructs (35, 52, 78 and 87kDa) linearised with PvuI followed by gel purification. 24 hours after transfection, the cells were selected with 2μg/ml puromycin (optimal conc. obtained from kill curve) for 2 weeks, and the surviving cells were picked up with the help of sterile glass tubes, trypsinized and transferred to new dishes. The transfectants were maintained in DMEM supplemented with 10% FBS and 1.5 μg/ml puromycin at 37°C.

Confocal Immunofluorescence Microscopy

After requisite treatments, i.e.,50μM & 100 μM H2O2, GFP- SG2NA variants stable cell lines were fixed in methanol (ice chilled) for 15 min followed by washing with PBS twice. Cells were then stained in Hoechst 33342 for 30 min and mounted in 10% glycerol after washing twice with PBS. Confocal images were obtained with a Laser Confocal Microscope, Olympus FluoView™ FV1000 at 60X magnification with oil immersion objective at AIRF, JNU and analysed by Image Pro Plus software. Images were processed using Adobe Photoshop CS3.

Polymerase Chain Reaction

Forward and reverse oligonucleotide primers flanking the desired region of interest were used for PCR. All PCR reactions were performed using either Taq polymerase (Fermentas), Phusion DNA polymerase (NEB). A typical amplification reaction contained 1X buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5μM primers, 5ng of template and 1U of Taq Polymerease or Phusion DNA polymerase in a reaction volume of 50μl. Cycling conditions were as follows:

1. 95°C for 10 minutes	Initial Denaturation
2. 95°C for 1 minute	Denaturation
3. 50°C - 60°C for 45 seconds	Annealing
4. 72°C for 1-6 minutes	Extension
5. Step 2-4	30- 35 times.
6. 72°C for 10 minutes	Final Extension

The amplification was carried out in a DNA thermal cycler (Eppendorf). The size and integrity of the products were checked by electrophoresing 10μl of the sample on a 1% agarose gel at 4V/cm for an appropriate time period.

Restriction Digestion

Restriction digestion was carried out according to the manufacturer's instructions (MBI Fermentas, Inc. USA). The digestion was carried out in water bath set at the recommended temperature. For analytical purpose, the reactions were set up in a volume of 20μl. For preparative purposes, the digestions were set up in a volume of 50μl. After incubation the reaction mixtures were loaded with 1X gel loading buffer (2.5% Ficoll type-400, 0.04 % Bromophenol blue, 0.04% Xylene cyanol FF) onto an agarose gel.

Dephosphorylation of DNA termini

Removal of 5'- phosphate groups from DNA fragments was carried out by using Fast Alkaline phosphatase (FAP). The digested vector DNA (1µg/µl) was resuspended in a 1X FAP reaction buffer and incubated with FAP at 37°C for 15 min. The enzyme was then heat inactivated at 65°C for 5 min and DNA was purified by gel purification using Qiagen kit as per the manufacturer protocol.

Elution of DNA from agarose gel

The agarose slice containing the band of interest was excised from the gel and trimmed. DNA in the gel was isolated using Gel Extraction Qiagen Kit following manufacturer's protocol. Eluted DNA was checked by agarose gel electrophoresis.

Ligation of DNA temini

Restriction digested vector and insert DNA were purified from agarose gel and ligated according to : [Amount of insert = (Size of insert/Size of vector) X 100ng of vector] in 20µl volume in 1X Ligase buffer and 5U of T4 DNA ligase (New England Biolabs, USA) at 16°C overnight.

Preparation of competent cells

Competent cells of E.Coli (DH10β) were prepared by the method described by Hanahan D et al., 1991. Single colony from LB agar plate was inoculated in 5 ml LB medium and grown overnight at 37°C. 1% of overnight culture was added to 50 ml LB and grown at 37°C to an OD_{600} of 0.38-0.42. The culture was then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was suspended gently in 10 ml of prechilled 0.1 M $CaCl_2$. The cells were incubated for 1 h on ice and then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml of 0.1 M $CaCl_2$ The competent cells were stored as 15% glycerol stocks in 100µl aliquots at -80°C.

Trausformation of competent cells

100µl of competent cells were thawed on ice and 5-10 ng of plasmid DNA or 50 ng equivalent of ligation reaction mix was added. The cells were incubated on ice for 30 min. Cells were then given a heat shock for 45 seconds at 42°C followed by a rapid chilling for

5 minutes. LB medium (900 µl) was added to the cells and the transformation mix was allowed to revive for 1 hour at 37°C at 220rpm. Transformants were plated on LB agar plate supplemented with appropriate antibiotic and incubated at 37°C for 14-16 hours.

Preparation of Plasmid DNA from E.coli transformants

Mini-preparation of plasmid DNA (Alkaline lysis method)

A single colony harbouring the desired plasmid was inoculated in 2 ml LB media containing appropriate antibiotic and grown overnight at 37°C. The cells were pelleted at 6000 rpm for 5 min and the supernatant was aspirated out. The pellet was suspended in 100µl of solution I (50 mM glucose; 25 mM Tris-Cl, pH 7.5; and 10 mM EDTA, pH 8). To the tube, 200µl of freshly prepared solution II (0.2N NaOH and 1% SDS) was added, mixed gently by inverting and incubated at RT for 2 min, 150µl of chilled solution III (3M Potassium Acetate, pH 5.5) was then added and the contents were mixed gently by inverting the tube and kept on ice for 5 min. The mixture was centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was transferred to a fresh tube and 0.7 volumes of isopropanol was added and centrifuged at 14,000 rpm for 5 min at 4°C. The pellet was washed with 70% ethanol by centrifugation at 14,000 rpm for 5 min at RT. The supernatant was discarded and the pellet was air-dried. The dried pellet was suspended in autoclaved distilled water or TE.

Midi-preparation of plasmid DNA (Alkaline lysis method)

Plasmid DNA was isolated essentially as described by Sambrook et al., (1989). One milli litre inoculum of E coli (DH10β cells containing the desired plasmid was added to 100ml of LB medium containing appropriate antibiotic. The culture was incubated at 37°C overnight in shaker incubator at 220 rpm. Cells were collected by centrifugation at 5000 rpm for 15 minutes at 4°C and the pellet was resuspended in 1.5 ml of ice cold Solution I (25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50mM Glucose) and incubated on ice for 10 minutes. 3 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added, mixed gently by inverting the tubes 3-4 times and incubated at room temperature for 10 minutes. 1.8 ml of ice cold Solution III (3 M Potassium acetate, pH 5.2) was added, mixed thoroughly and incubated on ice for 10 minutes. Cell debris was removed by centrifugation at 13,000 rpm for 15 minutes at room temperature. The supernatant was

transferred to fresh GSA bottles, 0.6 volume of isopropanol was added and the tube was incubated at room temperature for 15 minutes. Centrifugation was carried out at 13,000 rpm for 15 minutes and the supernatant was drained out. The pellet was washed with 70% ethanol, dried and resuspended in 1ml of TE with RNAse (final concentration of 10ug/ml). Following incubation at 37°C for 20 minutes, the DNA was deproteinated with phenol chloroform (1:1 vol/vol), precipitated by ethanol and finally resuspended in 500 ul of TE.

Agarose Gel Electrophoresis of DNA

DNA fragments of size >400 bp were resolved on 1% agarose gel, while those in the range of 100-500 bp were resolved on 1.5% agarose gel. The gels were electrophoresed in 1X TAE (40mM Tris Acetate; 1mM EDTA, pH 8.0) buffer containing 0.5 µg/ml ethidium bromide.

RNA Extraction, cDNA formation & RT PCR

Following requisite treatments and time periods, 1 ml of Tri Reagent was added to the cells (in 60 mm dish). The lysate was then incubated at room temperature for 5 minutes and 100µl of chloroform was added followed by a brief but vigorous shaking. Following incubation at room temperature for ten minutes, the mixture was centrifuged at 10,000g for 15 minutes at 4°C. The upper aqueous phase was carefully removed in a clean RNAse free tube and one starting volume of isopropanol was added and the mixture was then incubated at RT for 10 minutes. Thereafter, the samples were centrifuged at 10,000g for 20 minutes at 4°C to pellet down the RNA. The pellet was then washed with 75% ethanol (ice cold) followed by centrifugation at 10,000g for 5 minutes at 4°C. The pellet was air dried and resuspended in appropriate amount of formamide. First strand cDNA was generated using 3µg of total RNA, oligo(dT) primer, and MMLV reverse transcriptase in a total volume of 20µl at 37°C for 2 hours. Semi quantitative PCR reactions were performed using the Taq DNA polymerase and gene-specific primers for 20 cycles.

Preparation of Total Cell Extract and Immunoblotting

Following experimental treatments, adhering cells were washed with ice cold PBS, scrapped and collected by low speed centrifugation (3000 rpm) at 4°C followed by lysis in ice-cold lysis buffer containing 50 mM Tris pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM

EGTA, 1% NP-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, protease inhibitor cocktail and 1 mM PMSF. Cell lysates were then centrifuged at 13,000 rpm (at 4°C) and supernatant were saved at -80°C. Protein concentrations in the extracts were estimated by modified Bradford method (Bradford, 1976; Kruger, 1994). Appropriate aliquots of protein were diluted in 0.15M NaCl (100µl), One milliliter Bradford Reagent was added and the OD was recorded after 5 minutes at 595nm in a spectrophotometer (Cary, Eclipse). Equal quantities of protein samples (whole cell lysates, 100 µg) were resolved by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS/PAGE; 10% Acrylamide). Part of the protein gel (selected based on the molecular weight of the protein of interest) were sliced out and transferred to PVDF membranes in Towbin's buffer (25 mM Tris, 192 mM glycine and 20% methanol). Following transfer, the membranes were blocked for 4-5 h at RT with 5 % BSA (Bovine Serum Albumin) in TBS containing 0.05% Tween-20 (TBST). Blots were then incubated overnight at 4°C with respective primary antibodies at appropriate dilutions in TBST. Next day the blots were extensively washed with TBST (3 washes with each wash of 10 min) and incubated for 45 min at RT with respective secondary antibody (at appropriate dilutions) in TBST. Following removal of the secondary antibody, blots were extensively washed (3 washes with each wash of 10 min and one wash with TBS) and developed using the Enhanced Chemiluminescence detection system (ECL). When stripping was required, the membranes were incubated with 62.5mM Tris-HCL (pH 6.8), 2% SDS, 0.1M 2-mercaptoethanol for 20 min at 56°C and washed extensively with water and TBST before being subjected to reblocking and probing (Rebollo et al., 2000).

Subcellular fractionation:

A set comprising six dishes were harvested in phosphate buffered saline(pH 7.4) .after being grown to 80-90% confluency. The cells were pelleted at rmp 200g & 4°C for 7 min. The pelleted cells were resuspended in 5 ml of isolation medium containing(250 mM sucrose, 10 mM HEPES-Tris, pH 7.4, 1 mM EGTA-Tris). These cells were again centrifuged at rpm 200g & 4°C for 7 min. The pellet was again resuspended in 5ml of slightly hypotonic medium (100 mM sucrose; 10 mM HEPES-Tris pH 7.4; 1 mM EGTA-Tris) & homogenised using a syringe of 25 gauge pressure. The homogenisation was monitored using inverted microscope, as the homogenisation was complete 0.56µl of hypertonic medium (1.78 M sucrose.10 mM HEPES-Tris pH 7.4; 1 mM EGTA-Tris) was

added along with 0.44µl of isolation medium devoid of sucrose and supplemented with 2mM MgCl2. This was subjected to centrifugation at 200rpm&4°C for 7 min. The pellet thus obtained contained nuclear fraction & some unbroken cells in it. The supernatant was again centrifuged at 5000g for 10 min to obtain mitochondria as pellet. The resultant supernatant comprises microsome which was isolated as pellet after certifugation for 1hr at 40,000 rpm, 4°C. The supernatant thus obtained contains cytosol in it, which was concentrated using Millipore centricon 10,000 dalton; concentrating it to 400µl from 6 ml.

GST Pull down

Purification of bacterial protein:

pGEX4T3 containing various isoforms of SG2NA (vector only, 35kDa & 78kDa) along with GST tag were transformed in E.coli BL21(DE3). A single colony was picked & inoculated in 5 ml culture overnight at 37°C & 220 rpm. Next day 100ml of secondary culture was inoculated using 1% of primary culture. The secondary culture was induced by 1mM of IPTG O.D. at 600nm reaches 0.4. 2ml of uninduced culture was kept aside. Then lysozyme was added to a final concentration of 100µg/ml & kept for 15min at RT, along with 5mM DTT & 1mMPMSF. This was sonicated 12 times for a cycle of 30 sec with interval of 1 min. To above mix sarkosyl detergent was added to final concentration of 1.5% & incubated in ice for 15 min: this whole was centrifuged at 10,000rpm at 4°C for 10 min. Toabove triton X100 was added to final conc of 2%. This was kept at -20°C.

Preparation of nuclear extract for GST pull down assay:

NIH3T3 cells were harvested using 500µl of fractionation buffer comprising 250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA. Then cell lysate was passed through a 25 G (26 G) needle using 1 ml syringe. The homogenisation was monitored under phase contrast microscope. This was incubated for20 min in ice & then pelleted down at720g for 5 min at 4°C. The pellet was again resuspended in 500µl of fractionation buffer & centrifuged at 720g for 10 min. The supernatant was discarded & pellet was resuspended in nuclear buffer, e.g., STE buffer with 10% of glycerol.

Silver Staining for MALDI TOF/TOF Analysis

Silver staining was carried out according to Blum H et al., 1987, the gel is kept in fixative (50% CH3OH, 12% Glacial Acetic acid 100 µl HCHO for 200 ml solution) for 45 min to

overnight. Then keep the gel in 50% ethanol for 30 min followed by wash once with double distilled water. The gel was kept in Hypo solution (40 mg Sodium thiosulfate in 200 ml double distilled water) for 1 min. The gel was washed three times of 20 sec each with double distilled water. The gel was kept in silver nitrate solution (400 mg silver nitrate and 150 µl HCHO in 200 ml double distilled water) for 20 min followed by 3 washes with double distilled water each of 20 seconds. Then the gel was transferred to the developer solution (12 gm Na_2CO_3, 4 ml Hypo solution and 100 µl HCHO in 200 ml double distilled water) till the band appears. As the band appears the gel is immediately washed off with double distilled water. The staining is stopped with stop solution (40% ethanol, and12% acetic acid) and finally the gel is washed with washing solution (50% methanol). Finally the gel is kept in double distilled water.

Sample Preparation for Mass Spectrometric Analysis

Selected protein spots were individually excised into new tubes, and the isolated protein spots were destained with 100 mM sodium thiosulfate and 30 mM potassium ferricyanide and washed three times for 20 min with 50% acetonitrile/100 mM ammonium bicarbonate. After 5 min incubation with 50% acetonitrile, gel slices containing protein spots were treated with 10 mM DTT at 56 °C for 30 min and with 55 mM iodoacetamide at RT for 25 min in dark. They were then digested with trypsin at 37°C for 16h with shaking. The resulting peptide mixtures were extracted with 200 µl of 60% ACN and 0.1% trifluoroacetic acid (TFA) for 20 min with sonication, and the supernatant was removed. After pooling the supernatant into new tubes, peptide samples were dried completely using SpeedVac system for 4 h. Samples were dissolved in 50% acetonitrile/0.1% TFA, co-crystallized by mixing with matrix (α-cyano-4-hydroxy-cinnamic acid saturated with 0.1% TFA/50% acetonitrile) and loaded on the silcon-coated 396 well microtiter ground plate (Bruker Daltonics).

Mass Spectrometric Analysis

Mass Spectrometric Analysis of peptide fragments obtained by Tryptic digestion of purified recombinant GST tagged SG2NA variants were excised from CBB stained SDS-PAGE was done using Autoflex II MALDI-TOF/TOF, Bruker Daltonics, Germany.

Results

Results:

Generation of myc-SG2NA expression cassette selectable by puromycin (pcDNA3.1):

SG2NA has multiple splice variants that are modulated during the progression of cell cycle (Ph. D. thesis submitted by Pooja Chauhan, 2012). Therefore, to delineate the role of each variant in the process, we wanted to overexpress them one at a time in NIH3T3 cells. Accordingly, expression cassettes for 35, 78, 52 and 87 kDas were made in pcDNA3.1 harbouring neomycin as the selectable marker. However, the overexpressing clones upon several passages turned of the respective isoforms of SG2NA. Upon further investigation , it was shown that neomycin itself induces epigenetic modulation and silences the expression of certain genes (Datta et al., manuscript submitted). Hence to circumvent that problem, we decided to construct the expression vector in pcDNA 3.1 with puromycin as the selectable marker. Plasmids harbouring 35 kDa, 78 kDa and 87 kDa SG2NA cDNAs in pcDNA3.1 (neomycin) were used as templates for PCR amplifying the entire backbone devoid of neomycin casette using primers. (Table 1 and Fig 1).

The amplified fragments were then ligated to the puromycin expression cassette obtained from restriction digestion of digestion of pGFP-V-RS vector with *Hind III* & *Cla I* and filling the overhangs by the Klenow fragment (Fig 2). The recombinant plasmids were then confirmed by restriction digestion with *NcoI* enzyme generating fragments of 3868 bp & 2224 bp for the 35 kDa isoform and 3869 bp & 3456 bp for 78 kDa isoform respectively (Fig 3). The candidate clone for 87 kDa SG2NA turned out to be incorrect and not pursued further.

Hence, the two recombinant plasmids were generated as the expression vectors for 35 kDa and 78 kDa SG2NAs (myc tagged) with puromycin as the selectable marker. The plasmids were then transfected into CHO (Chinese Hamster Ovary) cell line using Escort IV reagent. Cells were harvested 48 hour after transfection and RNA was isolated. RT-PCR analysis was carried out after checking the integrity of RNA using puromycin specific primers (Table 1 and Fig 4, upper panel). To further confirm the expression of puromycin, western blot was also done with the cell lysate using anti-myc antibody confirming the expression of respective SG2NAs (Fig 4, Lower panel).

Table 1: Primers used for PCR amplifications:

AMPLIFICATION PRODUCTS	PRIMER PAIRS
SG2NA expression vector in pcDNA 3.1 devoid of the neomycin expression casette	_Puro (f): 5'GGTTCACGTAGTGTGGTGTGGAAAGTCCCCA GGC 3' _Puro (r): 5' CATTTCGAATCAGGCACCGGGCTTGCG 3'
Puromycin cDNA for confirming its expression (by RT-PCR)	puro forward: 5' GAGCTGCAAGAACTCTTCCTC 3' puro reverse: 5' GCTCGTAGAAGGGGAGGTTGC 3'

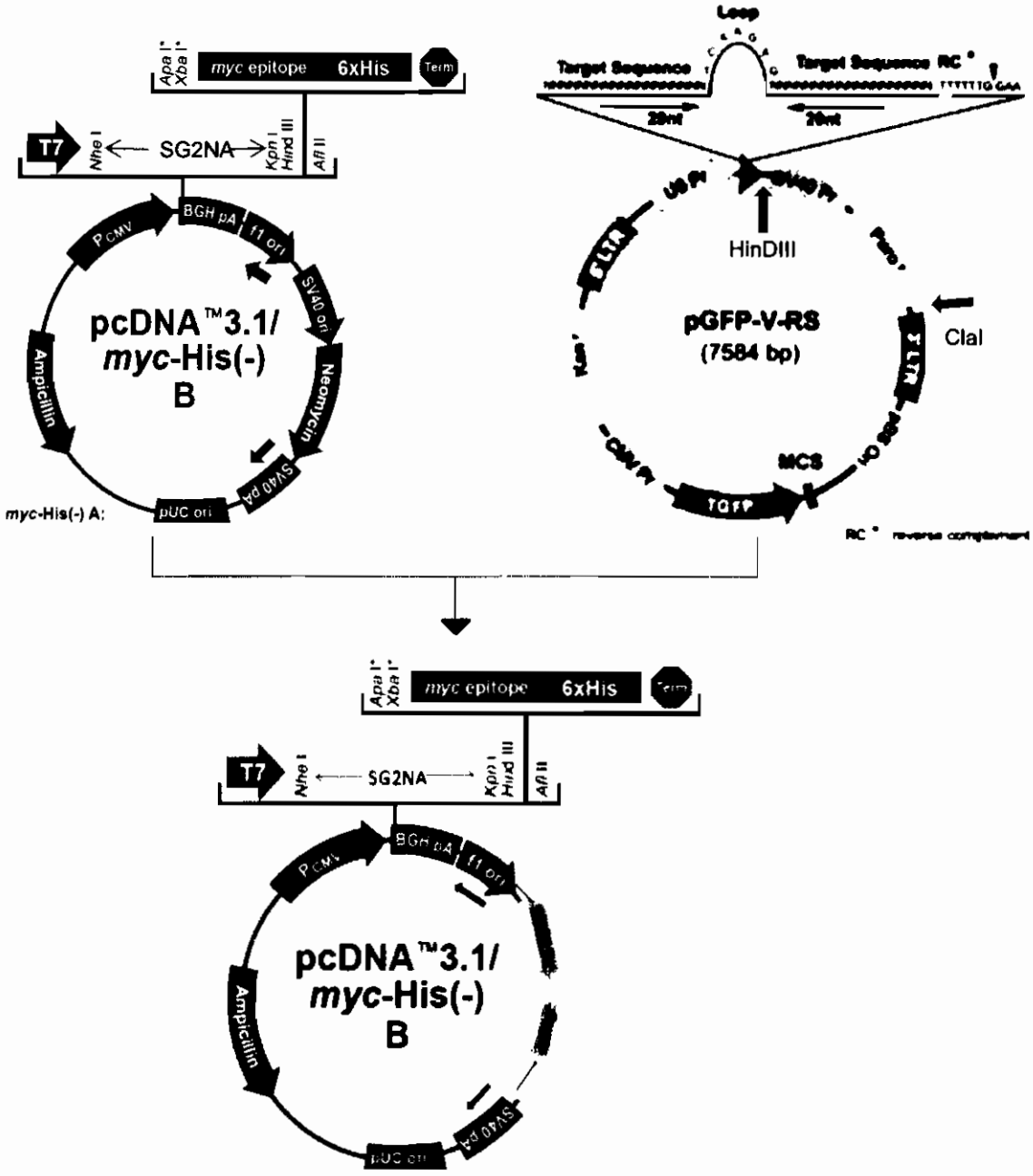


Figure 1: Strategy for the construction of expression vector for 35 and 78 kDa SG2NAs with puromycin as selection marker. Upper panel (left): expression vector for SG2NA in pcDNA 3.1 vector with neomycin as selection marker. This vector was used as a template for amplifying the expression cassette devoid of the neomycin expression system (forward primer spans the SV40 polyadenylation site and the reverse primer spans the f1 origin). Upper panel (right): plasmid pGFP-V-RS (used for expressing shRNAs) was digested with restriction enzymes *Hind-III* & *Cla-I* to release the puromycin casette followed by end filling by Klenow fragment to create blunt ends. Lower panel represents the recombinant expression vector for SG2NA variants with puromycin as the selection marker obtained after ligation of puromycin expression casette and the amplified vector backbone of SG2NA constructs devoid of neomycin casette.

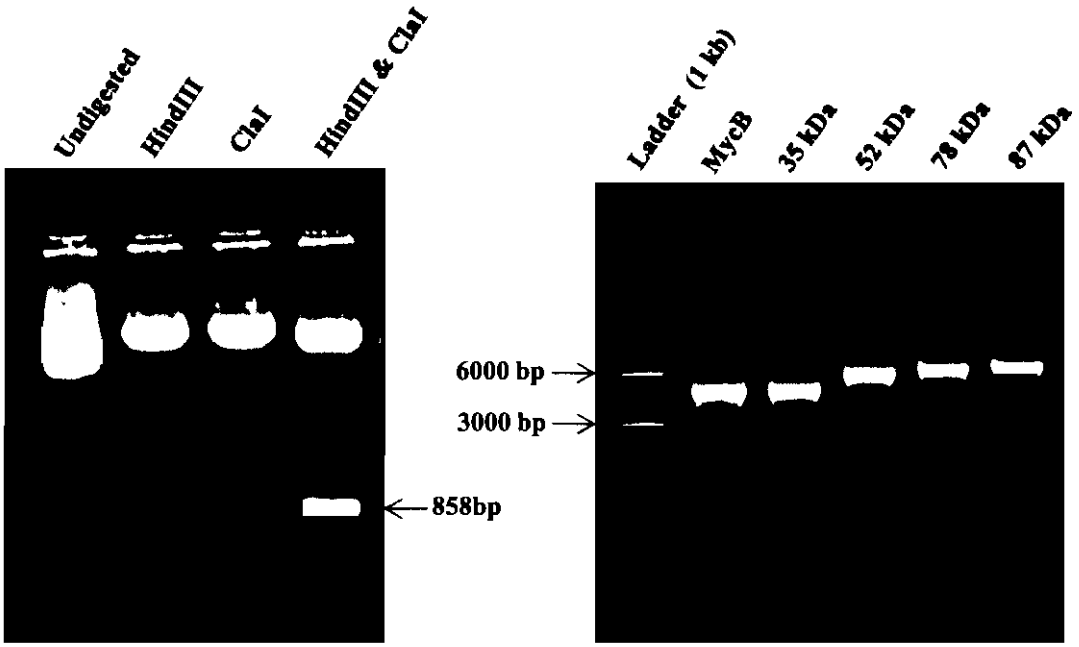


Figure 2: Cloning of puromycin expression casette in SG2NA-pcDNA 3.1 vector backbone. (Left) Restriction digestion of GFP-V-RS plasmid with *HindIII* & *ClaI*. Upon digestion with *HindIII* and *ClaI*, 858 bp fragment containing SV40 promoter and the puromycin ORF was released. (Right): SG2NA expression vectors for various isoforms were PCR amplified using forward primer spanning the SV40 polyadenylation site and the reverse primer spanning the f1 origin. The amplified product was devoid of neomycin expression casette.

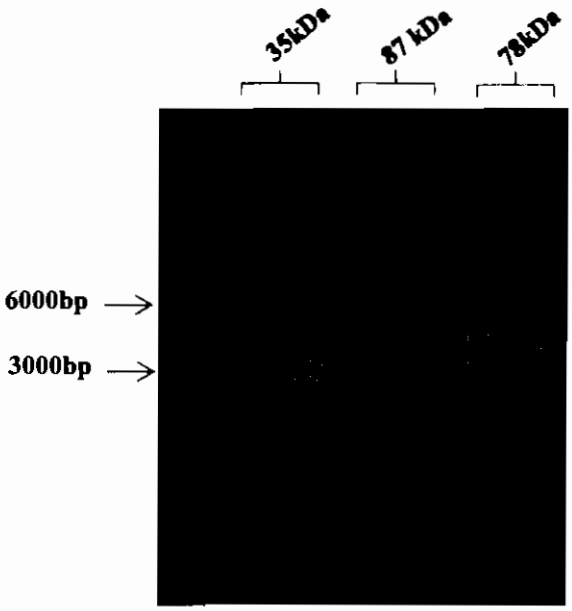


Figure 3. Confirmation of recombinant plasmids with expression cassettes for SG2NA and puromycin. The candidate recombinant plasmids were digested with restriction enzyme *Nco-I*. Two recombinants were identified as shown in lane 2 and 7 that gave correct size of fragments i.e., 3868 & 2224 bp and 3869 & 3456 bp for 35 and 78 kDa SG2NAs respectively. The candidate clone for 87 kDa SG2NA turned out to be incorrect and not pursued further.

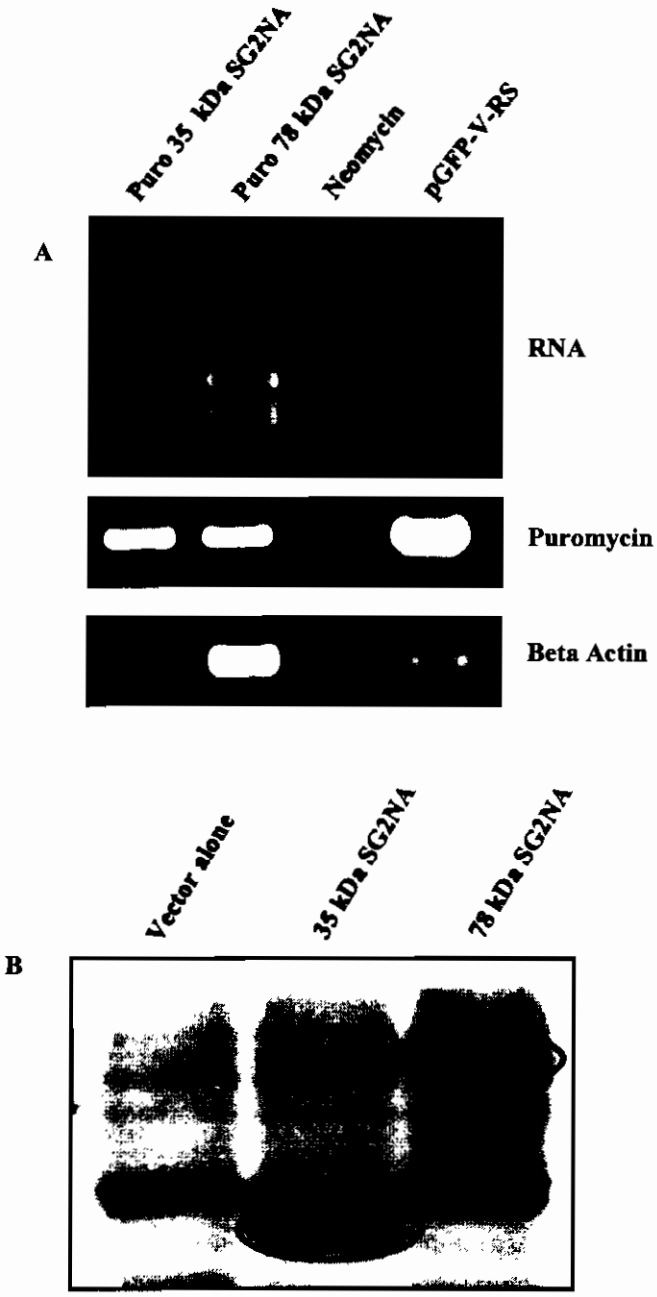


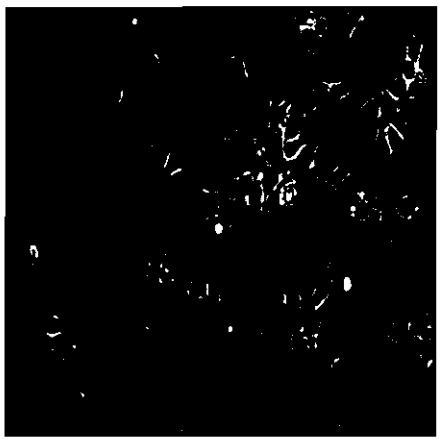
Figure 4: Expression analysis of recombinant puro-SG2NA pcDNA 3.1 constructs: (A) RT-PCR analysis of the RNA isolated from CHO cells transiently transfected with 35 kDa SG2NA-Puro, 78 kDa SG2NA-Puro, 35 kDa SG2NA-Neo (negative control) and pGFP-V-RS (positive control) expression vectors using primers specific for puromycin. Upper panel, integrity of RNA. Mid panel, amplification with primers specific for puromycin. Lower panel, control amplification with primers for beta-actin. (B) Western analysis using myc antibody confirms expression of myc tagged 35 kDa and 78 kDa SG2NAs as marked by circles.

Generation of cell lines stably expressing myc-SG2NAs:

The recombinant vectors, pcDNA3.1-puro harbouring 35 kDa and 78 kDa SG2NAs were linearized with *Pvu I* (6092 and 7235 bp respectively) and transfected into NIH3T3 cells (mouse embryonic fibroblasts). However, upon selection with puromycin (1μg/ml), no clones were obtained. To address this issue, another transfection was done along with linearised pGFP-V-RS plasmid as a tracker (expressing GFP). Forty eight hours after transfection, cells were scanned under fluorescent microscope and no fluorescence was seen in cells transfected with the vector for 35 kDa SG2NA, while that for the 78 kDa SG2NA, only a single cell in the field was seen expressing GFP (Fig 5). So, it was concluded that transfection efficiency was very poor in NIH3T3 cells. Thereafter, transfection was done in Hela cells. First, a kill curve was made for varying concentrations of puromycin and the optimal concentration required for the selection was determined to be 1.5μg/ml (Fig 6). Linearised 35 kDa and 78 kDa SG2NA-Puro plasmids were transfected into Hela cells using Escort IV reagent. 72 hrs post-transfection, 1.5 μg/ml of puromycin was added to the medium for selection. Untransfected cells started dying after 3-4 days and small isolated colonies of cells resistant to puromycin were seen. Those colonies were picked up by trypsinizing in glass well and transferred to 35 mm dishes (Fig 7). However, those clones did not survive for more than 2 weeks after transfer. Hence, the clonal cell lines stably expressing 35 kDa and 78 kDa SG2NAs could not be generated.

Subcellular distribution of SG2NA in serum starved and serum stimulated cells: Initial study claimed that SG2NA is a nuclear antigen with augmented expression during S to G2 phase of the cell cycle (Landberg & Tan, 1994). Upon identifying its splice variants, we also tested its modulation by serum, norepinephrine and insulin in NIH3T3 and H9c2 cells (mouse fibroblasts and rat cardiac myoblasts respectively) by immunoblot analysis. Isoforms of SG2NA except 82 kDa are upregulated upon growth stimulation (Sanghamitra et al, 2008; Talukdar I, Ph.D thesis., 2011). By using both immunocytochemistry and GFP tagging (of individual variants i.e., 35, 52, 78, 87 kDa SG2NAs), we also had observed that when NIH3T3 cells are stimulated by serum for 24 hours, all the isoforms partially translocate to the nucleus while the duration of their stay in the nucleus might vary from 4-8 hours (Chauhan P, Ph. D Thesis., 2012). We thus confirmed this observation by biochemical assays. NIH3T3 cells were grown in DMEM to 80-90% confluence, synchronized by serum starvation (0.5% FBS) for 24 hours and then stimulated with 10%

35 kDa SG2NA-Puro Plus pGFP-V-RS



78 kDa SG2NA-Puro Plus pGFP-V-RS

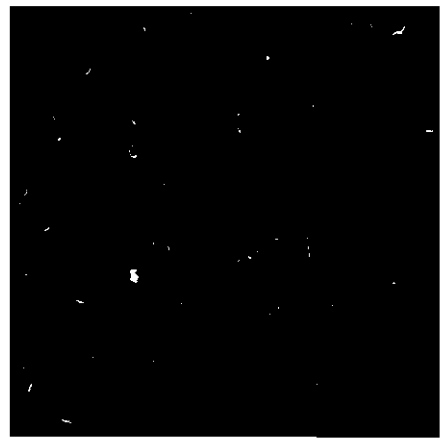


Figure 5: Expression of recombinant Puro-SG2NA pcDNA 3.1 constructs in NIH3T3 cells. Linearized 35 kDa- and 78 kDa SG2NA-Puro plasmids were transfected into NIH3T3 cells along with pGFP-V-RS as a tracker (GFP). No green fluorescent cells were seen in cells transfected with 35 kDa vector while in case of 78 kDa vector, one cell was seen in one field. It was thus concluded that the transfection efficiency was very low in NIH3T3 cells.

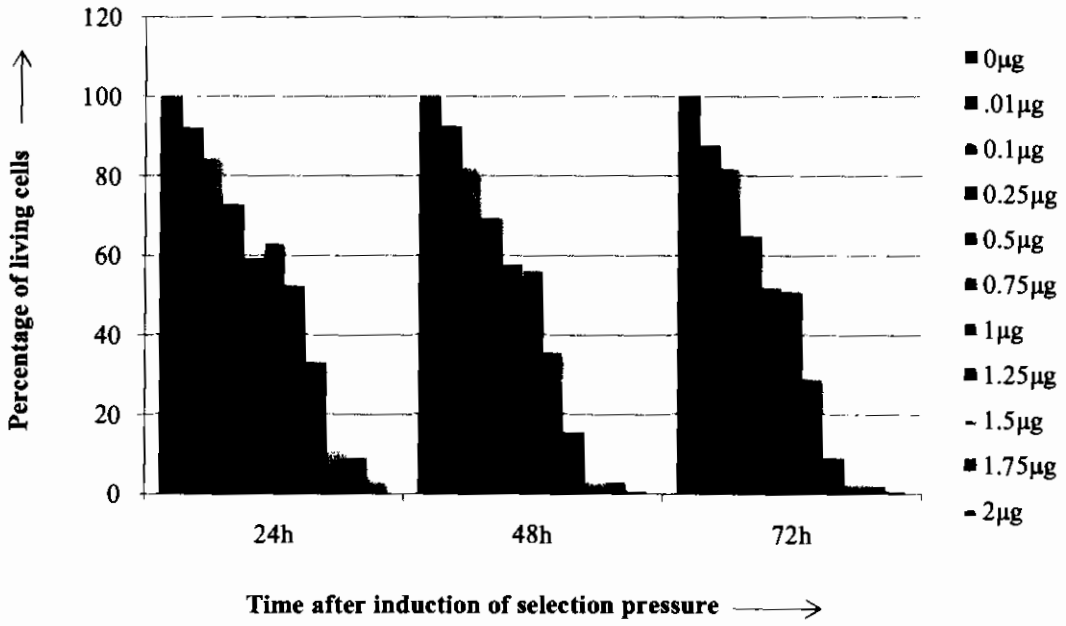


Figure 6: Puromycin kill curve for HeLa cells. HeLa cells grown on 35 mm dishes were treated with various concentrations of puromycin as indicated and cell viability was assayed by MTT reagent.

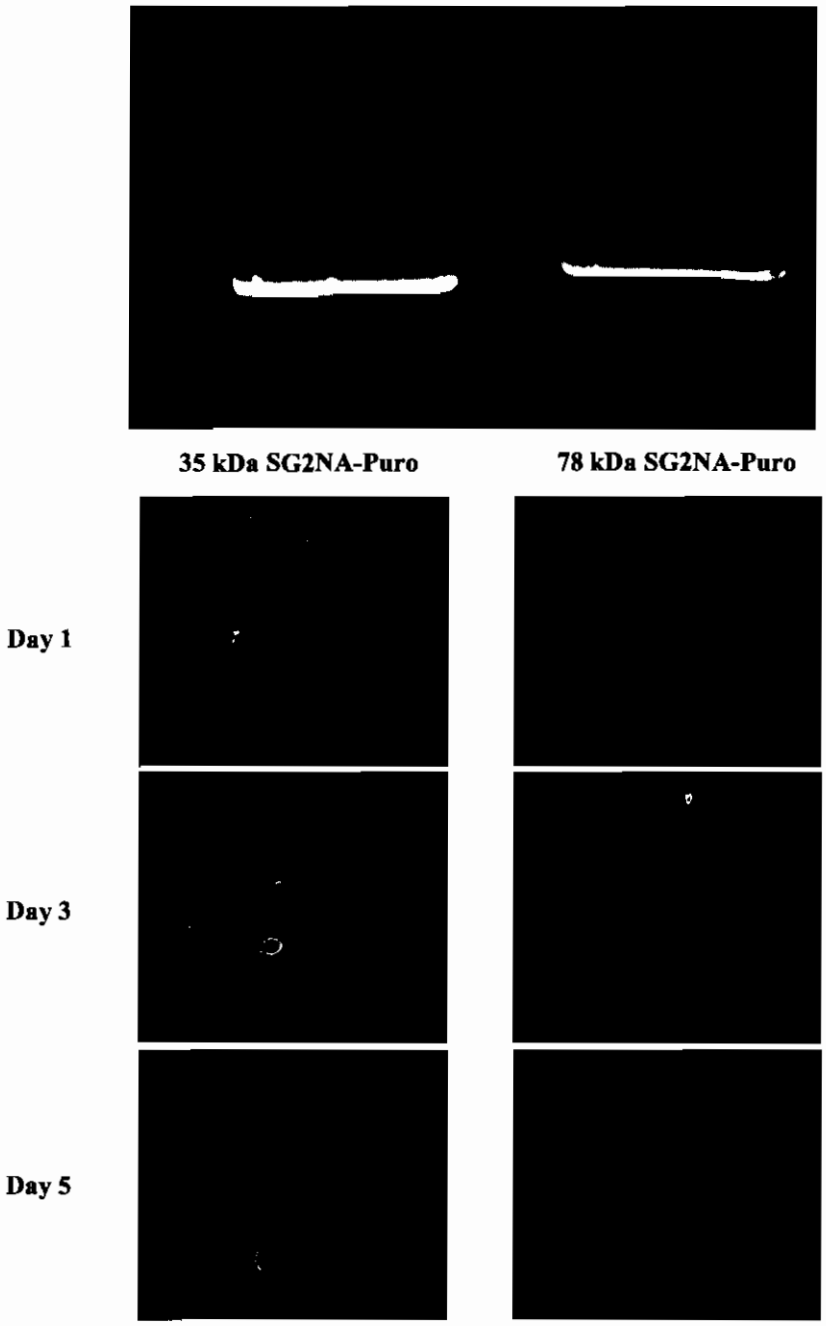


Figure 7: Generation of stable cell lines of recombinant Puro-SG2NA pcDNA 3.1 constructs in Hela cells. Linearised expression vectors for 35 kDa and 78 kDa SG2NAs (upper panel) were transfected in to Hela cells and selected in puromycin (1.5 μg/ml) containing medium. Untransfected cells died after 3-4 days and the surviving clones resistant to puromycin were transferred into new dishes which died thereafter (lower panel as depicted by the Phase Contrast images).

FBS for 6 hours to proceed through cell cycle phases. Cells were then harvested, fractionated into nuclear, microsomal, mitochondrial and cytosolic fractions, and analyzed by immunoblotting using mouse monoclonal anti-SG2NA antibody. As shown in Fig 8, such analysis demonstrated that serum stimulation results in the augmentation of expression of SG2NA in all the organelles but more so in microsomal and cytosolic fractions. Also, the 78 kDa isoform was the predominant isoform found in all the organelles, while its most robust increase was in the cytosolic extract followed by nucleus and microsomal fractions. On the other hand, the 87 kDa isoform was seen only in cytosol while the 82 kDa isoform was present solely in the mitochondria in serum stimulated cells. The lower molecular weight but yet unknown isoforms of SG2NA were more in the nuclei and in mitochondria under serum stimulated condition. These results thus suggest that upon serum stimulation, all isoforms of SG2NA get induced and are redistributed into various cellular compartments.

Identification of nuclear interacting partners of SG2NA at S phase:

As stated earlier, striatin family members are characterized by a WD-40 repeat domain at their C terminus, which enable them to act as an interaction platform for various proteins. Being a member of striatin family, SG2NA is expected to interact with a variety of proteins, which might play roles in membrane trafficking and cell cycle progression. Keeping it in mind, we attempted to identify the interacting partners of SG2NA in the nucleus in the S phase of cell cycle. Recombinant GST-35 kDa and 78 kDa SG2NAs variants were purified from *E coli* lysate using glutathione sepharose beads (Tanti G K, 2007; M Phil thesis) (Fig 9). NIH3T3 cells were grown to 80-90% confluence, synchronised to G1 phase of cell cycle by serum deprivation for 24 hours followed by serum stimulation for 6 hours. Nuclear extracts were prepared and allowed to bind to GST-SG2NAs on immobilized GST bead on a rotatory platform at 4°C. Protein bound to immobilized SG2NAs were then resolved on 10% SDS-PAGE followed by silver staining (Fig 10A). Individual bands specific for SG2NAs and lacking in vector control were excised, processed and analysed by MALDI-TOF/TOF (Fig 10B). The interacting partners thus identified are shown in Table 2.

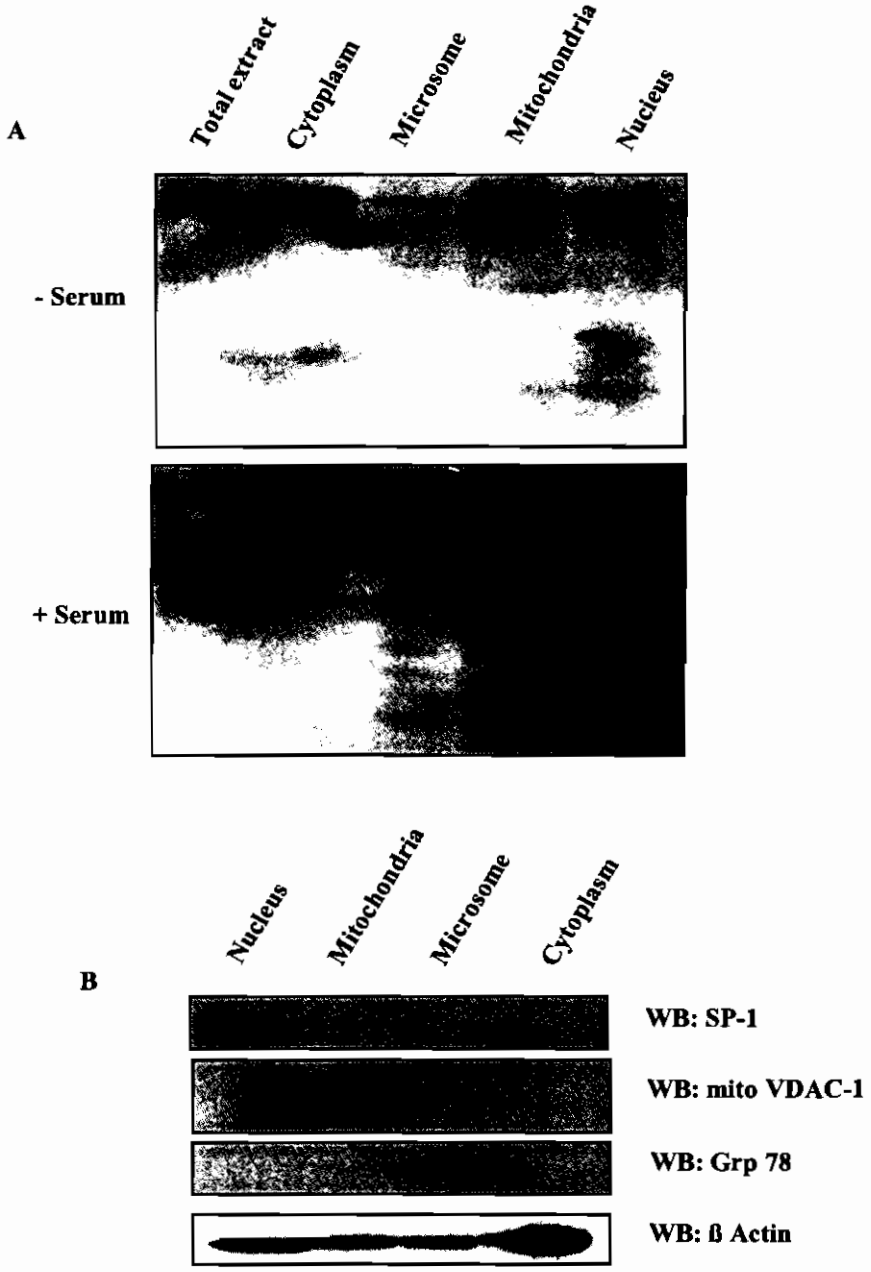


Figure 8. Subcellular distribution of SG2NA isoforms in serum starved and serum stimulated cells. (A) NIH3T3 cells were kept in serum free medium for 24 hours followed by serum (10%) stimulation for 6 hours. Cells were then harvested, fractionated into cytosolic, mitochondrial, nuclear and microsomal fractions and immunoblotted with antibody against SG2NA. (B) Validation of subcellular fractionation. The authenticity of the different fractions was tested by western blotting using organelle specific marker antibodies: Transcription factor SP1 for nucleus; Voltage-dependent anion channel-1 (VDAC-1) for mitochondria; GRP78 (ER specific) for the microsomes (ER and plasma membrae), and ß actin for cytosol.

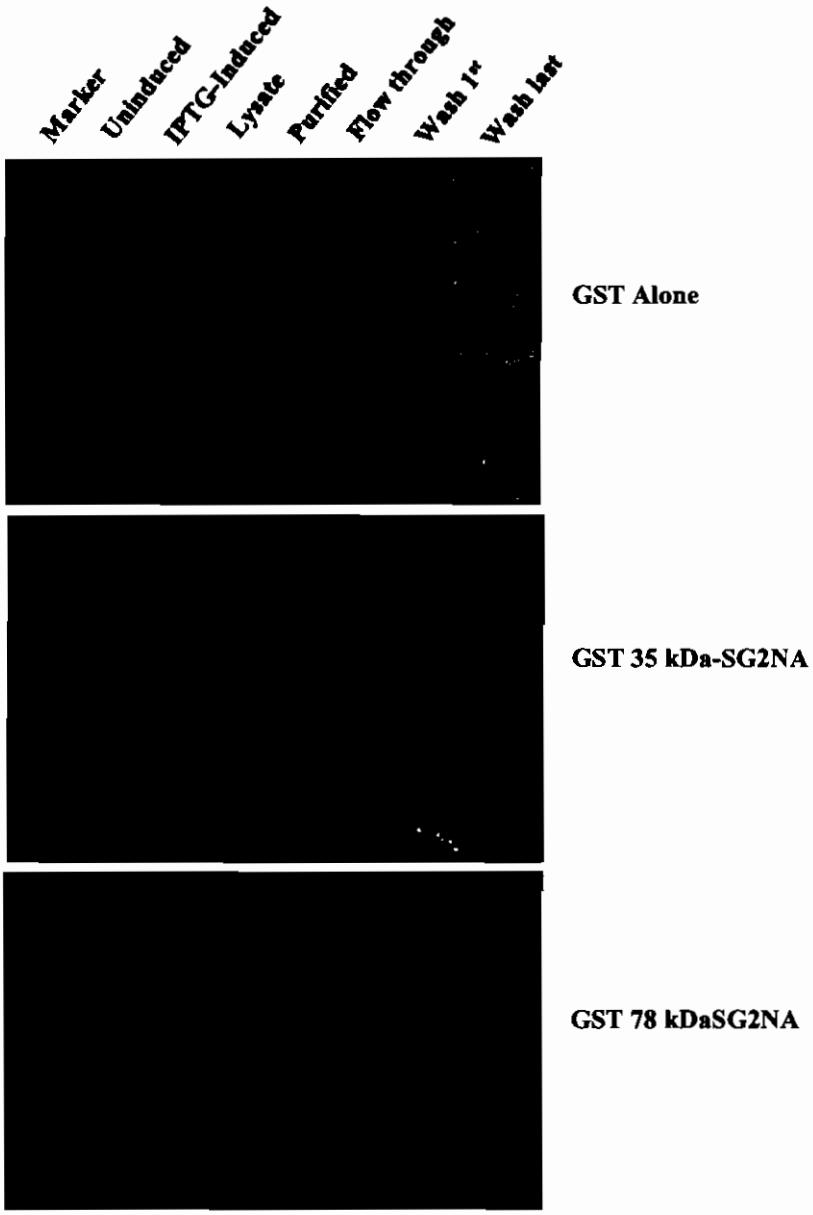


Figure 9. Purification of GST tagged 35 and 78 kDa SG2NAs for pull down assay. Recombinant GST tagged SG2NA variants were induced in *E.coli* by 1mM IPTG, lysates were prepared and allowed to bind to glutathione beads. Fractions from various steps of purification were loaded on 10% SDS PAGE & stained with CBB.

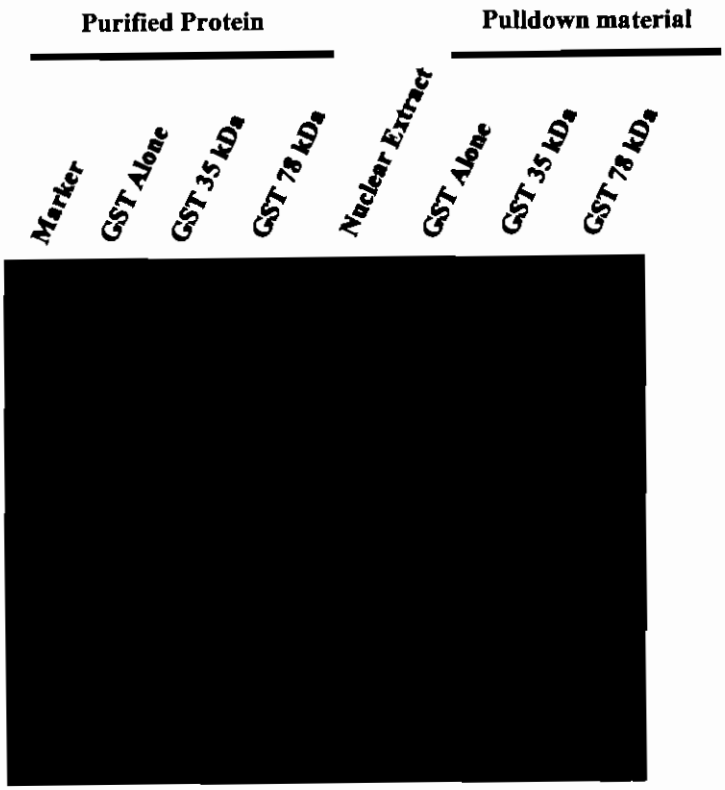
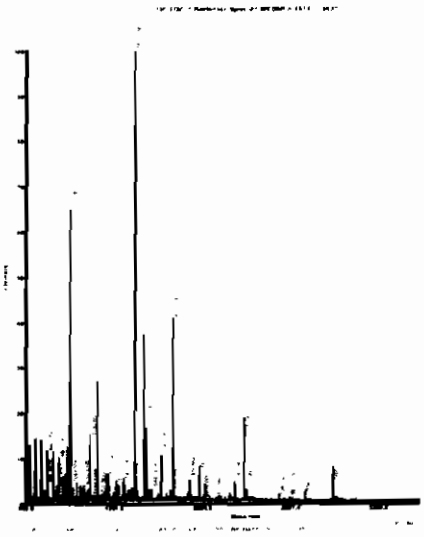


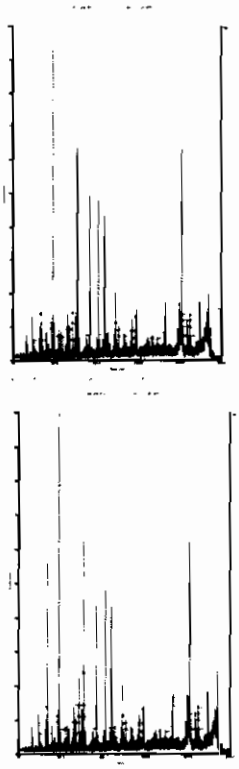
Figure 10A. GST pull down assay for identifying interacting partners of 35 and 78 kDa SG2NAs in the nucleus. Purified GST SG2NA immobilized on GST beads were incubated with nuclear lysate from NIH3T3 cells, resolved on 10% SDS and silver stained.

Table 2: SG2NA interacting proteins and their function as revealed by GST Pull down followed by Mass Spectrometry

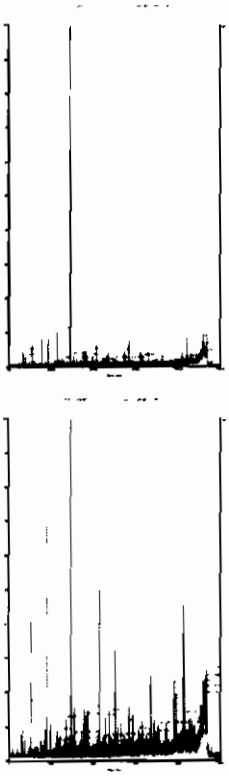
INTERACTING PROTEINS	35 kDa	78 kDa	FUNCTION	SCORE	SEQUENCE COVERAGE
Hemi desmosomal Plaque protein	+	+	Cytoskeletal protein.	45	7%
Rock2	-	+	Actin polymerization & cell migration by contraction.	44	13%
Neurofilament	+	+	Cytoskeletal protein in neurons.	43	24%
MARK 3	+	+	Phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding	39	21%
Anexin 3	+	+	Bind membrane in calcium dependent manner.	38	32%
HAOX2	+	+	This enzyme belongs to the family of oxido-reductases, specifically those acting on the CH-OH group of donor with oxygen as acceptor. The systematic name of this enzyme class is **(S)-2-hydroxy-acid:oxygen 2-oxidoreductase**	42	33%
Nucleophosmin	+	+	B23/Nucleophosmin is a major nucleolar phospho-protein mainly located within the nucleolar granular compartment, where preribosomes are assembled.	48	31%
UBTD 1	+	+	UBX domain-containing protein 1 (UBXN1 or SAKS1) is a component of a complex required to couple deglycosylation and proteasome-mediated degradation of misfolded proteins in endoplasmic reticulum that are retro-translocated in the cytosol	39	29%
GFAP	+	-	Glial fibrillary acidic protein is probably involved in controlling the shape, movement, and function of astroglial cells	40	32%



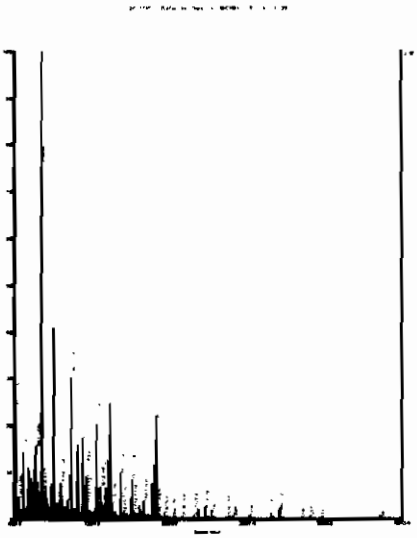
HAOX2 MS Spectra



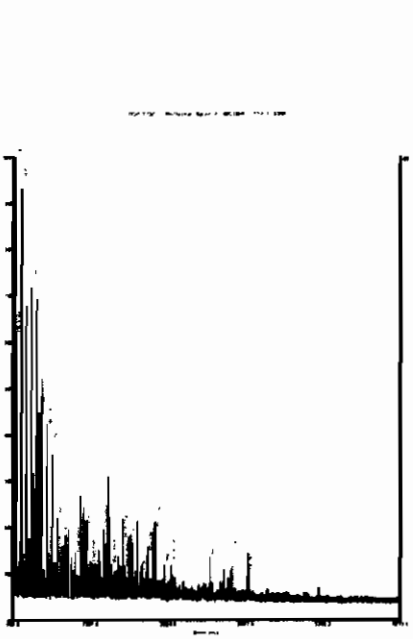
HAOX2 MS/MS Spectra



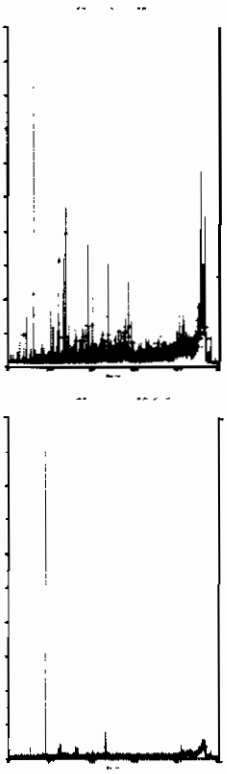
MS/MS Spectra of MARK3



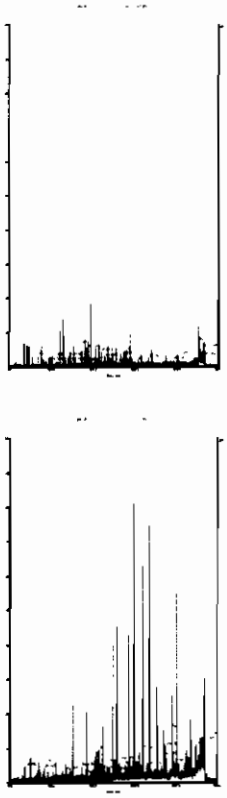
MS Spectra of MARK3



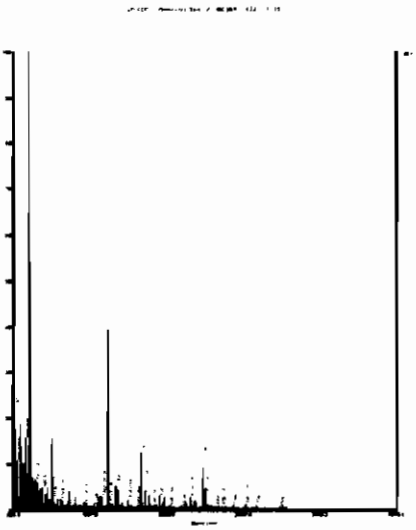
MS Spectra of GFAP



MS/MS Spectra of GFAP



MS/MS Spectra of Nucleophosmin

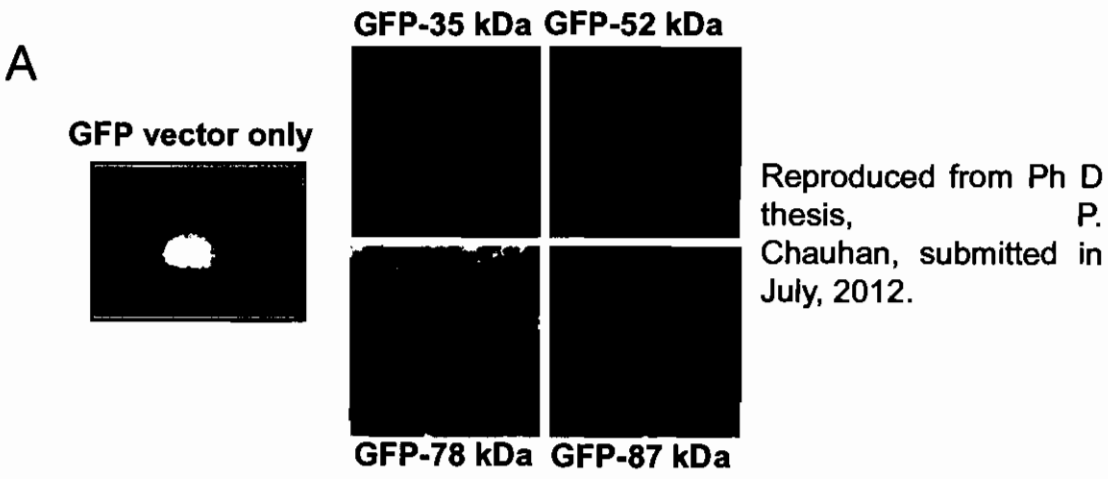


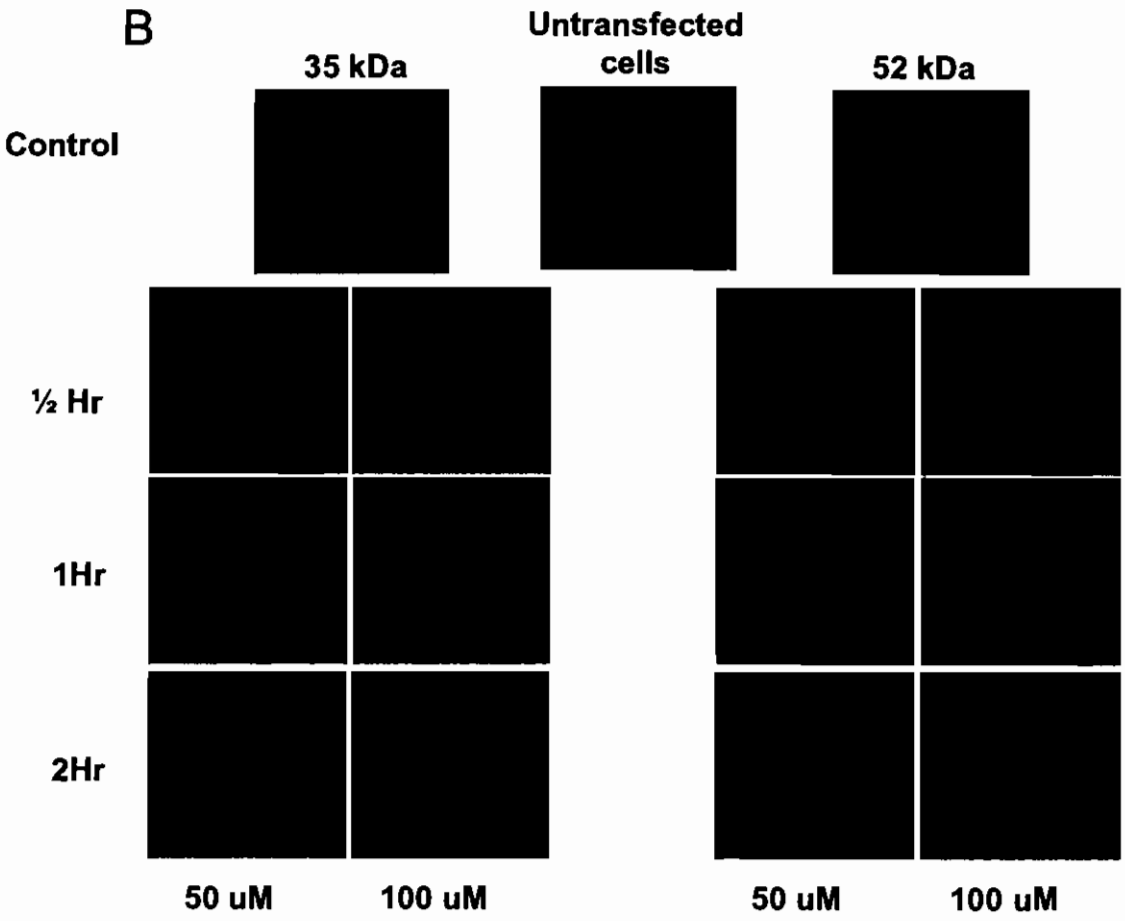
MS Spectra of Nucleophosmin

Figure 10B : Mass Spectra and MS/MS spectra of some of the identified interacting proteins of SG2NA.

Modulation of subcellular localisation of SG2NA by hydrogen peroxide:

We have observed that in serum deprived quiescent cells, SG2NA variants are also modulated and transiently localize in the nucleus (Chauhan P, Ph. D Thesis., 2012). Therefore, SG2NAs might have a role in both growth and stress responses. We thus monitored the dynamics of GFP tagged SG2NAs in NIH3T3 cells (stably transfected; Chauhan P, Ph. D Thesis., 2012) treated with hydrogen peroxide as a stressor. However, we noticed that those clonal cells developed in 2010, now showing much lower levels of GFP-signals, especially in case of GFP-78 and 87 kDas (Fig 11A). Incidentally, the other two cell lines that are expressing 35 and 52 kDas had slightly higher level of fluorescence than the background (Fig 11A). Nonetheless, we pursued with those cells by synchronizing them in serum free medium, followed by treatment with hydrogen peroxide (50 and 100μM) for 30min till 2 hour. As shown in Fig 11B, either doses of hydrogen peroxide induced the expression of GFP for all four variants but it was more robust in case of the higher dose. Also, the kinetics of induction was different for different isoforms (35 kDa: 1 and 2hr; 52 kDa: 4hr; 78 kDa: 1 hr; 87 kDa: ½ hr). Also, a small fraction of increased GFP was nuclear localized as seen in case of 35 kDa, 100 μM H_2O_2, 1hr; 52 kDa, 100 μM H_2O_2, 2hr; 78 kDa, 100 μM H_2O_2, 1-2 hr; 87 kDa, 100 μM H_2O_2, 1/2hr. Taken together, it appears that SG2NAs also play isoform specific distinctive roles in mediating stress response by partial nuclear localization. It would have been interesting to know their interacting partners in the nucleus under oxidative stress but such study could not be pursued due to time constraint.


A
GFP vector only
GFP-35 kDa
GFP-52 kDa
GFP-78 kDa
GFP-87 kDa
Reproduced from Ph D thesis, P. Chauhan, submitted in July, 2012.


B
35 kDa
Untransfected cells
52 kDa
Control
½ Hr
1Hr
2Hr
50 uM
100 uM
50 uM
100 uM

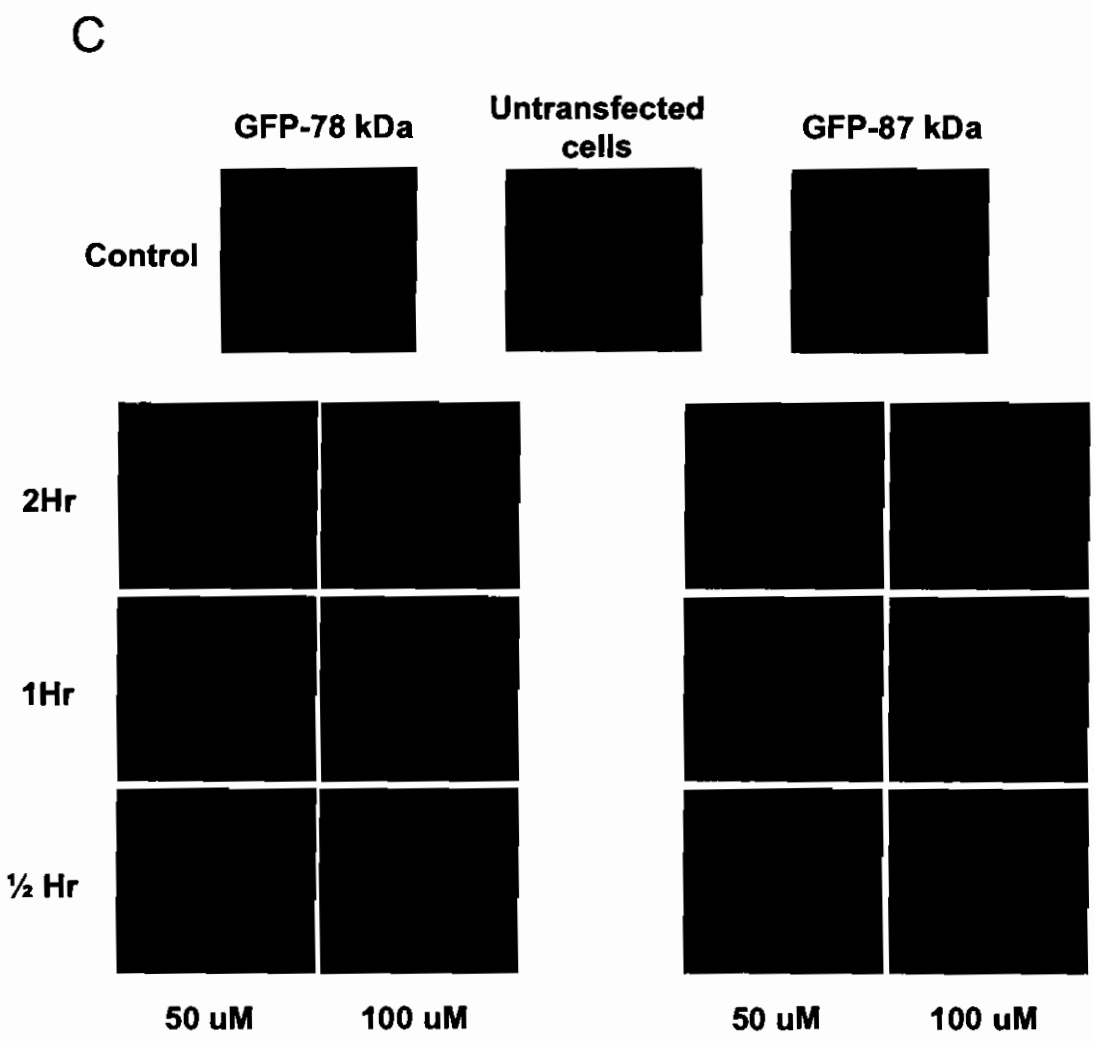


Figure 11: (A). Higher expression levels of GFP-SG2NAs in stably transfected NIH3T3 cells as originally developed in 2010. Speckle shaped expression of recombinants vis-à-vis diffused expression in vector control suggests specific localization of SG2NAs. Cells stably expressing GFP tagged (B) 35- and 52 kDa SG2NAs and (C) 78- and 87 kDa SG2NAs were synchronised by serum starvation for 16 hrs followed by treatment with different 50 & 100μM H_2O_2 for ½ till 2 hours. Cells were then fixed and analysed by confocal microscopy. The nucleus was counter stained with Hoechst 33342 (blue).

Discussions

Discussions:

Accumulating evidences over the past decade strongly suggest that Striatin family members play key roles in vesicular trafficking, cell cycle, signaling, and differentiation. Although in early studies, SG2NA was shown to be involved in cell cycle regulation and Striatin in signal transduction and vesicular trafficking (Benoist et al., 2006; Muro et al., 1995); a recent study has shown role of Striatin in cytokinesis as well (Hyodo et al., 2012). Taken together, such disparate functions by these proteins is suggestive of context specific activities as signal scaffolds.

Our interest in SG2NA came from the observation that it has multiple splice variants that are located in key organelles like mitochondria, endoplasmic reticulum, golgi body, as well as in plasma membrane and cytosol. Also, most of the isoforms are modulated during the progression of cell cycle. We thus hypothesize that isoforms of SG2NA might have similar but distinctive roles in cell function. To explore that hypothesis, attempts were made in developing cell lines stably overexpressing 35 and 78 kDa variants SG2NAs. Unexpectedly, those cell lines did not survive subsequent subcultures. One possibility could be higher level of overexpression might be toxic to cell in long term making them susceptible to death. Since stable integration of an ectopic cDNAs into the genome is not site-specific, and hence vary in expression levels; future attempts could be made to develop a repertoire of such cells with varying degree of expressions.

Nevertheless, potential role of SG2NA in cell cycle regulation was further explored by looking for its interacting partners in the nucleus. We, in our laboratory have already observed that larger variants of SG2NA (52, 78, 87 kDas, devoid of the WD-40 repeats) interact with multiple cytosolic proteins including DJ-1 (Tanti G, unpublished work), a redox responsive protein associated with Parkinson's and cancer (Wilhelmus et al., 2012). Hence, studies on interacting partners of SG2NA in the nucleus, wherein it only transiently localize, might further expand our knowledge of its versatile role in cell function.

The repertoire of interacting partners thus identified, is quite versatile and includes those known to be cytosolic, extracellular, ER localized etc. Nevertheless, seven out of nine were found to be associated with both 35 kDa and 78 kDa SG2NAs, and hence cannot be ruled out to be *in vitro* artifacts. Importantly, at least one, i.e., phosphoprotein

nucleoplasmin is a nuclear resident protein with highly versatile function including ribosome biogenesis, genomic stability, DNA repair, centrosome duplication and inhibition of caspase-activated DNase (Colombo et al., 2011). Although it is too speculative, considering the diverse function of nucleoplasmin, SG2NA might be a scaffold for certain specific functions in a given cellular context. Further validation by assays such as coimmunoprecipitation etc. might shed light on this possibility.

Summary & Conclusions

Cells exhibit an array of specific responses towards various stimuli which are mediated by a wide range of signalling networks. These networks comprise of disparate as well as similar pathways including numerous ligands, receptors, mediators, effectors etc. which are assembled into specific complexes via scaffold proteins (Buday and Tompa, 2010; Zeke et al., 2009). Amongst various frameworks of studying orchestrated cellular responses, is cell cycle regulation; that involves a large number of regulatory modules.

We, in our laboratory have demonstrated that SG2NA has multiple splice variants that are preferentially but not exclusively located in organelles like mitochondria, endoplasmic reticulum, golgi body; as well as in plasma membrane and cytosol. Most of these isoforms (except 82 kDa) are modulated during the progression of cell cycle and under quiescent condition. Also, in both growing and quiescent cells, isoforms of SG2NA transiently localize to the nucleus. The work embodied in this thesis was thus directed towards gaining insights into the role of SG2NA variants in cell growth/cell cycle progression and stress responses via developing cell lines (NIH3T3) stably expressing recombinant (myc tagged) SG2NAs and identifying its interacting partners in the nucleus.

The results reveal that higher level of over expression (35 and 78 kDa variants of SG2NAs) might be toxic to cell in long term making them susceptible to death. Potential role of SG2NA in cell cycle regulation was further strengthened by the identification of its interacting partners in the nucleus. MALDI analysis revealed one of the interacting partners to be phosphoprotein nucleoplasmin, a nuclear resident protein with highly versatile function including ribosome biogenesis, genomic stability, DNA repair, centrosome duplication and inhibition of caspase-activated DNase (Colombo et al., 2011). Although it is too speculative, considering the diverse function of nucleoplasmin, SG2NA might be a scaffold for certain specific functions in a given cellular context. Further validation by assays such as co-immunoprecipitation etc. might shed light on this possibility.

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