To understand the role of α-1-Syntrophin (SNTA-1) protein in signal transduction pathway involving RhoGTP'ase Rac1

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BY

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DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

This is to certify that the work contained in this thesis entitled ***"To understand the role of α-1-Syntrophin (SNTA-1) Protein in signal transduction pathway involving RhoGTP'ase Rac1,"*** is the bonafide research work of **Ms. Hina Fayaz Bhat,** and is a worthy of consideration for the award of Doctor of Philosophy in Biotechnology.

Dr. Firdous A. Khanday

Supervisor

DECLARATION

The research work entitled: ***"To understand the role of α-1-Syntrophin (SNTA-1) Protein in signal transduction pathway involving RhoGTP'ase Rac1,"*** presented in this thesis embody the original research work done by me for the **Doctor of Philosophy** (Ph. D.) degree in the Department of Biotechnology at University of Kashmir, Srinagar. This work has not been submitted in part or in full for any other degree or diploma.

Hina Fayaz Bhat

Dedicated to

My Beloved Parents

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ABSTRACT

Abstract:

Rho GTP'ase Rac1 is involved in the regulation of ROS generation, cellular migration as well as the actin/microtubule cytoskeleton organization etc. Although several mechanisms for the activation of Rac1 have been reported earlier, the exact mechanism for its activation is not yet clearly understood. Alpha-1-syntrophin (SNTA1) and P66shc proteins have both been implicated in the highly conserved mechanism that regulates Rac1 protein activation. The growth factor receptor bound (Grb2) protein that is considered to be crucial in the activation of Rac1 has been shown to form a stable complex with both these tyrosine-phosphorylated proteins i.e SNTA1 and P66shc. This study attempts to understand the possible involvement of these adaptor proteins in the Rac1 activation. We provide evidence for a novel complex formation between these proteins that leads to an effective increase in the activation of Rac1, while the decreased SNTA1/P66shc expression using small interfering RNA effectively reduced cellular active Rac1 levels. Our immune-precipitation experiments indicate that formation of a tri-complex containing SNTA-1, Grb2 and P66shc proteins constitute a switch over mechanism that functions to displace Sos1 from Grb2 and increase its availability for the E3B1/EPS8 to form Sos-E3B1-EPS8 complex, which has an intrinsic Rac1 activation property. We also provide evidence that this SNTA1/P66shc mediated Rac1 activation pathway exerts a downstream control on the reactive oxygen species (ROS) generation in human breast cancer cell lines and increases their migratory potential. Conversely, reduction of SNTA1 expression inhibits Rac1-induced migration, indicating that efficient Rac1 signalling requires involvement of both these proteins. Together, our results represent a new aspect of Rac1 signalling and emphasize the involvement of SNTA1 in Rac1-mediated ROS generation, cell migration/acquisition of malignancy. Our results support our previous data were we report an increase in the SNTA1 protein levels in human breast cancer samples and a possible involvement of this protein in signal transduction pathways involved in oncogenesis.

Introduction & Review of Literature

1. Introduction and review of literature:

This dissertation deals with the adaptor proteins involved in the Rho-GTP'ase-Rac1 activation pathway. In this dissertation, different *in vitro* interactions of alpha-1-syntrophin protein have been identified, characterized and their influence on Rac1 activation and the downstream pathway has been discussed. In this chapter, briefly the syntrophin family proteins have been reviewed and the proteins, which are found to be regulating Rac1 protein functioning as well as the signal transduction pathways operating via these proteins within the cell.

1.1. What are syntrophins?

Syntrophins are a multigene family of membrane associated adaptor proteins. They represent a biochemically heterogeneous group of 58-60 kDa intracellular membrane-associated proteins that are characterized by the presence of a PDZ (Post synaptic density protein-95/ Disc large/ Zona occludens-1) split N-terminal PH (pleckstrin homology) domain and a C-terminal SU (syntrophin unique) domain [1-4]. The term 'syntrophin' has been derived from the greek word 'syntrophos' meaning companion or associate [5]. Syntrophins were first identified in the post synaptic membranes of the Torpedo electric organ [6] and later shown to be present in many mammalian tissues [7,8]. Interest in the protein first came from its location at the neuromuscular junction and later from the demonstration that it is directly associated with dystrophin (Dys), the protein product of duchenne muscular dystrophy gene locus [9-11], within the dystrophin signaling complex (DGC), implicating its involvement in some important cellular mechanisms such as cell synapses and signal transduction.

The syntrophin family of scaffold proteins serves to provide a platform for the formation of signal transduction complexes, directing the various participating signaling components to their specialized membrane domains and thereby link various cell surface receptors, ion channels and downstream effectors to these signaling complexes. Primarily, they serve to link several cell components to the dystrophin protein complex via a direct interaction with the dystrophin protein [10,11] and dystrophin related proteins like utrophin and dystrobrevin [9,12-14]. The presence of multiple binding domains like PH,

SU and PDZ in syntrophins allow them to interact simultaneously with varied cellular components, clustering cell proteins, neurotransmitters, receptors, glycoprotein's and lipids etc into appropriate functional networks at specific sub cellular compartments and orchestrating these signal transduction complexes. By controlling their specific spatial and temporal distribution, these proteins thus help in coordinating and regulating various crucial cellular processes and intracellular signaling events [2].

1.2. Syntrophin family:

The syntrophin family of proteins consists of five known homologous isoforms i.e. alpha-1- (α-1-) syntrophin, beta-1- (ß-1-) syntrophin, beta-2- (ß-2-) syntrophin, gamma-1- (γ-1-) syntrophin, and gamma-2- (γ-2-) syntrophin proteins [7,12,15]. The nomenclature of these isoforms is based on the fact that while α-1-syntrophin is acidic, ß-1-syntrophin and ß-2- syntrophin are the basic forms of this protein [5]. Of all these known isoforms, alpha-1- syntrophin was the first to be discovered in post-synaptic membranes of torpedo, followed by ß-1, ß-2-syntrophin [3,6,7], while the two syntrophin isoforms γ-1-syntrophin, and γ-2- syntrophin have only been recently identified [16]. All isoforms of syntrophin family may exist as monomers or dimers within the cell and almost all of these isoforms have been shown to bind to each other.

Alpha-1-syntrophin, encoded in humans by the SNTA1 gene, represents a 58 kDa, acidic isoform with PI 6.7 and 505 amino acids length (unprocessed protein). This membrane protein is found mostly as a peripheral cytoplasmic membrane protein associated with dystrophin protein and dystrophin related proteins e.g utrophin, dystrobrevin, glycoproteins e.t.c in a complex called dystrophin associated protein complex (DAPC) [9,10,14] in muscle cells or concentrated at the neuro-muscular junction in brain. The beta isoforms i.e. ß-1- and ß-2-syntrophins are encoded by the SNTB1 and SNTB2 genes respectively in humans. While ß-1-syntrophin is 538 amino acids length protein, ß-2-syntrophin is 540 amino acids in length. The complete proteins represent nearly 58-59 kDa dystrophin-associated protein A1 basic component 1 and 2 respectively with PI in the range of 8.3-8.6. Both the ß-1- and ß-2- isoforms are ubiquitously expressed in mammalian tissues, however ß-1 is the predominant isoforms [14]. Weak levels of isoform ß-2 are present in all mammalian tissues, except in liver and heart where it is highly expressed. Both these isoforms have been shown to bind to dystrophin and the

related proteins of DAPC [14,17,18]. The gamma isoforms i.e. γ-1- and γ-2- syntrophin proteins are encoded by SNTG1 and SNTG2 genes in humans. The γ-1- and γ-2-syntrophin genes encode for 517 and 539 amino acids length proteins of nearly 58 and 60 kDa molecular weight respectively. While γ-1-syntrophin has been shown to interact with the dystrophin protein [16] and very few of its related proteins, the γ-2 isoform has not been shown to bind to dystrophin or its related proteins. Only a few of their interacting partners have been identified yet and there is huge scope for further investigation into their possible interacting partners or the functioning of these syntrophin isoforms.

Each of the five syntrophin isoforms has been found to have a unique tissue expression (Figure 1.1) and developmental pattern [4,7,12] and has also been shown to selectively interact with different dystrophin family proteins [9,13,19]. Alpha-1-syntrophin is primarily expressed in skeletal muscles [7] in addition to being expressed in other mammalian tissues like heart, brain, stomach, breasts, colorectal tissues e.t.c [20], ß-1-syntrophin and ß-2- syntrophin are ubiqutiously expressed and are present in almost all mammalian tissues [7] while γ-1-syntrophin has been shown to be expressed uniquely in the brain, specifically localized in hippocampal pyramidal neurons, purkinje neurons in cerebellum and cortical neurons [21], γ-2-syntrophin is also present in brain but is considered to have a broader distribution in mammalian tissues including skeletal muscles, liver, testis etc **[**16,21]. In addition to their difference in expression in mammalian tissues, these syntrophin isoforms have also been shown to reveal a marked difference in their sub-cellular location and distribution within the cells [18]. Thus although more than one syntrophin isoform protein has been shown to be expressed in a single cell type, their sub-cellular localization are effectively different and very tightly regulated [22]. For instance, in neurons/muscles, both the γ- isoforms are found to be localized to the endoplasmic reticulum and in skeletal muscle, alpha-1-syntrophin has been shown to be distributed over the entire sarcolemma, present throughout the folds at neuromuscular junctions, whereas ß-1 has found to be present only at the neuromuscular junction folds and ß-2-syntrophin is almost found to be exclusively restricted at the neuromuscular junctions and confined to the lower portion of these folds [18,21-23], while the γ-2-syntrophin localizes in the sub-synaptic space beneath the neuromuscular junction in skeletal muscles [21]. The tightly regulated cellular and subcellular localization of the different isoforms of syntrophin proteins is indicative of distinct

functional roles for these different isoforms within the cell [4,12-14,23]. For example alpha-1-syntrophin, via its association with DAPC in skeletal muscles, has been shown to serve as a link between the extracellular matrix and internal cell signalling apparatus and cell cytoskeleton [24], and is also important for the proper targeting of proteins like utrophin, nNOS etc to the postsynaptic membrane of skeletal muscle [22-24]. Similarly, alpha-1- syntrophin, beta-1 and beta-2-syntrophins have been shown to bind to neuronal nitric oxide synthase (nNOS) and microtubule-associated serine/threonine kinases (MAST) for the membrane association of these proteins in brain cells [24-28]. Similarly the gamma-1-syntrophin has been shown to bind to and regulate the sub-cellular localization of diacylglycerol kinase ζ (DGK- ζ) and is involved in cellular synaptic function [29].

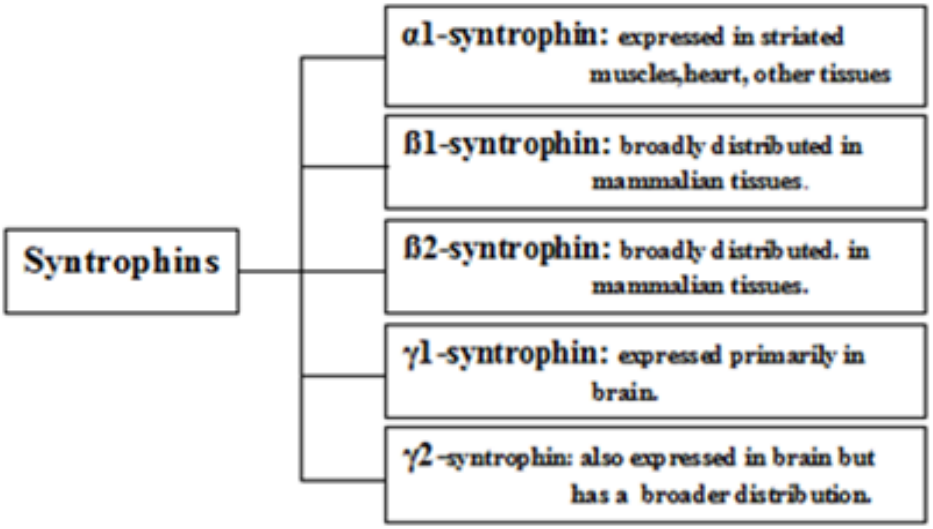


Figure 1.1: Syntrophin family of proteins: *Schematic representation of the various members of syntrophin family proteins and their general distribution in mammalian tissues.*

1.3. Chromosomal location and homology:

All the isoforms of syntrophin family have been identified as the products of different genes [12,13]. The full-length cDNA of alpha-1-syntrophin is 2136 bp long and encodes a single large open reading frame that maps to the chromosome 20q11.2 [7,12]. The Chromosomal location of β-2-syntrophin maps to chromosome 16q22-23 while β-1-syntrophin is located at 8q23-24. γ- 1 isoform of syntrophin maps to 8q11-12 and γ-2 isoform is located at 2p25.3.

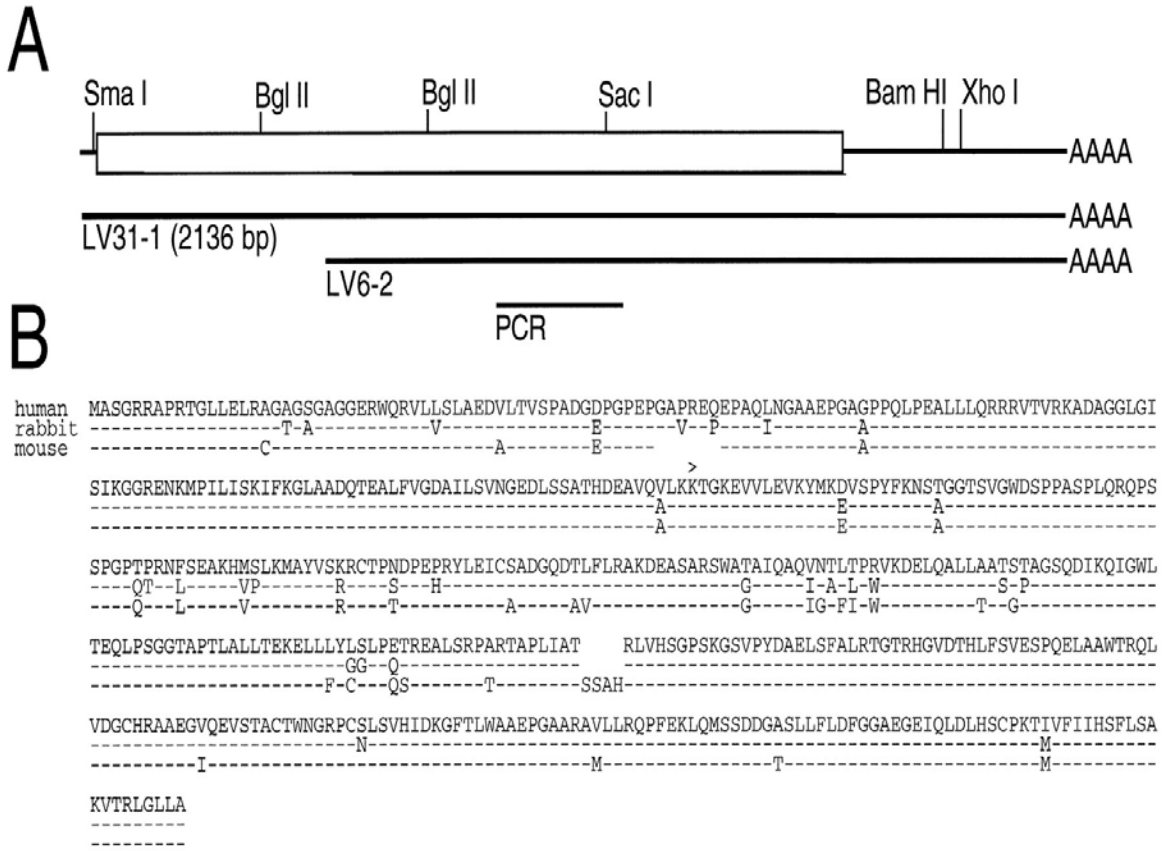


Figure 1.2: Restriction map and schematic representation of isolated α1-syntrophin cDNA clones. *A) The open reading frame is indicated by an open box. A dash indicates identity to the residue at that position for the β1-isoform, and a space indicates gaps introduced by Pile-Up software to optimize the alignment.* ***B)*** *Interspecies comparison of the deduced amino acid sequence of the α1-syntrophins, between human, rabbit, and mouse [7].*

The open reading frame begins with the first ATG start codon in the cDNA, which is in a favorable context for the initiation of translation, and is flanked at the 3′ end with a poly-adenylation signal at the appropriate distance from a poly-A tail (Figure 1.2). At the

amino acid level, the human alpha-1-syntrophin isoform is 94% identical to the mouse sequence and 93% identical to the rabbit sequence. All three sequences contain homologous start codons. In comparison to rabbit and human, the mouse cDNA bears an internal deletion of 6 amino acids near its N terminus (GAPREQ) while the 4-amino acid internal insertion in mouse (SSAH) is considered to represent a rare splicing event to a nearby splice acceptor [3].

The deduced amino acid sequences of human α-1and β- 2-syntrophin are nearly identical to their homologues in mouse, suggesting a strong functional conservation among the individual isoforms. At the amino acid level, the human alpha-1-syntrophin isoform is 94% identical to its mouse sequence and 93% identical to the corresponding rabbit sequence [4,7]. However it has been observed that the three human syntrophins i.e α-1, β-1 and β-2 syntrophins are less strongly conserved with respect to each other e.g. human alpha-1-syntrophin peptide sequence is nearly 54% and 50% identical to human β-1 and β-2-syntrophin respectively while the human β-2-syntrophin peptide has been shown to be 57% identical to human β-1-syntrophin [3,4]. The amino acid sequence of the two γ-syntrophin isoforms is found to be more similar to each other than to the α- or β-syntrophin isoforms. In fact the γ-1-syntrophin and γ-2-syntrophin isoforms have been shown to possess a C-terminal tail of nearly 23 and 14 amino acids respectively, extending their SU domain and their amino acid sequence shows only 15-22% substantial homology to the amino acid sequence of α- and β-syntrophin isoforms [21]. The difference of γ-syntrophin isoforms from α-/β-syntrophins in their amino acid sequence and in their interaction/association with other proteins, in fact qualifies them to be considered as a subfamily of syntrophins isoforms.

1.4. Structure:

Structurally all the members of syntrophin family share a common domain organization (Figure 1.3). All the syntrophin isoforms contain two tandem PH domains i.e. one N-terminal split PH domain (PH1), one central PH domain (PH2) and a C-terminal SU domain. However, the γ-1-syntrophin and γ-2-isoforms differ slightly in their structure from the other syntrophin isoforms in the sense that in addition to these conserved domains, they possess an extended 23 and 14 amino acid tail at their C-terminals respectively. This C-terminal tail has been shown to form a putative P-loop that could be

important in their dystrophin binding [21,30]. Since none of these domains contains any intrinsic enzyme activity, syntrophins are therefore thought to function as adaptors that serve to target their binding partners to specific locations (Figure 1.3) on the cell membrane [31].

1.4.1. PH-1 Domain:

Pleckstrin homology domains are abundant protein modules shown to play critical roles in cellular signaling and cytoskeletal organization [32,33]. They are called pleckstrin homology domains as they show homology to a region repeated in the pleckstrin protein. PH domains are regarded as lipid-binding modules that are capable of targeting several PH containing proteins to the cell membrane and are involved in recognition of lipid signal transduction [2,3,34-36]. There are two PH domains present within the syntrophin proteins. The PH-1 domain of syntrophin protein is split into two halves with one half i.e. N-terminal half of PH1 domain extending from amino acids 1-77 and other half i.e. C-terminal half of PH1 domain extending from amino acids 162-271 in length. Between these two PH1 halves, lies a highly conserved globular structure, of nearly 80 amino acids in length, called PDZ domain [32,37-40]. The NH2 terminus of this pleckstrin homology 1 domain and the NH2 terminus of the PDZ domain have been reported to bind calmodulin in a Ca^{2+} independent manner [40-42]. This domain has also been shown to be involved in the oligomerization of syntrophin proteins in vitro in a Ca^{2+} dependent manner. Calmodulin inhibits oligomerization in a Ca^{2+} independent manner [41,42]. The PH1 domain of syntrophins is also involved in binding to the membrane phospho-lipids and is implicated in lipid signaling mechanisms [42,43] e.g PH1 domain of alpha-1-syntrophin has been shown to interact with phosphotidylinositol 4,5-bisphosphate (Figure 1.4) for to its own membrane localization [43]. The NMR studies reveal that the PHN half is composed of three β-strand while the PHC half contains four β-strands and a C-terminal α-helix (Figure 1.3). The PDZ domain is inserted in the β3/β4-loop of the PH domain Thus the PHN-PDZ-PHC tandem forms a functionally unique supra-module structure in which the split PH domain and the PDZ domain function synergistically in binding to membrane lipids e.g. inositol phospholipid [39,43]. The split PH domain of syntrophin proteins adopts a canonical PH domain fold that has been implicated in several interactions with the PH and PDZ domains of several other proteins and may also interact

with the complementary partial PH/PH-like domains present hidden in the primary sequence of many proteins [7,39,44]. In alpha-1-syntrophin this intramolecular association between the two PH domain halves has been observed [36,39]. The insertion of PDZ domain does not affect the conformation or interactions of the PH1 domain in syntrophin [39,42-44]. However, the functional significance (if any) of the PDZ domain-mediated PH domain splitting has not yet been addressed.

1.4.2. PDZ Domain:

The name PDZ comes from the first three proteins found to contain repeats of this domain i.e Post Synaptic Density protein-95, Drosophila discs large protein, and the Zona occludens protein 1). These evolutionarily conserved domains have been shown to recognize certain amino acid regions, for their interaction with other proteins, known as PDZ binding motifs (PBM). The PDZ domain stretches nearly in between amino acids 75-170 of the PH1 domain of alpha-1-syntrophin. Consisting of seven β- strands and two α- helices the well folded PDZ domain is inserted at the β3-β4 loop within two long linker region of the PH1 domain of syntrophins [37-39]. Studies reveal that majority of PDZ interactions occur through the recognition of C-terminal PBM and that the terminal carboxylate group is important for binding to take place [45-48]. Generally, on the basis of the two amino acids present at the positions 0 and -2, these C-terminal PBM's are categorized into three major groups i.e. Type I PBM, Type II and Type III PBM respectively [2,48-52] e.g. Type-I PBM is of S/T-X-I/L/V type, were -2 position is occupied by either serine (S) or threonine (T), -1 position can be occupied by any amino acid (X) while 0 position is occupied by any of the three amino acids i.e. isoluecine (I), leucine (L) or valine (V). Type-II PBM is of φ-X-φ type, were -2 and 0 positions are occupied by any hydrophobic amino acid (φ) while -1 position is occupied by any amino acid X. Type- III PBM is of D/E-X-φ type, were - 2 position is occupied by either by aspartate (D) or glutamate (E) residue , -1 position is occupied by any amino acid (X) and 0 positions are occupied by any hydrophobic amino acid (φ) [47-52]. A classical example of PDZ domain binding with the C-terminal PBM's is exhibited by the PDZ domain of alpha-1-syntrophin protein. Its PDZ domain interacts with the carboxyl termini type-I consensus sequence -E(S/T)XV of kinase proteins such as stress-activated Protein Kinase-3 (SAPK-3) [52-55]. A large number of other signaling proteins possessing C-

terminal PBM that bind to the PDZ domain of alpha-1-syntrophin protein may include aquaporin-4 [56], voltage-gated sodium channels [57-58], Potassium channels [59-60], Calcium channel TRPC1 [61], serine/threonine protein kinases [28,62], the ATP-binding cassette transporter A1 [63] etc.

In addition to the C-terminal PBM, PDZ domains have also been reported to bind to certain internal motifs of their binding partners, however such interactions are governed by very strict pre-requisites e.g. proteins with internal sequences, structurally resembling a C-terminal end, such as a beta-hairpin finger-like structure or an aspartate residue, have been shown to bind to the PDZ domains [45-48, 49]. Intermolecular binding of such an internal PBM with a PDZ domain has been characterized for the interaction between neuronal nitric oxide synthase i.e nNOS or that of G-protein coupled receptors (GPCR) [64-67] and the PDZ domain of alpha-1-syntrophin [64, 49, 65].

1.4.3. PH-2 Domain:

The second Pleckstrin homology i.e. PH-2 domain lies to the C-terminus of syntrophin proteins. The PH-2 domain stretches between the amino acids 281 to amino acid 406 of syntrophin protein and consists of a conserved core structure composed of a partially open, two-sheeted β-barrel with one end of the barrel capped with a C-terminal α- helix [68-71]. The first β-sheet is composed of four anti-parallel β-strands (β1–β4), and the second β-sheet contains three strands (β5–β7). The PH2 domain of syntrophins has been shown to bind several lipid/proteins bound to the plasma membrane and the best characterized function of PH2 domains is binding to inositol phospholipids [32,36-39]. The PH-2 domain in syntrophins is mostly involved in the targeting of PH domain containing proteins via PH-PH interaction, in detecting the lipid signals generated in cellular processes, and in the recruitment of signaling proteins to the sarcolemma [71-72].

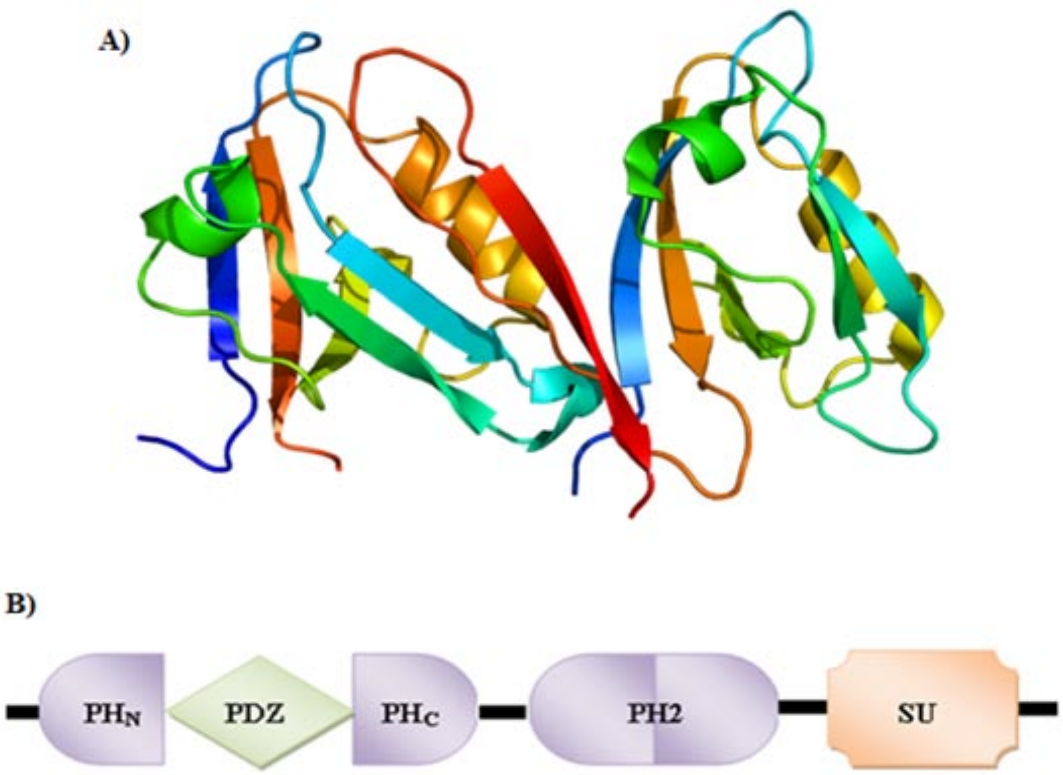


Figure 1.3: SNTA1 structure and domain organization: *Diagrammatic representation of the A) Structure of alpha-1-syntrophin protein and B) the domain organization in the protein [12].*

1.4.4. SU Domain:

The C-terminal 57 amino acids, within all isoforms of this family of proteins, exhibit a region of strong homology lacking homology to other characterized proteins. This highly conserved amino acid sequence is hence known as syntrophin unique or SU domain [4,12,13] and has predicted to consist of from three to five strands of β-sheet separated by as many turns [7]. The PH2 and SU domain of syntrophins are responsible for binding to the dystrophin, utrophin, and dystrobrevin (Figure 1.4) and it is this SU domain that lingers syntrophin proteins to the membrane via its interaction with the DGC [9,17,73-74].

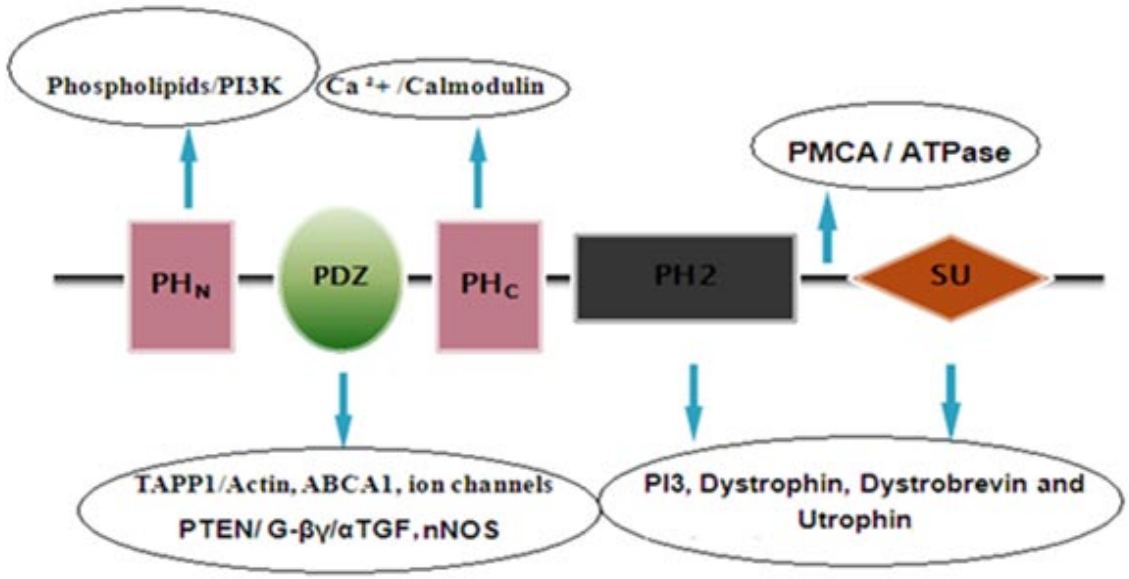


Figure 1.4: SNTA1 mediated interations within the cell: *Diagrammatic representation of the various interactions/bindings of α-1-syntrophin protein within the cell and the various binding domains mediating these interactions.*

1.5. Alpha-1-syntrophin in cytoskeletal organization:

The dystrophin associated protein complex (DAPC) is thought to be a signal transduction complex that serves as a mechanical link between the extra-cellular matrix and inner signaling pathways and cell cytoskeleton [75]. The presence of as much as four syntrophin binding sites in close proximity within a single DAPC indicates that syntrophins play an important role in tethering of multiple signalling molecules together to form signalosomes at specific membrane sites [3]. In muscle cells DAPC provides stability against the forces of contraction or relaxation [76] and has been implicated in modulating the actin cytoskeleton by effecting the recruitment of proteins involved in actin cytoskeleton organization at the membrane [76-79]. Alpha-1-syntrophin being an important component of DAPC has been shown to play a critical role in executing this function of DAPC. Alpha-1-syntrophin in itself has been characterized as an actin binding protein (Figure 1.5) whose sub-cellular localization is in turn regulated through cytoskeletal reorganization within the muscle cells [80]. Alpha-1-syntrophin has also been shown to bind to F-actin [80]. Although this binding involves several sites on alpha-1-syntrophin yet, the second PH domain and the SU carboxyl-terminal is specially considered to be important.

Alpha-1-syntrophin has been implicated in the actin stabilization at membrane and inhibition of the actin-activated myosin ATPase activity [80]. It serves to regulate the intracellular localization and activity of the downstream signaling targets of DAPC e.g the lipid proteins such as phosphoinositol-3-phosphate (PI3K) and diacylglycerol-ζ (DGK-ζ) have been shown to bind to syntrophins on the inner surface of the cell membrane [43,77]. Both these lipid proteins are involved in the actin remodeling and cytoskeletal dynamism. Changes in the actin organization are tightly linked to the production of signaling phosphoinositides such as phosphoinositol 3, 4-Bisphosphate (PI(3,4)P2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), which recruit PH domain-containing proteins to the sites of receptor activation at the plasma membrane [78]. These lipid signaling proteins control the actin cytoskeleton organization of the cells in response to growth factor stimulation. PI3K is a key component of multiple signaling pathways, including those that regulate cell survival, growth, and motility [79]. PI3K catalyzes the transient production of phosphatidylinositols like phosphatidylinositol 3, 4,

5-trisphosphate (PI(3,4,5)P3), which triggers the activation of downstream signaling pathways by recruiting PH domain-containing signaling proteins to the plasma membrane.

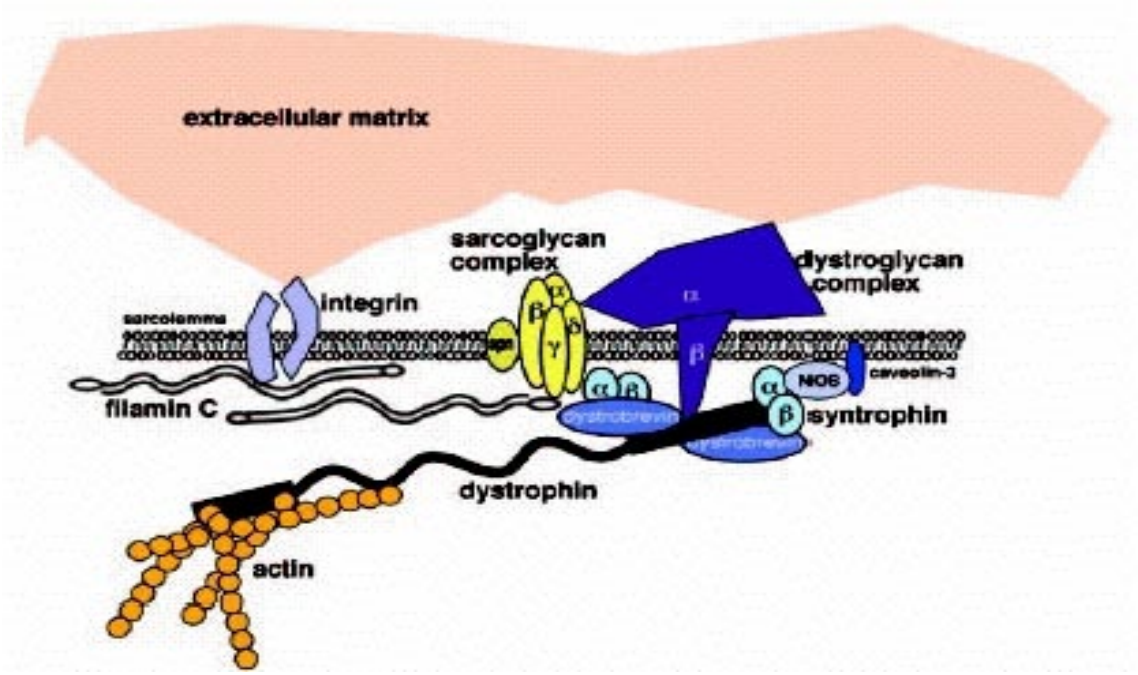


Figure 1.5: SNTA1 as a component of DGC: *Diagrammatic representation of the dystrophin glycoprotein complex (DGC) showing the α-1-syntrophin as its component towards the inner cell membrane, serving as a link between DGC, extracellular matrix as well as the cell cytoskeleton (F-actin) (Angela, et al., 2004).*

TAPP1 (i.e. tandom PH domain-containing proteins1) is one of the PH domain-containing adapter proteins recruited to the plasma membrane of cells in response to phosphoinositol 3-kinase (PI3K) activation by growth factors like PDGF. The C-terminal PH domain of TAPP1 has been shown to interact with PI(3,4)P2 and translocate it along with itself to the plasma membrane [81], thereby activated and thus initiate various local responses like reorganization of the actin cytoskeleton. The C-terminal PDZ binding sequence of TAPP1 has been shown to preferentially bind to the PDZ domain of alpha-1-syntrophin [81-85]. Both TAPP1 and alpha-1-syntrophin are localized to PDGF-induced circular membrane ruffles in NIH-3T3 cells [78] and it has been shown that the ectopic

expression of TAPP1 potently blocks PDGF-induced formation of dorsal circular ruffles without affecting peripheral ruffling while co-expression of alpha-1-syntrophin with TAPP1 prevents the blockade of circular ruffling. Thus suggesting that alpha-1-syntrophin regulates the localization of TAPP1, important for remodeling the actin cytoskeleton in response to growth factor stimulation (Figure 1.6).

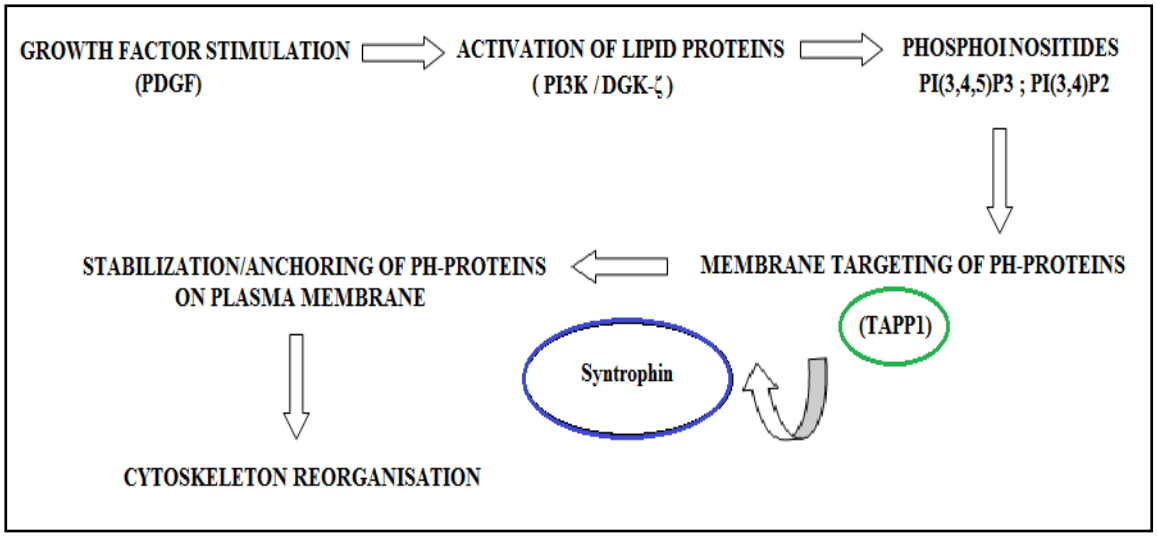


Figure 1.6: SNTA1 regulated cytoskeletal dynamism: *Diagrammatic representation showing α-1-syntrophin mediated cytoskeletal reorganization.*

The PI3K-driven signaling pathways downstream of PDGF stimulation have been shown to induce a rapid reorganization of the actin cytoskeleton which may manifest as plasma membrane specializations such as lamellipodia, filopodia, and membrane ruffles and it has been shown that DGC components are involved in the regulation of actin organization in response to PDGF signaling [86-88]. Thus DGC helps in organizing signaling components that remodel the actin cytoskeleton and in recruiting signaling proteins having PDZ binding motifs to the PDGF-induced circular ruffles on the membrane [19,86]. Together DGC and syntrophins work to act as a scaffold to promote the formation of signalling complex, which includes TAPP1 and the other downstream effectors of pathway, and stabilize their association with the membrane [78,87], playing a central role in modulating the cytoskeleton dynamism within the cell (Figure 1.6).

1.6. Regulation of channel proteins:

Alpha-1-syntrophin has been implicated in regulation and localization of various ionic channels and membrane binding proteins (Figure 1.7). The presence of a PDZ domain bestows syntrophins the ability to interact with several receptor molecules, muscle, and nerve voltage-gated Na+ channels and link them to the actin cytoskeleton and extracellular matrix via the DAPC [57]. A few of these channel proteins will be discussed below:

1.6.1. Sodium channels: The PDZ domain of alpha-1-syntrophin interacts with the C-terminal consensus sequence motif E(S/T)XV in various skeletal muscle Na^+ channels [56-58]. Two voltage-gated sodium channels of skeletal and cardiac muscle, i.e. SkM1 and SkM2 possessing this consensus sequence in their C-terminal, have been shown to bind to the PDZ domain of α-1-, ß-1-, and ß-2-syntrophin [57]. Moreover, the sodium channels in brain (NaChs) which lack this E(S/T)XV consensus sequence have also been shown to interact with syntrophins, although this interaction is not mediated via the PDZ domain of syntrophins. Nav1.5 is the main cardiac voltage-gated sodium channel that generates the fast depolarization of the cardiac action potential, and plays a key role in cardiac conduction. Nav1.5 is part of a multi-protein complex in which dystrophin and syntrophin proteins play an important role in determining its expression levels [88] as the C-terminal last three residues (Ser-Ile-Val) of Nav1.5 have been shown to interact with the PDZ domain of α- and β-syntrophin proteins [88].

1.6.2. Aquaporin-4 (AQP4): is a type of integral membrane channel protein that regulates the homeostasis and flow of water in brain. The cellular distribution of this channel protein is in close agreement with the distribution of the dystrophin protein complex [89-91]. AQP4 has been shown to possess type-I C-terminal PDZ domain binding sequence, i.e. Ser-Ser-Val (-SSV) that associates with the PDZ domain of the alpha-1-syntrophin protein [91-92]. It has been observed that the subcellular localization of AQP4 is altered in alpha-1-syntrophin knocked out skeletal muscles ($\alpha\text{-Syn}^{-/-}$) but the total level of AQP4 protein in $\alpha\text{-Syn}^{-/-}$ skeletal muscles does not change [56, 93], implicating that the subcellular localization and thus the functioning of these AQP4 channel proteins depends on the C-terminal-SSV/PDZ interaction between AQP4 and alpha-1-syntrophin protein [55].

1.6.3. Inward rectifier potassium channels (Kir): represent a family of channel proteins, involved in the transport of potassium ions that play important roles in the maintenance and control of cell excitability. Many members of this family, e.g., Kir2.1, Kir2.2, Kir2.3, and Kir4.1, have been shown to bind to the components of DAPC [60]. One of the major physiological roles of these Kir potassium channels in glial cells is to promote potassium spatial buffering in the central nervous system, a process necessary to maintain an optimal potassium concentration in the extracellular environment [94-96]. Kir4.1 has been shown to be localized in glial cells by its association with PDZ domain of alpha-1-syntrophin. The presence of consensus PDZ domain binding motif (SNV) at the last three amino acids of the Kir4.1 potassium channel allows it to interact with the DGC complex, via the PDZ domain of alpha-1-syntrophin [59, 95, 60]. The interaction between Kir4.1 and alpha-1syntrophin is considered to be very important for their precise distribution in discrete sub-domains of cell membranes and are suggestive of an important role for the DGC and syntrophins in nervous system physiology.

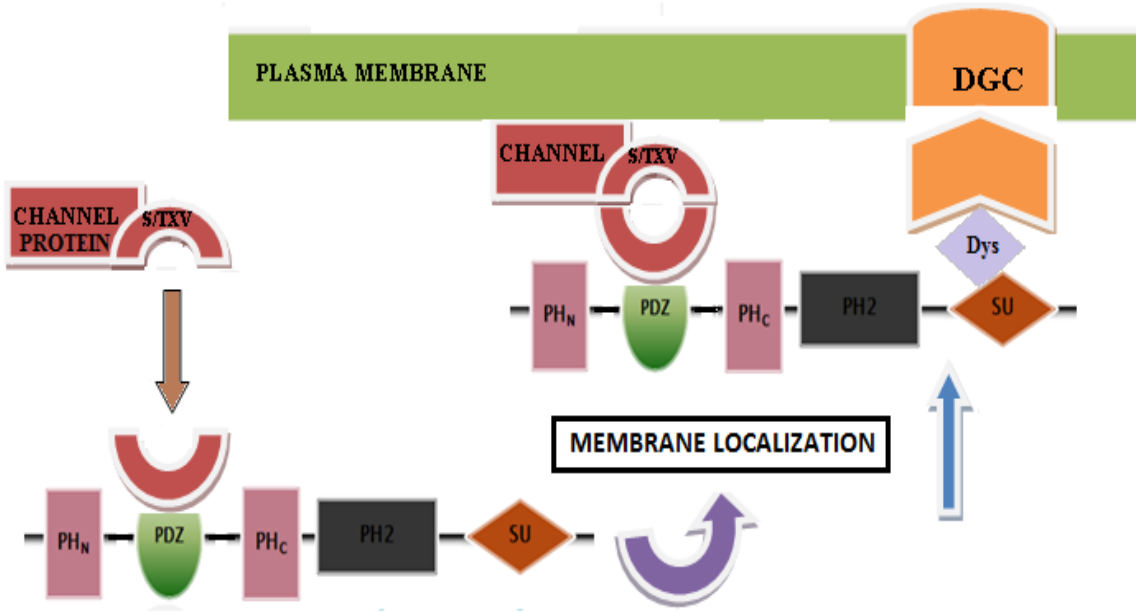


Figure 1.7: Role of syntrophins in the membrane localization of ionic channel: *Diagrammatic representation of the membrane localization of ionic channel proteins by syntrophin mediated by the interaction between its PDZ domain and the S/TXV motif of these channel proteins.*

1.6.4. Transient receptor potential canonical channels (TRPC): are a family of membrane channels that belong to the TRP channel superfamily [97]. There are seven TRPC channels (TRPC1-TRPC7) known to play an important role in maintaining calcium homeostasis in striated muscles and in cardiac muscle cells [97, 98]. In skeletal muscles TRPC channels, particularly TRPC1 and TRPC4 have been shown to form a costameric macromolecular complex along with alpha-1-syntrophin and dystrophin proteins. This complex has been implicated in regulating the normal calcium entry into the skeletal muscle cells [99]. The PDZ domain of alpha-1-syntrophin associates with TRPC channels as they possess a C-terminal consensus PDZ binding domains. Alpha-1-syntrophin serves as an adaptor that anchors the TRPC1/TRPC4 channels to the dystrophin-associated protein complex (DAPC) which in turn may connect them to the cell cytoskeleton or extra-cellular matrix, assuring the normal activation and modulation of these cation channels or via its multiple binding domains, it may also aid in recruiting other proteins to this complex, thus building a whole signalosomes responsible for regulating the activity of TRPC channels and maintaining normal calcium homeostasis in muscle cells.

1.7. Role within the cell:

Alpha-1-syntrophins has been shown to play a central role in protein trafficking and organization of molecular architectures containing several signal transduction proteins. It has been seen to be involved in the precise localization and/or regulation of many transduction proteins e.g SAPK-3, ARMS, Calmodulin, G-proteins, n-NOS, ABCA1, Grb2 e.t.c. within the cell (Figure 1.8). Some of these proteins are discussed below:

1.7.1. Stress-activated protein kinase 3 (SAPK-3): belongs to the family of mitogen-activated protein kinases (MAPK). These MAP Kinases are essentially protein kinases that are activated by a dual threonine/tyrosine phosphorylation elicited by certain conditions such as cellular stresses, bacterial endotoxins, or inflammatory cytokines [100]. SAPK-3 (also called ERK6/P38γ) is a more recently identified stressactivated protein kinase that is abundantly expressed in the skeletal muscles. The downstream targets of this MAP kinase are yet to be identified. However, alpha-1-syntrophin has been recently identified as a substrate for SAPK-3 [62]. In skeletal muscles, both these proteins are found in abundance at the neuromuscular junctions. SAPK-3 has been shown to bind to the PDZ domain of alpha-1-syntrophin through its C-terminal-KETXL

sequence. SAPK-3 has been shown to phosphorylate alpha-1-syntrophin at two different serine residues, S193 and S201, in vitro [62]. The SAP kinases regulate the cell proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis within the cell, and the finding that SAPK-3 binds to the PDZ domain of alpha-1-syntrophin and that the phosphorylation of alpha-1-syntrophin depends on this interaction thus supports the involvement of alpha-1-syntrophin in these important cellular processes [62, 100]. Also, SAPK-3 is unique in the sense that it is the only member of its family having a carboxyl-terminal PDZ binding motif. The presence of a C-terminal PDZ binding sequence (-KETPL) in SAPK-3 thus provides a mechanism for its selective targeting to the specific subcellular sites, like cell synaptic junctions in skeletal muscles, as well as its specificity for alpha-1-syntrophin protein as a substrate pointing towards a more distinct physiological role for this isoform of stress-activated protein kinases within the skeletal muscles [62].

1.7.2. Ankyrin repeat-rich membrane spanning (ARMS): is a novel downstream substrate for protein kinase D and receptor tyrosine kinases, like ephrin (Eph) receptors and tropomyosin-related kinase (Trk) receptors that play a central role in neural development and cell synaptic function [101–103]. ARMS is a 220-kDa multidomain protein and, just like certain syntrophins isoforms, it is also expressed in muscles and found concentrated at the neuromuscular junction (NMJ) [104]. The C-terminus of ARMS possesses a typical class-I PDZ-domain binding conserved motif (-RESIL) that has been shown to bind to the PDZ domain of alpha-1-syntrophin and β-2-syntrophin isoforms. These syntrophin isoforms thus help to anchor ARMS proteins to the synaptic DGC and stabilize ARMS protein clusters at the NMJ and are also implicated in regulating the localization of ARMS during NMJ differentiation. Since ARMS functions as an important RTK downstream target and has been shown to enhance the Eph receptor signaling by increasing EphA4-induced Jak and Stat phosphorylation, it is likely that these syntrophins are also involved in enhancement of Eph signaling by effecting the specific subcellular localization of these protein complexes [102-104]. Over-expression of alpha-1-syntrophin induces ARMS clustering in a PDZ domain-dependent manner, while co-expression of ARMS enhances EphA4 signaling [104]. EphA4 signaling has recently been implicated in the regulation of synapse formation and plasticity, and, recently, ARMS has been shown to mediate sustained MAPK signaling, elicited by neurotrophins,

implicating ARMS as an important target for receptor tyrosine kinase (RTK) signaling [105]. Thus, syntrophins play an important role in RTK-signaling events by aiding the localization of ARMS [104] which is essential for the NMJ development.

1.7.3. Guanine nucleotide binding proteins (G-proteins): are an important class of signal transducing proteins that are bound to the inner surface of the cell membrane and are associated with a transmembrane receptor. These proteins along with their coupled receptors, i.e. G-protein-coupled receptors (GPCR), function by transmitting the outer cell signals, e.g., hormones, neurotransmitters, etc., to the inner cell compartment and by communicating these signals to downstream signaling targets, leading to the desired changes within the cell [106]. Heterotrimeric G-proteins, also belonging to G-protein family, are composed of two subunits, i.e. one Gα subunit and a Gβγ heterodimeric subunit. Studies have revealed that alpha-1-syntrophin plays a major role in the regulation of these heterotrimeric G-protein signaling mechanisms. Interaction of the alpha-1-syntrophin protein with heterotrimeric G-proteins has been confirmed before [107, 108]. Also, laminin α1, an extracellular matrix component, has been shown to induce G-protein binding to the PDZ domain of the alpha-1-syntrophin protein which has the consequences of altering the intracellular Ca^{2+} concentration in the muscle cells. In the absence of laminin, when the heterotrimeric G-proteins hydrolysis into its constituting subunits, Gα and Gβγ, Gα remains free to be activated by guanine nucleotide exchange factors (GEF). In its active form, i.e. GTP-bound form, the Gα subunit can bind to and cause activation of the L-type Ca^{2+} channels called dihydropyridine receptors (DHPR), thereby stimulating the Ca^{2+} influx into the muscles cells [109]. However, laminin binding to the α-dystroglycan protein of the dystrophin complex on the outer membrane induces an association of these trimeric G-proteins with the PDZ domain of alpha-1-syntrophin. The Gβγ heterodimeric subunit in G-proteins has been shown to bind to the PDZ domain of the alpha-1-syntrophin protein. The β component of the Gβγ subunit has a binding motif -LWL- or -IWN- at its C-terminus, while several Gγ components of this subunit, e.g., Gγ4, Gγ12, etc., have TIL similar to the S/TXL type class-I binding motif as their C-terminus. The binding between the PDZ domain of alpha-1-syntrophin and Gβγ may thus be involving the C-terminus of the Gγ component or Gβ component or may even involve some internal PDZ binding motif present within any of the two components of the Gβγ subunit. Laminin binding increases the Gβγ binding to alpha-1-syntrophin, sequestering

the G protein in its inactive form on the cell membrane. Thus, the Gα subunit is no longer free to bind to or activate DHPR Ca^{2+} channels, due to which the Ca^{2+} concentration within the muscle cell decreases drastically. This regulation of G-proteins by alpha-1-syntrophin has important implications in muscular dystrophies, such as ducchennes muscular dystrophy, which predominantly involve irregularities in cell Ca^{2+} concentrations. Further, the Gβ subunit is in itself involved in modulating the activities of several protein kinases, cyclases, or ion channels, e.g., it regulates the activation of some isoforms of the PI3K (PI3Kγ, PI3Kβ). Thus, G-protein binding to alpha-1-syntrophin could be regulating several other kinds of cell signalling pathways such as the Akt/PI3K pathway. Interaction of alpha-1-syntrophin protein with the Gα subunit of heterotrimeric G-proteins has also been confirmed [108]. The Gαi, Gαs, Gαo and Gαq subtypes of the Gα component of the G-protein have been shown to associate with the alpha-1-syntrophin protein, which suggests that alpha-1- syntrophin may be involved in regulating G-protein signalling via a wide variety of GPCRs. The N-terminal half of the first PH domain in alpha-1-syntrophin as well as the C-terminal SU domain have been shown to contribute to the binding with the Gα subunit of heterotrimeric G-proteins [108]. The N-terminal three β-strands of PH_N are thought to be involved in binding to the Gα subunit of G-proteins. Along with PH_N, the SU domain of alpha-1-syntrophin has also been shown to bind to the Gαs subunit, pointing towards a possible cooperative binding mechanism. Also, the binding has been found to be independent of the activation state of the Gα subunit, i.e. both the GTP- as well as the GDP-bound forms of Gα can form a complex with the alpha-1-syntrophin protein in the presence or absence of the GPCR ligands. Alpha-1-syntrophin may thus form a complex with G-proteins, by interacting with either its Gα or Gβγ heterodimeric subunit, and regulate the efficiency of signal transduction mediated by the GPCRs [108, 109]. The fact that the PDZ domain binds to both the C-terminal as well as the internal PBM sequences in GPCRs points towards the possibility that syntrophin proteins could possibly have diverse functions in the regulation of GPCR trafficking.

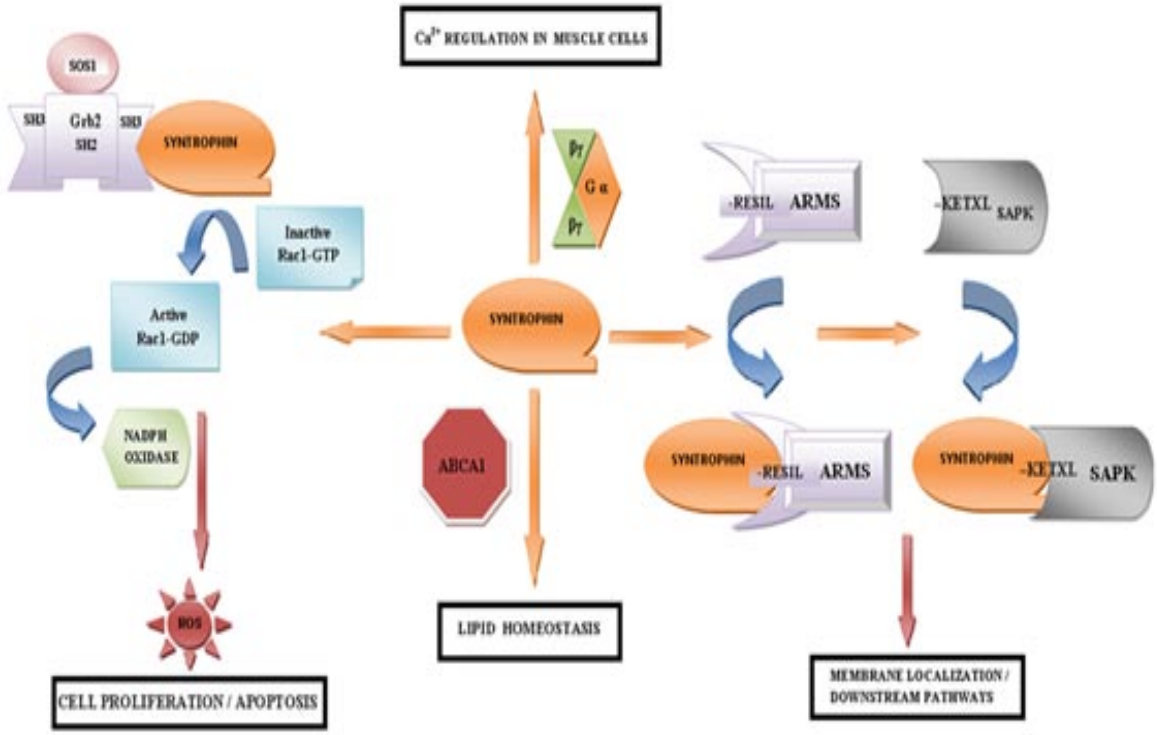


Figure 1.8: Roles played by SNTA1 within the cell: *Schematic representation of the roles played by alpha-1-syntrophin protein in cell signalling.*

1.7.4. Nitric oxide synthases (nNOS): have been characterised as neural NOS, i.e. nNOS or NOS-1, inducible NOS, i.e. iNOS or NOS-2, and endothelial NOS, i.e. eNOS or NOS3 [110]. While nNOS and eNOS are expressed constitutively throughout the heart, the inducible one, iNOS, is expressed under patho-physiological conditions. In the human body in general, and within the heart in particular, NO has several functions, e.g., it affects contractility and normal muscle functioning [111], and thus the importance of NOS-1 signaling in the heart is well established. NOS-1 ablation enhances basal contractility [111, 112] and NOS- 1-derived NO has been shown to increase as a consequence of experimental and pathological human heart failure [113]. In skeletal muscle cells, the muscle-specific isoform of neuronal nitric oxide synthase (nNOS), i.e. NOS-1μ, has been shown to bind to alpha-1-syntrophin protein which ensure its localization to the sarcolemma via the dystrophin complex (DGC) [113]. In fact, these proteins are thought to be a part of a macromolecular complex in which the sarcolemmal calcium pump, syntrophin proteins, and the neuronal nitric oxide synthase form a ternary association with each other. A physical interaction between the plasma membrane

Ca^{2+}/calmodulin-dependent ATPase 4b (PMCA4b), alpha-1-syntrophin, and NOS-1 in cardiac cells has been confirmed [114, 115]. The interaction between nNOS and alpha-1-syntrophin is, however, not dependant on the recognition of a COOH-terminal motif, rather it involves a PDZ-PDZ interaction [49]. The PDZ domain of nNOS specifically heterodimerizes with the PDZ domain of alpha-1-syntrophin protein [26, 114], and this interaction is a typical representative of an alternate class of PDZ domain binding, i.e. via the recognition of internal PBMs [114-116]. The interaction between the PDZ domains of these two proteins takes place in a linear head-to-tail fashion having a two-faced unusual polarized structure with a receptor face and a ligand face. The ligand face has a β-hairpin structure that acts as the PDZ ligand, binding the receptor face, i.e. the peptide binding site of the syntrophin PDZ domain [49], while the peptide binding groove of the nNOS PDZ domain remains available for binding with additional proteins. Such PDZ–PDZ interactions are proof that a terminal carboxylate group is not an absolute requirement for binding to the PDZ domains. Also, alpha-1-syntrophin interacts with the distal region of the large intracellular loop within PMCA4b, and the linker region between the PH2 domain and SU domain, corresponding to amino acids 399-447 of alpha-1-syntrophin, have been shown to be very important for this interaction to take place [116]. Thus, a ternary complex interaction between PMCA, alpha-1-syntrophin, and NOS-1 in cardiac cells has been proposed [115]. In this complex, PMCA acts as a negative regulator of NOS-1, and an over-expression of alpha-1-syntrophin and PMCA4b has been shown to strongly inhibit the NO production [113–115]. PMCA4b is shown to be linked to NOS-1 through interactions between the COOH-terminal tail of PMCA and the PDZ domain of NOS-1 [117], and alpha-1-syntrophin is shown to be bound to this complex via an interactions between its linker region (i.e. the region inbetween the PH2 and SU domains) and the large intracellular loop (between regions four and five) of PMCA4 (Figure 1.9) [115-117]. PMCA and alpha-1-syntrophin negatively regulate the NOS-1 activity in a synergistic manner (Figure 1.9), which further supports some significant functional implications for this complex formation on nitric oxide-regulated signalling pathways.

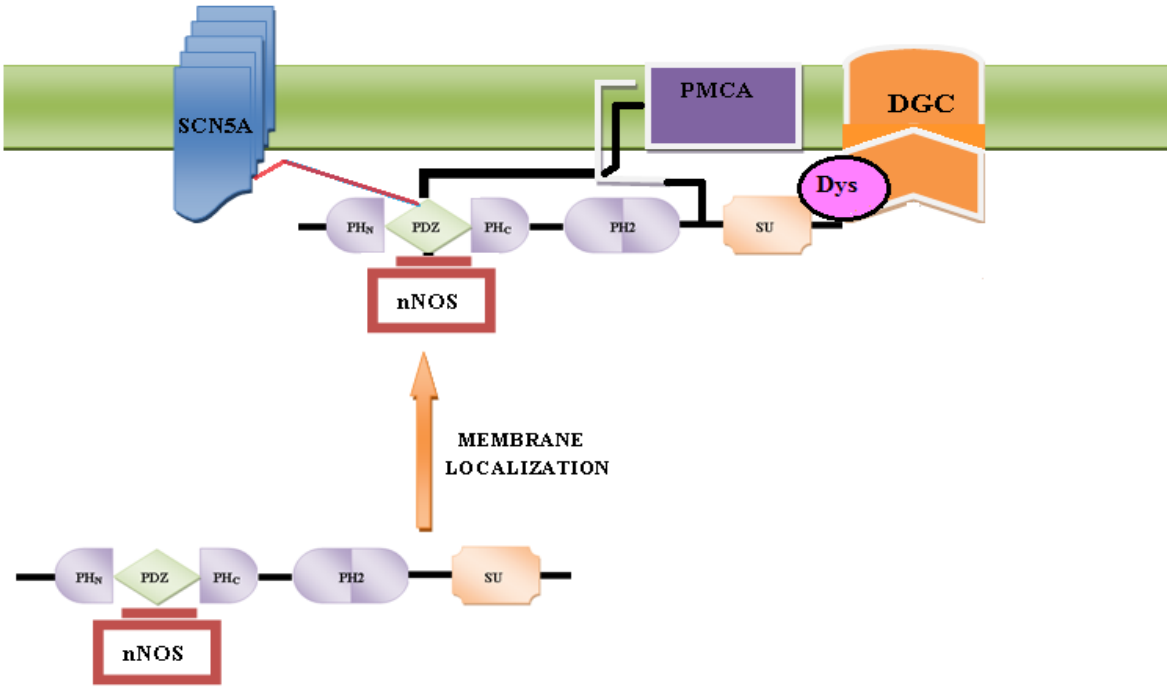


Figure 1.9: Multi-complex formation between PMCA, α-1-syntrophin, NOS, and the SCNA5 sodium channel: *PMCA via its intracellular loop, located between trans-membrane domains 4 and 5, binds to the linker region of syntrophin, and via its COOH terminus binds to the PDZ domain of NOS-1. Syntrophin also connects the SCN5A sodium channel to the nNOS–PMCA complex and regulates NO concentration at the membrane.*

1.7.5. The ATP-binding cassette transporter A1 (ABCA1): mediates the release of cellular cholesterol and phospholipids to form high density lipoprotein [118,119]. ApoAI-mediated cholesterol efflux is a major event in reverse cholesterol transport which serves to generate HDL and transports the excess cholesterol from the peripheral tissues to the liver for biliary secretion [120-122]. The PDZ domain of the alpha-1-syntrophin protein has been shown to interact strongly with ABCA1 via the C-terminal three amino acids SYV of ABCA1. Co-expression of alpha-1-syntrophin has been shown to increase the half-life of ABCA1, downregulating the degradation process of ABCA1, and also to increase the apoA-I-mediated release of cholesterol in human cell lines [123-126]. Binding of alpha-1-syntrophin to the C terminus of ABCA1 has been proposed to cause a conformational change in ABCA1 making it resistant to proteolysis by calpain [123-125]. Syntrophins have thus been implicated to be involved in lipid homeostasis in the brain

[124]. Also, a novel interaction of ABCA1 with the PDZ protein β1-syntrophin has been identified. SiRNA mediated down-regulation of β1-syntrophin has been shown to decrease ABCA1 protein levels, whereas over-expression of β1-syntrophin increases ABCA1 cell-surface expression and stimulated efflux to apoA-I [121-124]. These findings indicate that syntrophins are involved in the intracellular signaling pathways that regulate the cellular distribution, stability, and activity. All these associations have led to the conclusion that the syntrophins function to recruit signaling proteins to the dystrophin based cytoskeletal complex and thereby regulate its downstream signaling.

1.7.6. Growth factor receptor bound 2 (Grb2): is an important adaptor protein identified as a potentially important mediator of several signaling pathways that may elicit crucial cellular responses during cellular growth and development. Grb2 consists of two SH3 (Src homology 3) domains flanking a SH2 (Src homology 2) domain within its structure. The presence of these binding domains, SH2 and SH3, has important functional implications for Grb2. Grb2, via its SH3 domains, can interact with proteins possessing proline-rich motif (PXXP), e.g., P66shc, while its SH2 domain allows it to interact with phosphor–tyrosine proteins. The Grb2 SH2 domain binds to different growth factors and receptors directly through tyrosine phosphorylated motifs as well as binding to other adapter proteins which mediate the indirect docking of Grb2 to cell receptors. Alpha-1-syntrophin, via its proline-rich regions, has been shown to bind to this important adaptor protein [127]. In syntrophins, there are two different PXXP sequence-containing regions between amino acids 44–75 and 181–229, and both of these have been shown to bind each of the two Grb2 SH3 domains in vitro with high affinity [127]. Interaction of syntrophins with Grb2 has served as the basis of its involvement in the activation of an important Rho family GTPase Ras-related C3 botulinum toxin substrate 1, i.e. the Rac1 protein activation pathway.

1.8. Alpha-1-syntrophin mediated Rac1 activation:

Recently, a signaling pathway has been described that links the matrix laminin binding on the outside of the sarcolemma to the binding of Grb2 to alpha-1-syntrophin on the inside surface of the sarcolemma [128]. The downstream signalling pathway has been elucidated as Grb2-Sos1-Rac1-PAK1-JNK. This ultimately leads to the activation of c-jun via the phosphorylation of its Serine residue, S65, and the downstream pathways thereby (Figure

1.10). The DGC connects to the extracellular matrix laminin via α-dystroglycan (αDG) and spans the sarcolemma by way of proteins such as β-DG, α-, β-, γ-, and δ-sarcoglycan (δ-SG), and sarcospan (SSPN) [107, 128-131]. In cell signaling mediated by Grb2, normally Grb2 binds to a phosphorylated tyrosine-containing sequence, via its SH2 domain, while the N-terminal SH3 (N-SH3) domain of Grb2 binds Sos1 and activates it [132]. Syntrophin binding to Grb2 has been shown to activate the Sos1 protein and Rac1 protein [131-132].

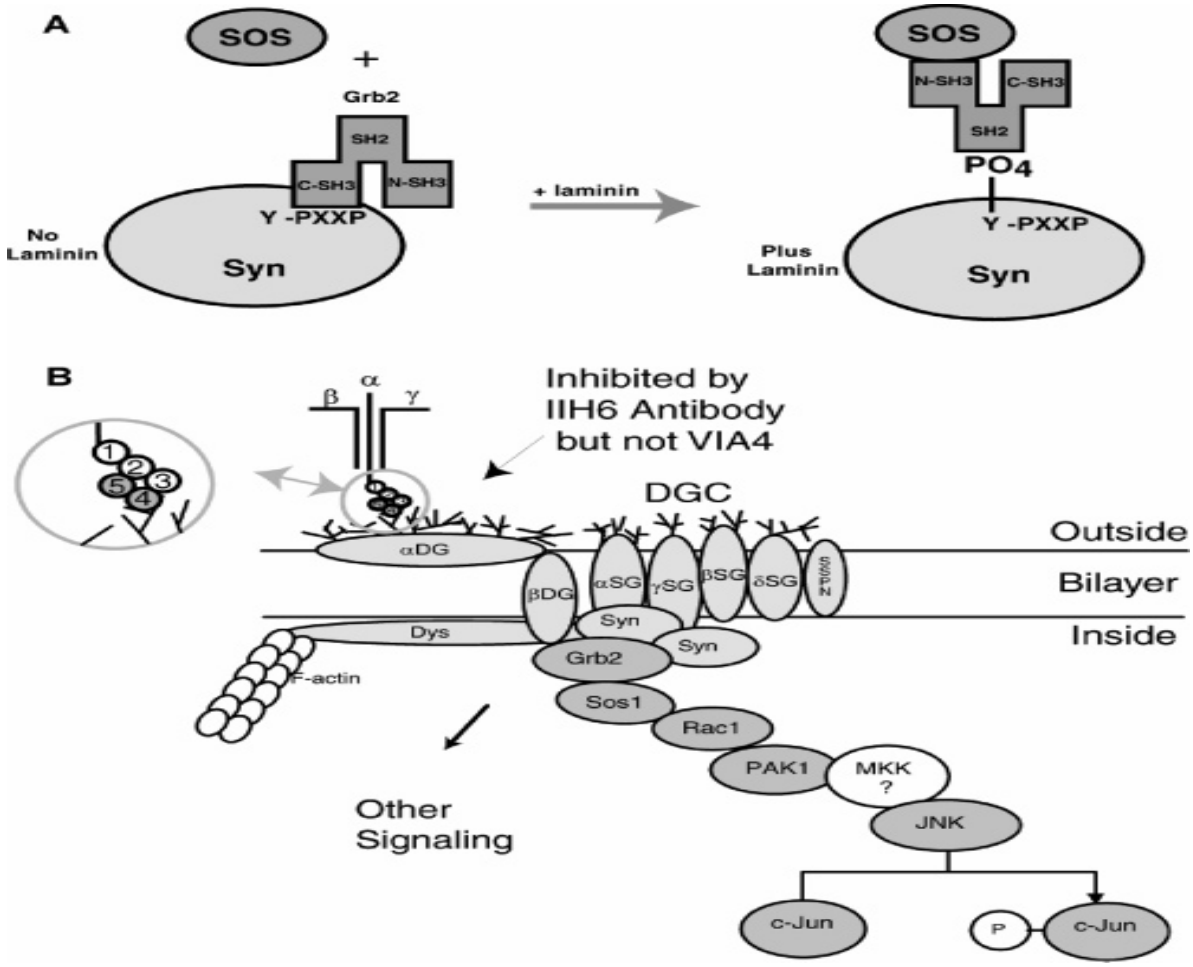


Figure 1.10: Diagram showing the Rac1 signaling model: *Laminin LG4-5 region binding to α-dystroglycan is required. Laminin-induced syntrophin phosphorylation alters Grb2-binding to initiate the downstream signalling [128].*

The Grb2 protein has also been shown to bind to both alpha-1-syntrophin protein [127, 128] and βDG via its SH3 domain [133]. Laminin binding on the outer sarcolemma has been shown to induce a conformational change in alpha-1-syntrophin on the inner surface of the membrane leading to the phosphorylation of alpha-1-syntrophin on a tyrosine residue leading to an altered Grb2 binding. When syntrophin is tyrosine-phosphorylated, it no longer binds to the C-SH3 domain but this phophorylated syntrophin now binds to the SH2 domain of the Grb2 adaptor protein, [128] and this complex is envisioned as being unable to bind Son of Sevenless homolog 1 protein (Sos1), leaving the Sos1 protein free from Grb2 to interact with the E3B1/EPS8 protein complex which in turn results in Rac1 activation and the remainder of the pathway. It has also been confirmed that the presence of syntrophin proteins is important for the recruitment of Rac1 to this complex as well as the functioning of this signaling complex [107, 127-132].

1.9. P66shc mediated Rac1 activation/redox balance:

P66Shc is a ubiquitous vertebrate protein, encoded by the Shc locus. The mammalian proto-oncogene SHC locus encodes three proteins with relative molecular masses of 52K (P52shc), 46K (P46shc) and 66K (P66shc). All these family members share a highly conserved modular organization consisting of an N-terminal phospho-tyrosine binding (PTB) domain, a central proline-rich collagen homology (CH1) domain and a C-terminal Src-homology2 (SH2) domain (Figure 1.11). However in P66shc, the PTB domain is preceded by a unique amino-terminal (CH2) domain that contributes to its diverse role(s) including apoptosis and oxidative stress as against the other isoforms that are predominantly involved in mediating cell proliferation and Ras activation [134,135].

Figure 1.11: P66shc domain structure: *Diagram showing the P66shc domain structure and the important tyrosine residues within the CH1 domain.*

This N-terminal CH2 domain of P66Shc contains serine 36 residue which has been shown to be phosphorylated upon oxidative stimulus, mediating its role in oxidant sensing and apoptosis [136]. P66Shc functions in the intracellular pathway by converting oxidative signals into apoptosis. P66shc acts as a sensor for ROS production and as the downstream target of activated p53, in p53-dependent apoptosis [136]. It has been shown that the tumor suppressor p53 induces P66Shc up-regulation by increasing its stability and the increase of the P66Shc mitochondrial fraction. P66Shc has been proposed as one of the lifespan determinants by virtue of its apoptotic signaling in mouse models [134]. P66Shc is known to mediate oxidative stress through diverse mechanisms including its role at transcriptional level [137]. At mitochondrial level it has been demonstrated to generate ROS and acts as an oxido-reductase enzyme, able to oxidize cytochrome C and to induce H_2O_2 production, causing the opening of permeability transition pores (PTP) and the release of the cytochrome C [135].

P66Shc manages the cytosolic oxidative stress by mechanisms involving reciprocal regulation through RhoGTPase Rac1/Sos-specific pathway [138,139]. P66shc has also been identified as a mediator of Rac1-induced oxidative stress. Expression of constitutively active Rac1 increased phosphorylation, reduced ubiquitination and increased stability of P66shc protein [138]. P66shc becomes tyrosine phosphorylated upon activation of growth factor receptors and forms a stable complex with Grb2 i.e the adaptor protein for Ras exchange factor Sos (Figure 1.12). However, it does not affect mitogen activated protein kinase (MAPK) activity and inhibits c-fos promoter activation, indicating that P66shc may not be involved in Ras activation [140-142]. Conversely, P66shc activates Rac1 through the mediation of exchange factor Sos1 [138,139]. It has been shown that P66Shc mediates the displacement of Sos1 from Grb2 to Eps8 and E3b1 pools. This happens because of competition between P66Shc [139] and Sos1 for binding to Grb2. This competition eventually displaces Sos1 to Eps8 and E3B1 pools. Thus by virtue of its proline motif interaction with Grb2, P66shc is able to displace Sos1 from Grb2 pool to Eps8 and E3b1 pool. The proline rich motif PxxP found at residues inbetween 47 to 50 of P66shc interacts with SH3 domain of Grb2 and thus displaces Sos1 protein which acts as an exchange factor for Rac1. This leads to an increase in Rac1 activity [139] which itself is an activator of ROS generating enzyme NADPH Oxidase [143]. Increasing oxidative stress (ROS generation) contributes to the elevated levels of

P66shc protein and its phosphorylation at serine 36 residue, which promotes further generation of ROS [137] while increased production of ROS in turn increases the P66shc protein levels to mediate oxidative stress signals which indicates the existence of a feedback mechanism between P66shc, Rac1 and ROS generation within the cell.

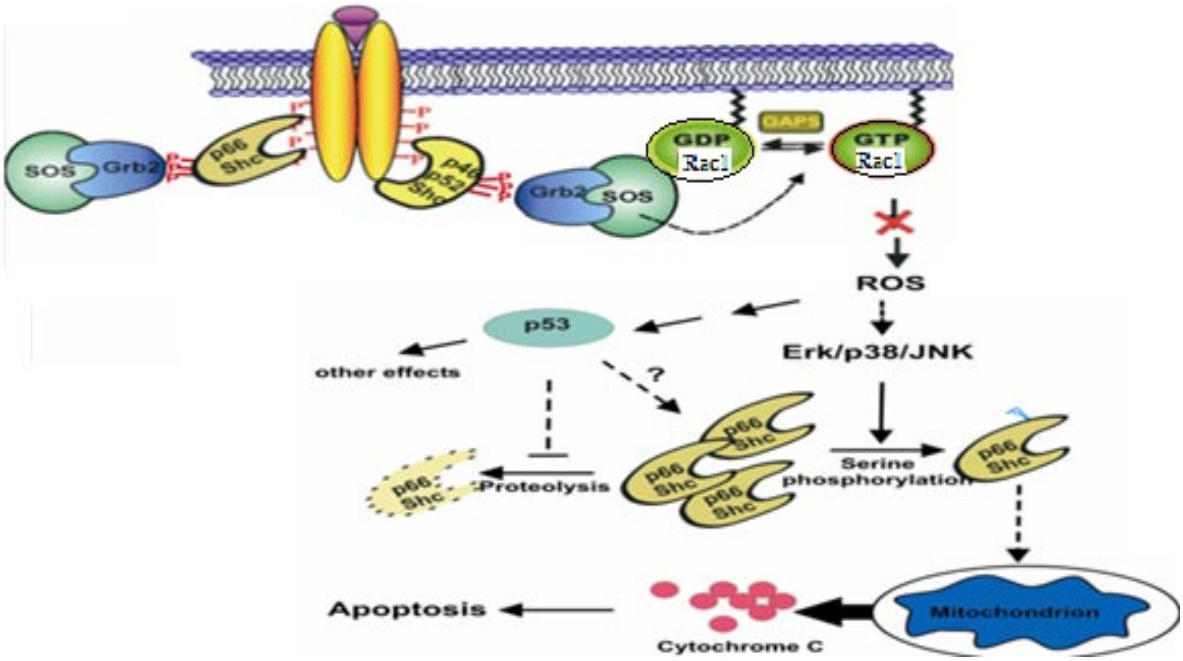


Figure 1.12: P66Shc regulated cytosolic oxidative stress*: Diagrammatic representation showing the P66Shc regulated cytosolic oxidative stress by mechanisms involving reciprocal regulation through RhoGTPase Rac1/Sos-specific pathway within the cell.*

1.10. Rac1 induced cell migration:

Cell migration plays a central role in many biological and pathological processes, including embryonic development, wound repair, the inflammatory response or tumor metastasis. Cell migration can be considered as a highly integrated multistep cycle process [144-146]. The migration cycle includes cell polarization, extension of protrusions in the direction of migration, formation of stable adhesion near the leading edge of the protrusions, and detachment of the adhesion and retraction at the rear.

It is already well known that members of the Rho family of small GTPases are key regulators of the actin cytoskeleton in diverse cellular functions including cell migration [147-150]. Like other small GTPases, Rho-family GTPases serve as molecular switches

by cycling between an inactive GDP-bound state and an active GTP-bound state, and activated GTPases can bind to their specific effectors that lead to a variety of biological functions. Activation of Rho-family GTPases requires GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs). Of Rho GTPases, Rac is activated at the leading edge of motile cells and induces the formation of actin-rich lamellipodia protrusions, which serve as a major driving force of cell movement [151] and induces rapid polymerization of actin and the formation of the branched actin filaments present in lamellipodia (Figure 1.13) [152,153]. However, precise mechanisms that lead to Rac activation during cell migration are not fully understood. In contrast, several studies have shown a role for alpha-1-syntrophin protein in cytoskeleton regulation. Alpha-1-syntrophin in itself has been characterized as an actin binding protein whose sub-cellular localization is in turn regulated through cytoskeletal reorganization within the muscle cells [80]. Alpha-1-syntrophin has also been shown to bind to F-actin [80], and is involved in the membrane recruitment of lipid proteins that are involved in the actin remodeling and cytoskeletal dynamism.

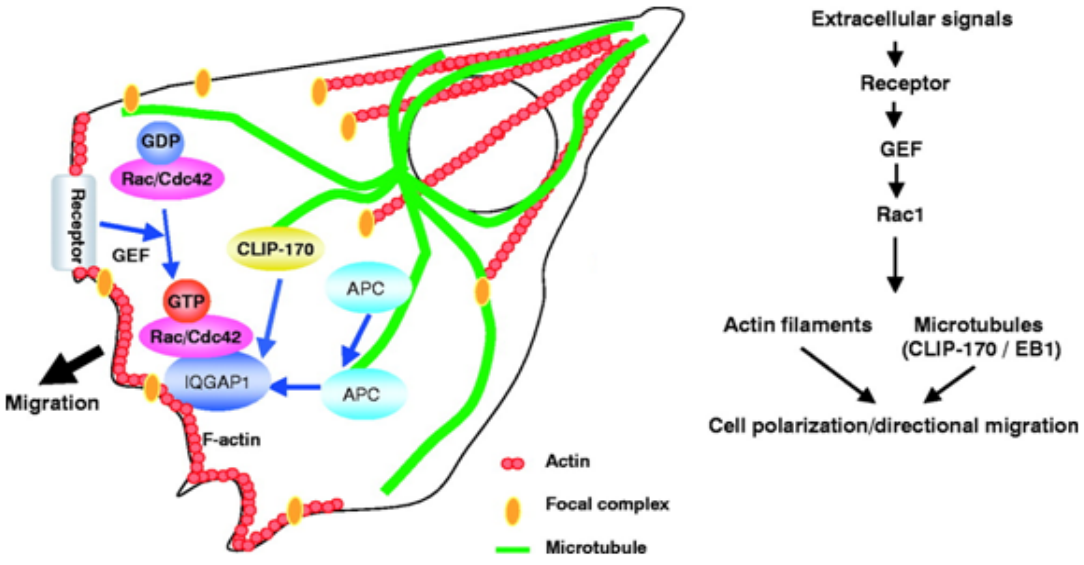


Figure 1.13: Rac1 regulated cell migration: *Involvement of Rac1 protein in regulating cell Polarization and/or directional migration in response to extracellular signals such as growth factors.*

Rationale

2. Rationale of the Study:

Alpha-1-syntrophin is found in a complex with other proteins in the dystrophin glycoprotein complex (DGC), proposed to be involved in Rac1 signal transduction. Also, accumulating evidence has indicated that SNTA-1 is involved in Rac1 activation.DGC recruits Rac1 via alpha-1-syntrophin through Grb2-Sos1 complex and by way of this SNTA1-Grb2-Sos1-Rac1-Pak1 pathway it ultimately results in the activation of Rac1 [128]. An antibody used against alpha-1-syntrophin prevents recruitment of Rac1 by DGC, suggesting that the complex requires alpha-1-syntrophin as essential component of the pathway [128]. Syntrophin binding to grb2 has been shown to activate Rac1 protein [127-128]. The Grb2 protein binds to both α-1-syntrophin protein [127] and βDG via its SH3 domain [129]. Laminin binding on the outer sarcolemma has been shown to induce a conformational change in syntrophin, leading to its phosphorylation which binds to the Grb2 SH2 domain [138], leaving Sos1 protein free to interact with the E3B1/EPS8 protein complex to activate Rac1. Also, P66shc, an adaptor protein that promotes oxidative stress, has been shown to increase Rac1 activity through the mediation of the Sos1 protein [138-139]. P66shc decreases Sos1 bound to the Grb2 protein and increases the formation of the Sos1-eps8-e3b1 tri-complex which eventually leads to Rac1 activation. This P66shc-induced dissociation of Sos1 from Grb2, formation of the Sos1-eps8-e3b1 complex and Rac1 activation have been shown to be mediated by the proline rich (PPLP) motif in the CH2 domain of the P66shc protein.

Thus Alpha-1-syntrophin as well as P66shc have been implicated in the highly conserved mechanism that regulates Rac1 activation and both have been shown to form a stable complex with GRB2. This complex relationship between p66shc, Grb2, SNTA1 and Sos1 provided us with a chance of proposing a novel mechanism for the activation of Rac1. We were intrigued by the possibility that the syntrophin-mediated Rac1 activity might involve the role of P66shc. Overall in this study, an attempt to understand the role of alpha-1-syntrophin and P66shc proteins in Rac1 activation was made. While trying to investigate the possible mechanism of its activation, we also tried to look into the effect of these proteins on the Rac1 mediated downstream implications/signalling such as the ROS generation and migration in human breast cancer cell lines.

Methods

3. Methods:

In this part of the dissertation, briefly the various experimental procedures that were followed in carrying out this study will be described and the name of companies, source of purchase etc will be mentioned for the majority of reagents/chemicals and instruments etc that were used during the tenure of this study.

Experimental procedures:

3.1. Cell line Maintenance:

M.C.F-7, H.B.L-100 and MDA-MB 235 cells were obtained from NCCS, cultured in Dulbecco's modified Eagles medium supplemented 10% (v/v) fetal bovine serum with 50 µg/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich) in a humidified atmosphere of 5% CO2/95% air at 37°C. Cells were routinely passaged by trypsinisation, twice a week. Cells were passaged at 80-90% confluency by adding with 2-5 ml of trypsin/ethylenediaminetetraacetic acid (EDTA) (Sigma) at 37°C for 2-3 minutes. Once all the cells had detached, an equal volume of media containing 10% FBS was added to the cells. The cells were centrifuged at 1500 rpm for 4 min at 4˚ C. The supernatant was aspirated. Cells were resuspended in fresh media and then seeded into new flasks or petri dishes at appropriate densities.

3.2. Liquid nitrogen cell stocks preparation and recovery:

Liquid nitrogen stored cell stocks were prepared by trypsinizing 80-90% confluent cultures and centrifuging the dissociated cells (1500 rpm, 5 min at room temperature). Cell pellets were re-suspended in medium containing 90% FCS and 10% dimethyl sulfoxide (DMSO). 1 ml aliquots were pipetted into cryotubes, which were brought to -80°C slowly. The cryotubes were then placed in liquid nitrogen for long term storage. Cell stocks were recovered by thawing for 1 minute in a water bath at 37°C. Stock mixture was resuspended in 2-3 ml of fresh media, centrifuged at 1500 rpm for 5 minutes and the pellet was again suspended in 2-3 ml of media. These cells were placed in a fresh sterile T-75 flask together with 10 ml pre-warmed culture medium and grown under standard growth conditions (i.e., 37°C and 5% CO_2 in a humidified incubator).

3.3. Transfections:

Cells were transiently transfected with the desired plasmid constructs using the standard calcium phosphate method. Briefly, cells seeded in the requisite culture dishes were allowed to grow to more than 70% confluency overnight. These were incubated with the DNA-Phosphate complex (2.5M $CaCl_2.6H_2O$+DNA+HBS) mixture i.e the required DNA and calcium chloride mixture in presence of Hepes buffer saline (HBS) overnight. Cells were then washed twice with complete media and allowed to grow for 48 hrs and then processed for the protein extraction, while the expression studies were monitored by western blotting.

Alternatively, transfections were carried out using cationic lipid-based transfection reagent Lipofectamine 2000 (Invitrogen) reagent as per manufacturer's instructions. Cells were seeded at cell density of nearly $2x10^6$ cells per 100mm tissue culture plate and serum starved in DMEM containing 2% serum before transfections was carried out. Cells were grown overnight to 90% confluence and culture medium was removed from the culture dishes and replaced with fresh Opti-MEM medium (Gibco). Requisite DNA-lipofectamine transfection mixture was applied dropwise to the culture dishes and after 4-6 hours, fresh serum containing medium was added to the cells. Cells were grown for 48 hours before harvesting them. For SiRNA Transfections, the standard protocol provided by the manufacturer was followed (Sigma Inc., USA). Cells were transiently transfected with 10 μM of control siRNA or with siRNA (Sigma Inc., USA) specific for the gene of interest (Sigma Inc., USA). The transfection was done as per the protocol provided by the manufacturer using lipofectamine 2000 (Invitrogen) as transfection reagent.

3.4. Preparation of cell lysates:

Cells after being transfected with the required plasmid constructs successfully were then processed for the requisite experiments. 48 hours after transfection, culture media was removed from cell culture dishes and washed with pre-chilled PBS twice. To obtain cell extracts, lysis buffers used were:

- RIPA buffer
- NP-40 buffer

3.4.1. RIPA buffer for western blot:

For analysis in which the preserved conformation of the proteins was not necessary, as in western blot, cell lysate was prepared by using RIPA buffer. For the experiments, the media was removed from the cells, the cell monolayer was washed twice with ice cold PBS, followed by addition of ice-cold lysis buffer. The cells were detached from the plate using plastic scraper and transferred to an eppendorff tube . The cells were incubated for about 30 min at 4˚C followed by centrifugation at 13000 rpm for 10 min. The supernatant was transferred to a fresh tube and protein concentration was estimated. The estimated protein was further used for experimental work or stored at -80˚ C.

RIPA Buffer Composition:

1% Igepal or NP-40

0.5% Sodium deoxycholate

0.1% SDS

PBS 49.5 ml

Just prior to use add:

100mM β-mercaptoethanol

1mM PMSF

10µl/ml protease inhibitor cocktail (1000xstock)

5-10mM sodium fluoride

15µl/ml triton X-100.

3.4.2. N-P buffer for immuno-precipitation:

For immunoprecipitation the cell monolayer was homogenized in NP -40 buffer without EDTA, incubated at 4˚ C for 30 min and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to a fresh and chilled eppendorff tube and protein concentration was estimated.

NP-40 buffer Composition:

20 mM Tris Cl pH 8

137 mM NaCl

20% glycerol

1% Nonidet P-40

2 mM EDTA

Just prior to use add:

5-10mM sodium fluoride

1 mM Sodium orthovanadate

1mM PMSF

10µl / ml of lysis buffer, protease inhibitor cocktail (1000x stock).

3.5. Protein Estimation:

A Bio-Rad protein assay based on Bradford's method was used for measuring protein concentration. It is a rapid and accurate method for estimation of protein concentration. The assay relies on the binding of the dye coomassie blue G250 to protein. The quantity of the protein can be estimated by determining the amount of dye in the blue ionic form. This is achieved by measuring the absorbance of solution at 595nm or 625nm. When compared with Lowry's method, it is subject to less interference by common reagents and non-protein components of biological sample. It is only a relative method, as the amount of dye binding appears to vary with the content of the basic amino acids arginine and lysine in the protein. The protein concentration of the protein lysates was determined spectrophotometrically at 595nm with the Bio-Rad protein assay kit as suggested by the manufacturer. Briefly, 1mg/ml of stock solution of BSA was made. From stock, 0.1 mg/ml working solution was prepared using double distilled water. The standard BSA 0.1 mg/ml, samples and distilled water were used according to the below mentioned protocol and the volume was adjusted to 0.1 ml. To each tube, then 1.5 ml of Bradford's reagent

was added, mixed and kept for 10 minutes. Optical density was read on spectrophotometer at 595 nm**.** Sample concentration = x / v mg/ml, where x = value from standard graph (µg) and V = volume of sample (µl).

3.6. Immuno-precipitations:

Protein G sepharose or agarose beads were used for immune-precipitation assaye. Beads were washed twice with cold P.BS (centrifuged for 2 min at 2000 rpm at 4◦C) before use. Immunoprecipitations were typically carried out by incubating 3 µg of antibody with 1 mg of cellular protein lysates overnight at 4°C, followed by incubation with 50 µl of protein A-Sepharose slurry for 4 h 4°C. Beads were washed thrice (centrifuged for 2 min at 2000 rpm at 4◦C) with ice cold PBS, boiled in 2X loading buffer for 5 minutes. The samples were run along with Mol. wt. standards with known concentrations on 10% SDS-PAGE, transferred to PVDF (Sigma) membrane and detection was done with specific antibody.

3.7. Western blotting:

SDS-PAGE and Western blotting was done by standard methods. The sample and the stacking gel contained Tris-glycine (pH 6.8), the upper and the lower reservoirs contain Tris-glycine (pH 8.3), and the resolving gel contains Tris.Cl (pH 8.8). All components of the system contained 0.1% SDS. Briefly, the protein lysates were prepared as described above and diluted in sample buffer (composition mentioned below) in the presence of reducing agent, separated using 10% SDS-PAGE and then transferred onto a PVDF membrane pre-activated in methanol using wet blotting procedure at 84V constant current for 90 minutes. Membranes were washed three times with TBS and then blocked using TBSTM (5% Non fat dry milk in T.B.S containing 0.05% tween-20) for 2-3 hrs. The membranes were probed with the specified primary antibody diluted in TBSM as per the recommended dilutions provided by the manufacturer and incubated overnight at 4°C on a rocking plate. Thereafter, membranes were washed three times with TBS-T (TBS with 0.05% Tween 20), followed by an incubation for 2 hr at RT with a 1:10,000 dilution of the appropriate peroxidase-conjugated secondary antibody. After subsequent washes with TBS-T, chemiluminescent signal was developed using Super Signal West Femto substrate (Pierce Chemical, Rockford, IL). Blots were imaged with a Chemi Doc system, Flour-

chem E (Cell Biosciences), and bands were quantified using Alpha imager (Cell Biosciences) using Alpha-View software version 3.2. For re-probing, blots were stripped in stripping buffer (10 mM glycine, 2% SDS, pH 2.0) and incubated for 30 minutes at room temperature with occasional agitation. Membranes were washed for 2 x 10 minutes in TBS-T at room temperature using large volumes of washing buffer. Membranes were then blocked and re-probed with the required antibody.

Sample Buffer Preparation (1X)

50 mM Tris-Cl (pH 6.8)

2% SDS (electrophoresis grade)

0.1% bromophenol blue

10% glycerol

5% β-mercaptoethanol

3.8. Rac1 activation assays:

The magnitude of GTP-bound Rac1 was determined using a commercial assay kit (Upstate Biotechnology) that affinity precipitates GTP-Rac1 in cell lysates using agarose conjugated Rac1 binding domain of PAK. This GST-PBD construct consists of amino acids 67-150 of human PAK1 bound to glutathione-agarose. Assay was performed according to the protocol provided by the manufacturer (Upstate Biotechnology) and the precipitates were then immunoblotted with anti-Rac1 antibody provided with the kit. Briefly, cells that were transfected with the desired plasmid construcs, SiRNA's etc successfuly, were then processed for the requisite experiments. Cells were lysed using ice cold 1x MLB buffer (preparation mentioned below). The fresh lysates were quantified for determining the protein concentration of samples using bradford's assay. These lysates were then incubated for 2 h with 10 µl of the agarose conjugated reagent provided in the kit. Beads were washed thrice with with cold MLB, boiled in 2X loading buffer for 5 minutes, subjected to SDS-PAGE, transferred to PVDF membrane. The GTP-bound form of rac1 associated with GST-PAK1 was detected using a monoclonal antibody against rac1, also provided with the kit.

1x MLB: To prepare 5ml working (1x) MLB buffer, combine the following:

- 1 ml of MLB stock solution provided.
- 4 ml of dd.H_2O containing 10% glycerol.

3.9. Cell Proliferation Assay:

An MTT assay (Sigma) was used to measure cell proliferation. Briefly, cells were transfected with the desired plasmid constructs, SiRNA's e.t.c in 6-well tissue culture plate, the medium was changed from DMEM-15 to DMEM for serum starvation. The serum-starved cells were then maintained for 36 hours in each culture condition. At the end of culture, the MTT (50 μg/mL) was added and further maintained for 3 hours at 37°C. The MTT-containing medium was discarded, and acidic dimethyl sulfoxide was added to the cells. After 30 minutes incubation at room temperature, absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

3.10. ROS generation assay:

H_2O_2 was quantified in cell media using the Amplex Ultra Red reagent (Molecular Probes, Invitrogen), as was described in the protocol provided by the manufacturers. Briefly, cells were seeded in 6-well plates at a density of $0.5x10^6$ cells/well and transfected with the required plasmid/SiRNA constructs and incubated at 37 °C in the incubator. After 38 hrs, 50μl of the working solution of Amplex ultra red /HRP mixture (preparation mentioned below) was added to each well containing the control as well as samples to initiate the reaction. The cells along with the mixture were protected from light and incubated at room temperature for 15-30 min. The fluorescence signal was then detected using spectro-fluorophotometer (Shimadzu, RF-5301) with excitation at 568nm and emission at 581nm.

Working solution of Amplex ultra red: To prepare 5 ml of working solution of Amplex ultra red /HRP mixture, combine the following:

- 50 μl of 10mM Amplex ultra red reagent stock solution (add 340 of fresh DMSO to one vial of Amplex ultra red reagent and vortex well to dissolve. Store at -20°C, protected from light).

- 100 of 10U/ml HRP.
- 4.85 ml of reaction buffer.

3.11. *In Vitro* Wound Healing Assay:

Standard protocol for in vitro scratch assay was followed [154]. Briefly, cells were plated in 60-mm dishes. At 70% confluence, cells were transfected with the plasmid encoding gene of interest or SiRNA along with an empty vector (EV) as control. Cells were incubated at 37 °C until cells reach 100% confluence to form a monolayer. P-10 pipette tip was used to create a scratch. After Washing the plate once with media, cells were fed with complete medium (10% FBS). Acquire images for the same scratched region until the scratch completely close or within a desired time frame. Pictures were taken with a Nikon camera fitted to a microscope. The gaps were measured and calculated into % wound closure by the available computing software (Image J software) that measures the distance travelled during the desired time frame. Data were expressed as mean ±SE and accompanied by the number of experiments performed independently, and analyzed by t-test. Differences at $P<0.05$ were considered statistically significant.

3.12. Cell migration assay:

Migration of cells was studied using a Migration assay chamber (Millipore QCM) based on Boyden chamber principle. 24-well transwell chambers with 8.0-μm pore size polycarbonate membrane were used for this experiment. This pore size supports the optimal migration for most of the cell types. Transfected cells were plated at a density of 5×10^4 per well in 0.3 mL of starvation media (without FBS) in the upper well, which was placed into a lower well containing complete medium (10% FBS). After 24 h at 37°C, 5% CO2 incubator, the experiment was stopped by wiping the cells from the inner well with a cotton swab. Cells that had migrated through the membrane were fixed, stained and destained using the stain/destain solutions provide with the kit (Millipore QCM). Absorbance was read on a standard mico plate reader (Epoch Biotek Instruments) at 565 nm. Each experiment was repeated at least thrice and the results were averaged. All values are provided as the mean ± SEM. p Value of ≤ 0.05 was considered to indicate the significant difference.

3.13. Mutagenesis:

Xpress-tagged p66Shc WT cloned in PCDNA 3.1/His A vector was a kind gift from Dr. Shaida Andrabi at Harvard medical School (Boston MA). PCDNA 3.1 was obtained from Addgene. Org. while Myc tagged SNTA-1 plasmid vector was a kind gift from Dr. Marvin Adams at University of Washington, (Seattle, WA). They were confirmed by sequencing of the plasmids using the services of Sci-genome labs (Kerala, India).

3.14. Generation of Mutant forms of SNTA1:

Site directed mutagenesis was carried out to generate various point mutants using Quick change II mutagenesis kit (Stratagene Inc. USA). Mutagenesis was performed by designing the below mentioned primers and incorporating the mutated base in place of the residue that needs to be mutated by P.C.R. This was followed by Dpn-1 treatment and transformation into XL-1 e.coli strain. Reaction mixture was transformed into XL1-Blue chemically competent cells supplied with the kit by heat shock method on ampicillin-LB Agar plate. Five transformants were selected at random for plasmid isolation and sent for sequencing. Mutagenesis efficiency was recorded at above 90%. The use of these dominant negative/mutant plasmid constructs helped us in understanding the mode of these interactions as well as the functional implications of the pathway executed by the SNTA-1 protein. The general protocol followed for inducing the point mutations is briefly mentioned below:

3.15. Primer Design: the primers for the site directed mutagenesis were designed using "*Primer-X"* site directed mutagenesis primer designing software. For introducing the triple mutations in the SYV (amino acid 426-428) motif of P66shc, we used 3 different sets of primers, each set used to introduce the desired mutations sequentially, so that the product of first P.C.R reaction would serve as the template for second reaction using the second set of mutant primers and the second P.C.R product was used as the template for third i.e final triple mutant (SYV to AFA) generation. Before using any P.C.R product in the next following reaction, the introduced mutation was sequence confirmed first. The 3 sets of primers thus used were as mentioned below:

3.15.1. 1Y SNTA1 (229 Tyrosine – Phenylalanine):

SNTA1 variant carrying a point mutation at amino acid 229, such that tyrosine was changed to phenylalanine residue i.e $\mathbf{Y^{229} = F^{229}}$

Primers:

Forward: CTTTGAAGATGGCGTTTGTGTCAAGGAGGTG

Reverse: CACCTCCTTGAGACAAACGCCATCTTCAAAG

3.15.2. 2Y SNTA1 (215 Tyrosine – Phenylalanine):

SNTA1 double point mutant (DM) variant i.e carrying a point mutation at amino acid 229, such that tyrosine was changed to phenylalanine as well as at amino acid 215, such that tyrosine was changed to phenylalanine residue i.e $\mathbf{Y^{215,229} = F^{215,229}}$

Primers:

Forward: CCAGAGCCCAGGTTTCTGGAGATCTGTGC

Reverse: GCACAGATCTCCAGAAACCTGGGCTCTGG

3.15.3. P66shc (426 Serine – Alanine):

P66shc point mutant variant i.e carrying a point mutation at amino acid 426, such that serine was changed to alanine i.e $S^{426} = \mathbf{A^{426}}$

Primers:

Forward 426: TTGATGATCCCGCCTATGTC

Reverse 426: TTGACATAGGCGGGATCATC

3.15.4. P66shc (427 Tyrosine – Phenylalanine):

P66shc point mutant variant i.e carrying a point mutation at amino acid 427, such that Tyrosine was changed to phenylalanine residue i.e $T^{427} = \mathbf{F^{427}}$

Primers:

Forward 427: ATGATCCCGCCTTTCTCAAG

Reverse 427: CTGGACGTTGACAAAGGCG

3.15.5. P66shc (428 Valine – Alanine):

P66shc point mutant variant i.e carrying a point mutation at amino acid 428, such that valine was changed to alanine i.e $V^{428} = A^{428}$

Primers:

Forward 428: ATCCCGCCTTTGCCAACGTC

Reverse 428: TTCTGGACGTTGGCAAAGGC

All generated mutants were then sequence confirmed.

3.16. Transformation:

Plasmid DNA was transformed into 50 µL of XL-1 competant cells by Transform Aid Kit (Fermentas) according to manufacturer's instructions. 2-5 µl of plasmid DNA was added to 50 µl competent bacteria and incubated on ice for 30 min. The bacteria were heat shocked for 90 seconds at 42°C and chilled on ice for 5 min. 1 ml LB was added to the transformed bacteria and the cultures were incubated for 1 hour at 37°C with constant shaking at 240 rpm. 200 µl of the culture medium were spread onto each ampicillin containing LB agar plate (50µg/ml). Agar plates were placed inverted in a 37°C incubator for 16-20 hours, after which single colonies corresponding to transformants were inoculated into LB broth and plasmid was isolated subsequently by mini prep plasmid isolation kit (Stratagene, USA) according to manufacturer's protocol and run on 0.8% agarose gel for confirmation. Gel electrophoresis was carried out in tris-acetate ethylenediamine-tetraacetic acid (TAE) buffer at 100V for 30 minutes. Analytical gels were visualized with a UV lamp and photographed by a Chemi Doc system, Flour-chem E (Cell Biosciences).

Results

4. Results:

4.1. SNTA1, P66shc protein expression in human breast cancer cell lines:

Before carrying out the protein interaction studies we intended to test the expression of our protein of interest i.e alpha-1-syntrophin (SNTA1) protein in various human cell lines**.** In agreement with our earlier studies [20], the expression of SNTA1 protein was found to be higher in human breast cancer cell lines i.e. HBL-100, MDA MB-235 and M.C.F-7 (Figure 1.14, 1.15). We then looked for the expression of P66shc protein in these breast cancer cell lines. We looked for the endogenous as well as exogenous (externally transfected) expression of these proteins in HBL-100 and M.C.F-7 human cell lines (Figure 1.16).

4.2. Alpha-1-Syntrophin forms a trimeric complex with P66shc and Grb2:

We intended to test the interaction of alpha-1-syntrophin protein with proteins that are thought to be involved in Rac1 activation like P66shc and Grb2 adaptor proteins. The association between SNTA-1 and other proteins i.e P66shc and Grb2 was tested using co-immunoprecipitation assays. Our results indicate that SNTA1 is co-immunoprecipitated alongwith P66shc. To confirm the physical interaction between human P66shc, Grb2 and SNTA-1, plasmids containing full length inserts were transfected into MDA-MB and HBL-100 cells. First we used anti-SNTA1 monoclonal antibody to pull down the complex from protein lysates of transfected HBL-100 cells, while the presence of P66shc among the precipitated proteins was detected by Western blot using rabbit monoclonal antibody recognizing human P66shc specifically and rabbit monoclonal antibody against Grb2 respectively (Figure 1.17).

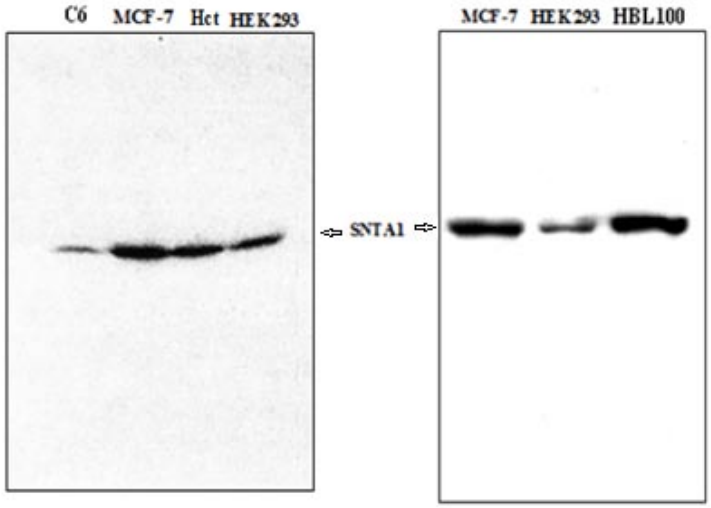


Figure 1.14: Expression of SNTA1 protein in different human cell lines: *representative immunoblots showing the expression of SNTA1 protein in 30µg each of whole cell lysates from C6, M.C.F-7, HCT, HEK-293 and HBL-100 human cell lines respectively.*

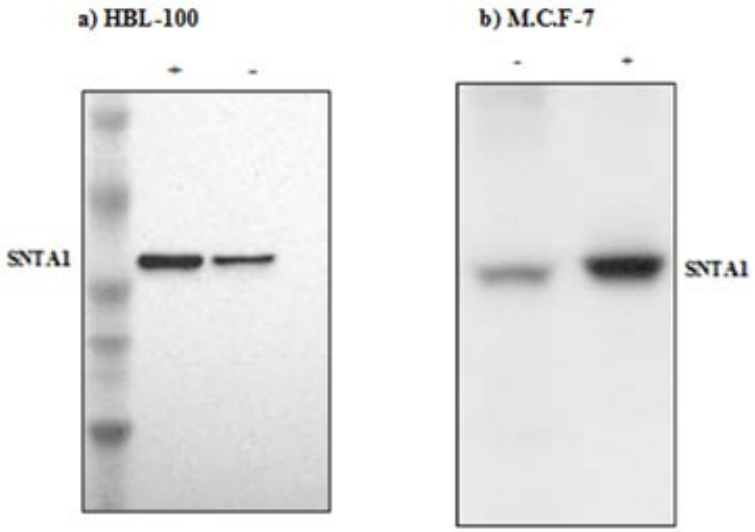


Figure 1.15: Expression of SNTA1 protein in HBL-100 and M.C.F-7 human cell lines: *representative immunoblots showing the endogenous (-) SNTA1 and exogenous (+) i.e. transfection of SNTA1 plasmid in a) HBL-100 and b) M.C.F-7 breast cancer cell lines using Lipofectamine 2000.*

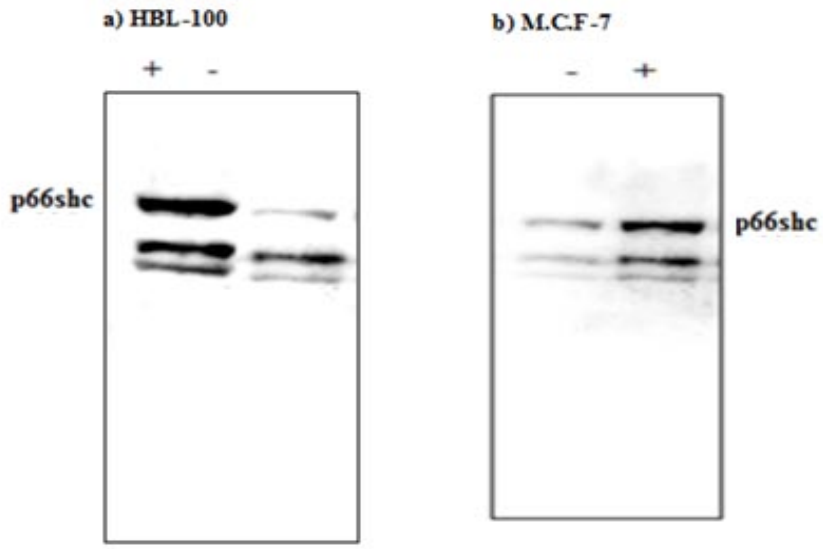


Figure 1.16: P66shc expression in HBL-100 and M.C.F-7 cell lines: *representative immunoblots for the expression of endogenous (-) P66shc and exogenous(+) i.e. transfection of P66shc bearing plasmid in a) HBL-100 and b) M.C.F-7 breast cancer cell lines using Lipofectamine 2000.*

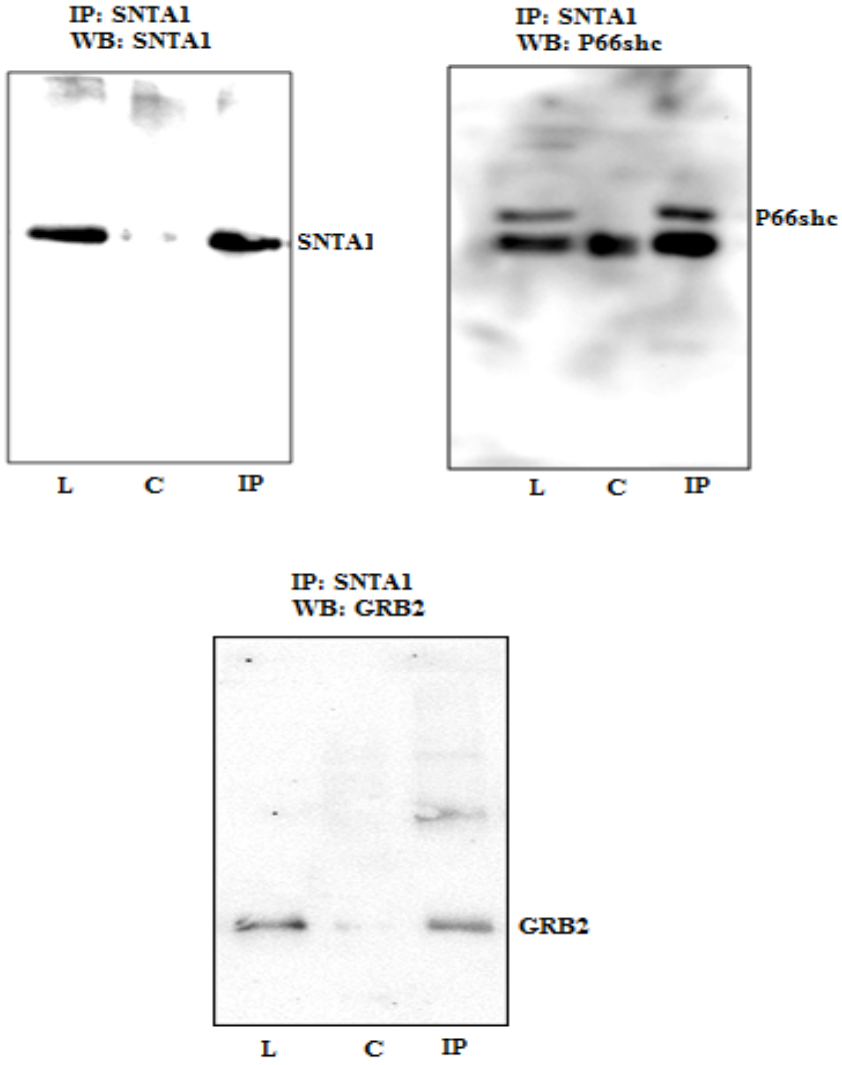


Figure 1.17: SNTA1, Grb2 and P66shc form a trimeric complex: *Immunoprecipitated SNTA1 protein along with P66shc and Grb2 protein co-precipitated. C = IgG control; IP = Protein A pull-down complex (anti-SNTA1); L = Whole cell lysates.*

Conversely, protein lysates from transfected HBL-100 cells were immunoprecipitated with a rabbit polyclonal antibody against P66shc and immune-precipitated proteins analyzed by Western blot using anti-SNTA-1 and anti-Grb2 antibody (Figure 1.18).

Similarly, we tried to test whether pull-down with anti-Grb2 antibody co-precipitates these two proteins as well. Thus protein lysates from transfected HBL-100 cells were immune-precipitated with antibody against Grb2 and immune-precipitated proteins analyzed by Western blot using anti-SNTA-1 and anti-P66shc antibody (Figure 1.19).

Thus our preliminary results indicated that SNTA1 is co-immunoprecipitated with P66shc. Along with these two proteins, we were also able to detect the Grb2 protein. We surmise a possible trimeric complex formation between SNTA1, P66shc and Grb2 proteins that play a role in regulation of Rac1 activity.

In order to confirm the trimeric complex formation between SNTA1, P66shc and Grb2 proteins within the cell, we repeated the co-immunoprecipitation assay using anti-myc monoclonal antibody. Since SNTA1 gene was myc tagged in the plasmid, we used anti-myc antibody for pull down of the proposed trimeric complex (Figure 1.20). As per our results, we were able to pull down both P66shc and Grb2 protein along with SNTA1**.**

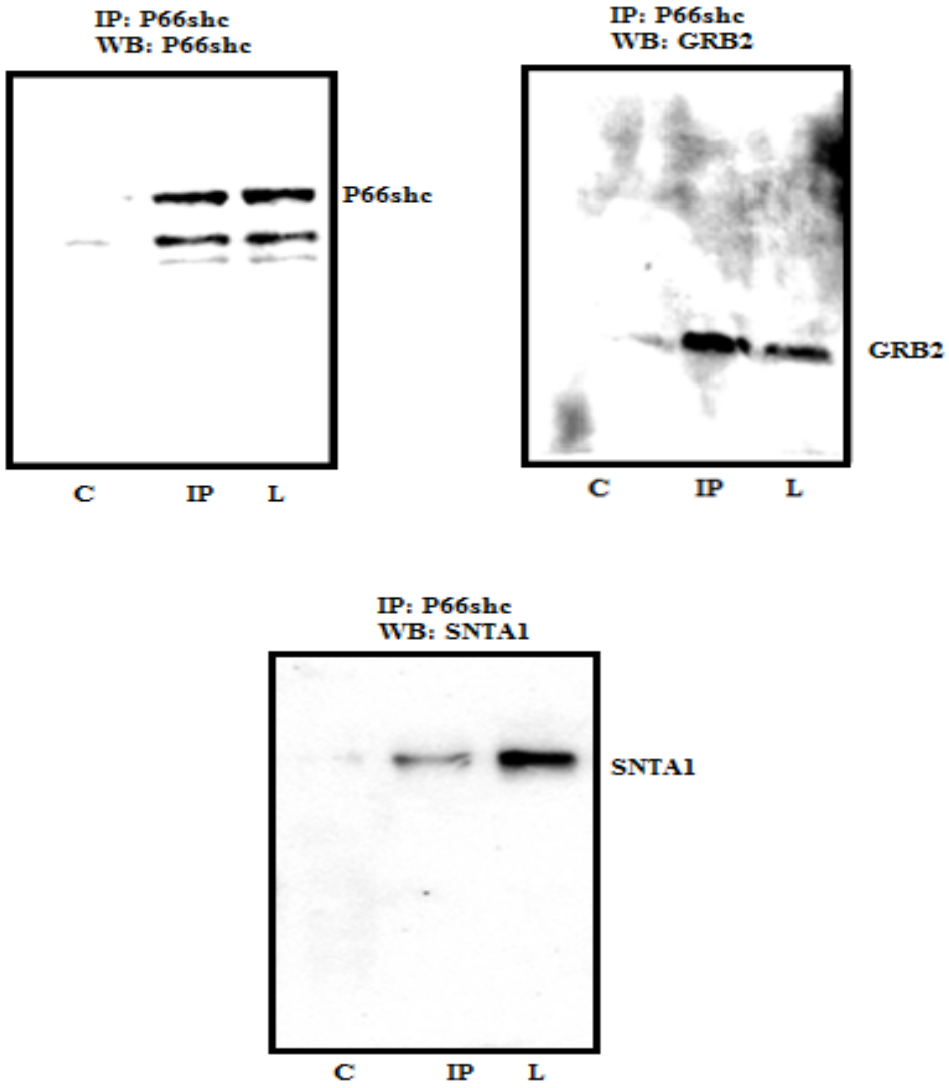


Figure 1.18: SNTA1, Grb2 and P66shc form a trimeric complex: *Immunoprecipitated P66shc protein along with SNTA1 and Grb2 protein co-precipitated. C = IgG control; IP = Protein A pull-down complex (anti-P66shc); L = Whole cell lysate.Co-immunoprecipitated SNTA1 protein along with P66shc and Grb2 proteins.C = IgG control; IP = Protein A pull-down complex (anti-P66shc); L = Whole cell lysate.*

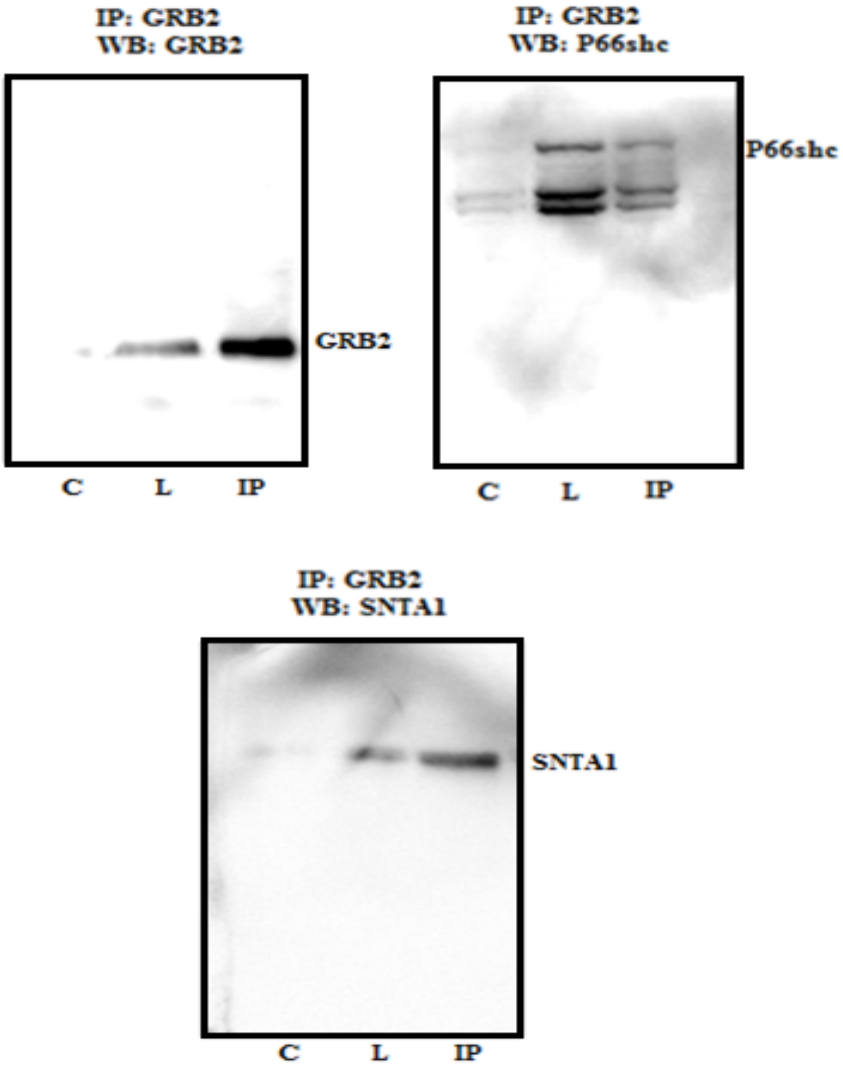


Figure 1.19: SNTA1, Grb2 and P66shc form a trimeric complex: I*mmunoprecipitated Grb2 protein along with P66shc and SNTA1 protein co-precipitated. C = IgG control; IP = Protein A pull-down complex (anti-Grb2); L = Whole cell lysate.*

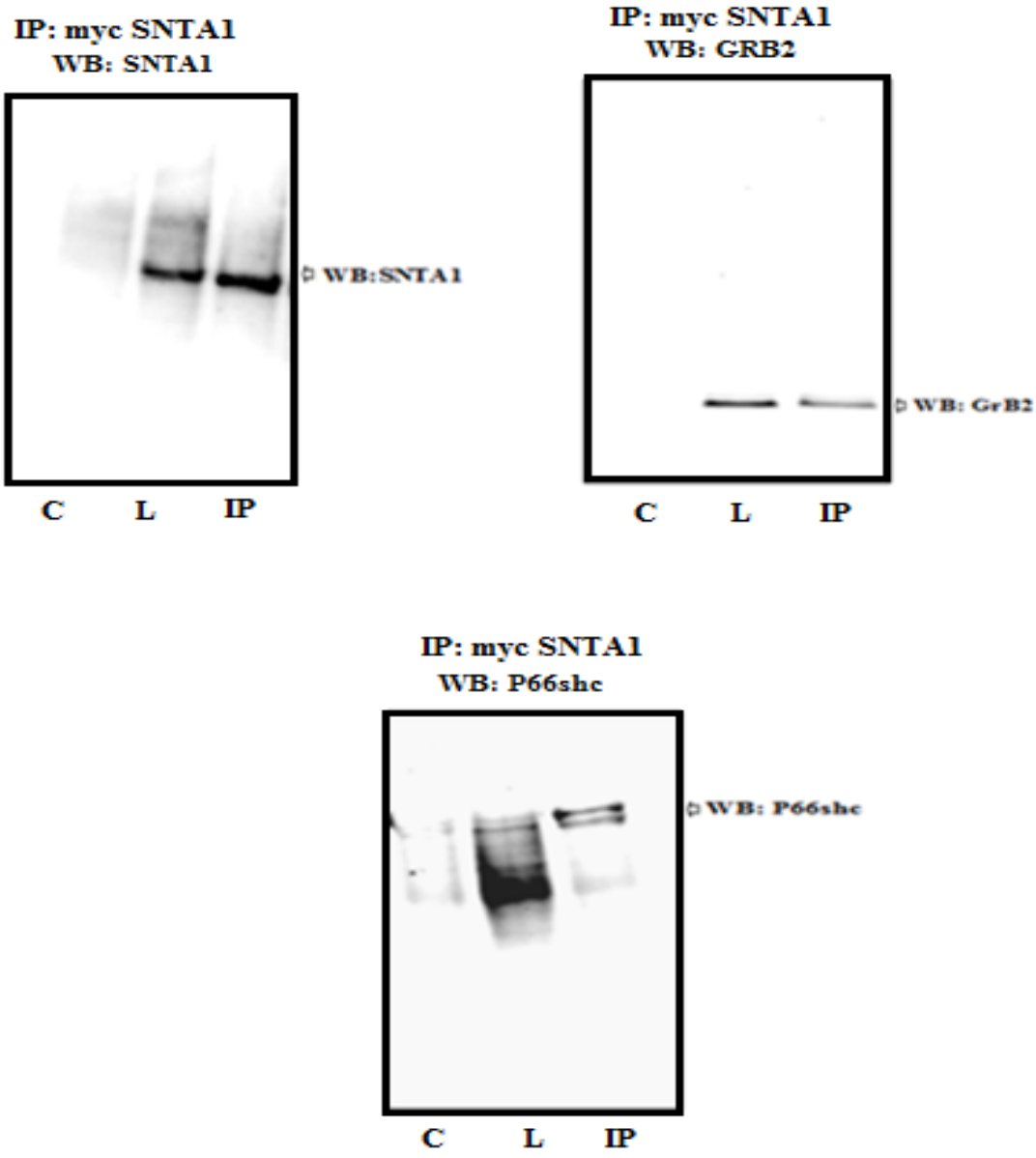


Figure 1.20: Pull-down using anti-myc antibody: *Anti myc(SNTA1) antibody was used to pull down SNTA1 protein. P66shc and Grb2 were detected from the western blotting of these co-immunoprecipitates using their respective antibodies. Normal IgG antibody was used as a control for pulldown. C = IgG control; IP = Protein A pull-down complex (anti-myc); L = Whole cell lysate.*

4.3. Knock-down of Alpha 1 Syntrophin and P66shc using SiRNA/ShRNA:

We planned to knock down the expression of SNTA1 and/or P66shc and look for its effect on this trimeric complex formation. To that end we first needed to standardize the transfection experiment with SiRNA of SNTA1 and ShRNA of P66shc. We were able to find a significant decrease in the expression of SNTA at 10μM concentration of SiRNA as compared to the control (Scramble SiRNA) or the cell lysates (Figure 1.21). Similarly for P66shc we were able to detect a significant decrease in expression at 10μg concentration of ShRNA plasmid used as compared to the empty vector control (EV) or the cell lysates (L).

4.4. Trimeric complex formation enhances rac1 activity:

Accumulating evidence has indicated that SNTA1 is involved in Rac1 activation but the mechanism is still not understood. To determine the effect of SNTA1 expression in human breast cancer cells, we compared the Rac1 activity in MCF-7 and HBL-100 cells transfected with either SNTA1 or P66shc full length plasmid constructs alone or co-expressed along with the respective controls i.e empty plasmid vehicle (EV) (Figure 1.22, 1.23). We carried out Rac1 activation assay as described in material and methods to look into the effect of these proteins on activation levels of Rac1. The Rac1 activation assay results showed that SNTA1 over-expression induced an approximately 3-fold increase in activation of Rac1 (Figure 1.24). Also, the activation level of Rac1 protein was maximum when SNTA1 was co-expressed with P66shc. We examined whether downregulating SNTA1/P66shc expression using small interfering RNA would have any effect on Rac1 activation levels in H.B.L-100 cells. Our results demonstrated that indeed Rac1 activation level was significantly decreased in cells transfected with SiRNA against SNTA1. Further when P66shc was over-expressed in presence of SiRNA against SNTA1, activation level of Rac1 was found to be much lower as compared to its activation levels when transfected with P66shc alone or when P66shc and SNTA1 were co-expressed. Also the activation of Rac1 was found to be least when expression of both these proteins was reduced (Figure 1.22, 1.23). This indicates that the two proteins SNTA1 and P66shc cooperate to function in the activation of the Rho GTPase Rac1.

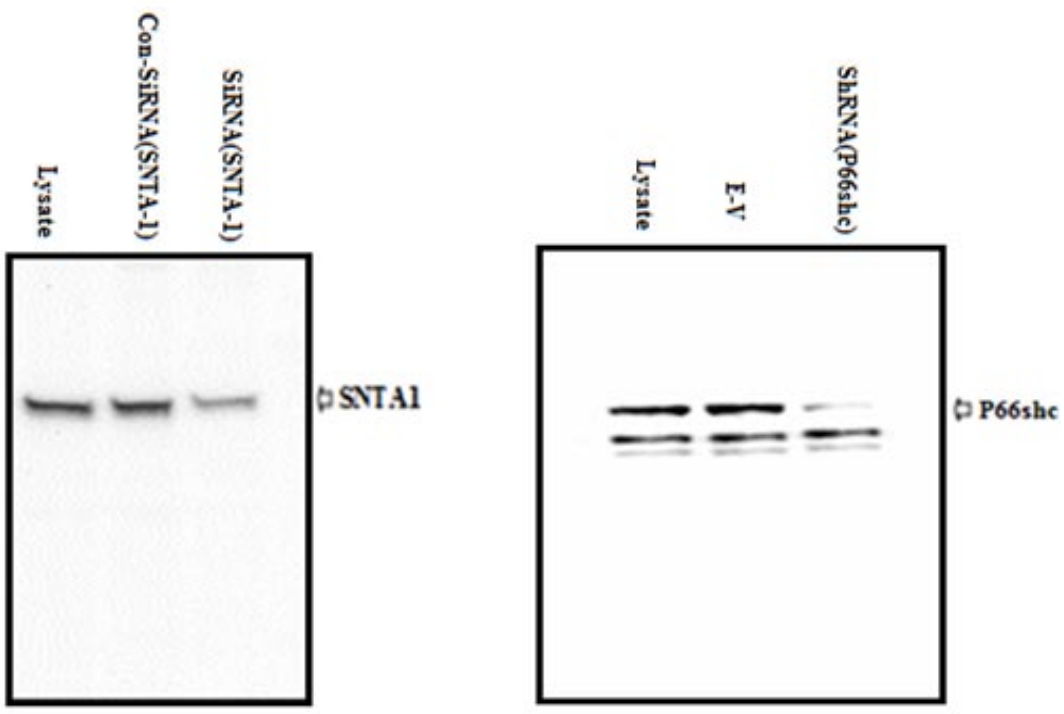


Figure 1.21: Use of SiRNA/ShRNA against SNTA1 and P66shc: *SiRNA (SNTA1) was used to knock down SNTA1 expression with scramble SiRNA as a control (Con-SiRNA). ShRNA (P66shc) was used to knock down P66shc expression in cell lines. Other two isoforms of P66shc (P46 shc & P52shc) were also detected in the blot. Empty vector (EV) control and whole cell lysates were also used to check for the efficiency of SiRNA/ShRNA.*

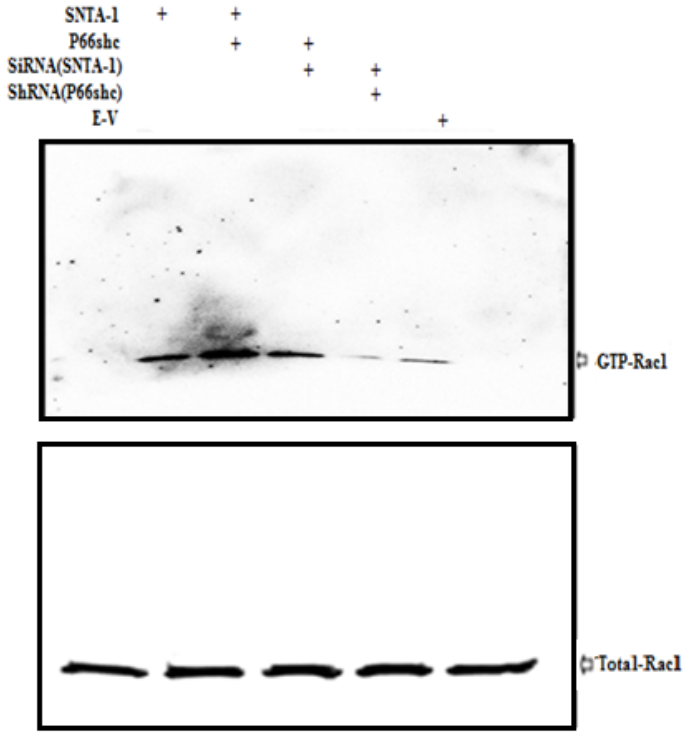


Figure 1.22: SNTA1 and P66shc enhance Rac1 activity in MCF-7 cells: *Comparison of active Rac1 levels in immune-precipitated lysates from MCF-7 lysates transiently transfected with empty vector (EV) or the indicated plasmid constructs.*

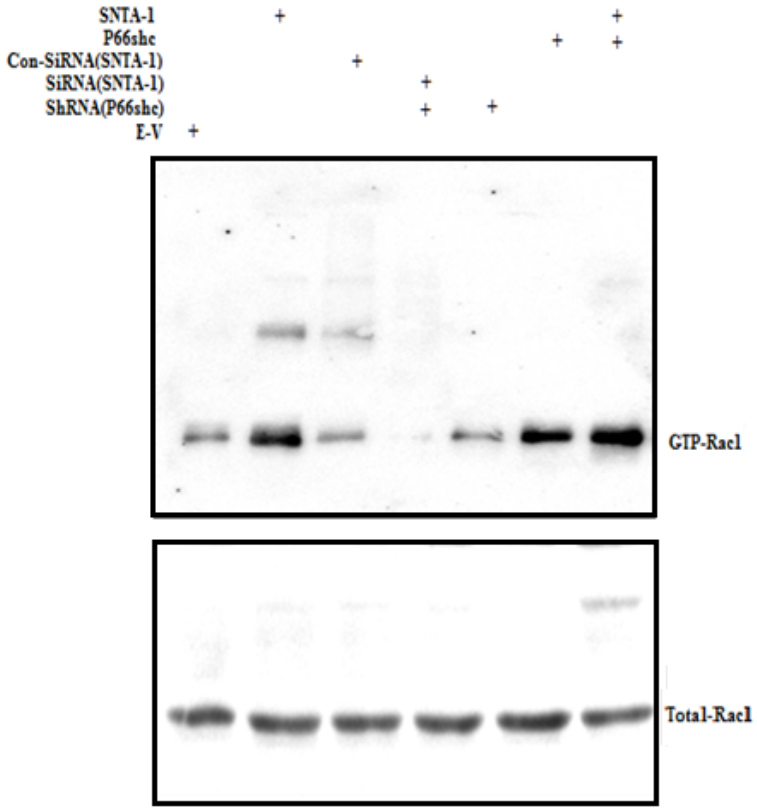


Figure 1.23: SNTA1 and P66shc enhance Rac1 activity HBL-100 cells: *Comparison of active Rac1 levels in immune-precipitated lysates from HBL-100 lysates transiently transfected with empty vector (EV) or the indicated plasmid constructs.*

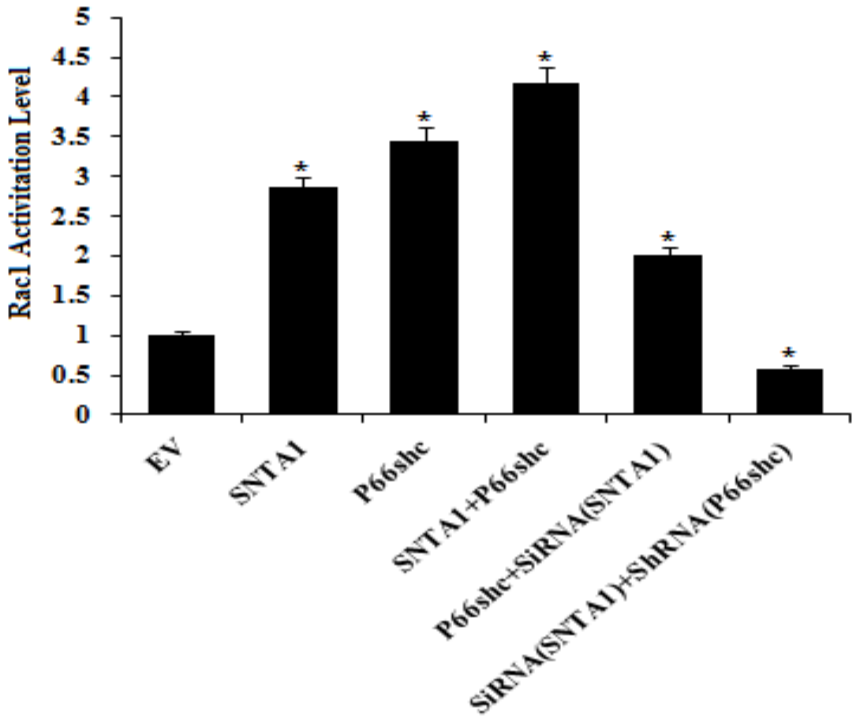


Figure 1.24: Bar graph for the showing the effect of SNTA1 and P66shc on Rac1 activation: *Error bars depicting the difference in Rac1 activity in cells transfected with the mentioned constructs as compared to the empty vector control. Results are mean values ± SE from three separate experiments. *Differences were calculated against EV and considered significant at $p < 0.05$.*

4.5. Site directed mutations of SYV motif of P66shc and Tyrosine ($Y^{215,229}$) of SNTA1:

Once the interaction between SNTA1, P66shc and Grb2 proteins was confirmed by pull-down assays, we further intended to look into the nature of these interactions. The PDZ domain of SNTA1 plays a very critical role in its binding with other proteins and it has been shown to binds to certain proteins through their C-terminal SYV motif. P66shc adaptor protein contains one such SYV motif near its C-terminal, spanning the amino acid residues 426 to 428. Further the Y residue within this SYV motif has also been shown to be important for the interaction of P66shc with Grb2 protein. We surmised a possible role of this SYV motif in the interaction between these proteins and the trimeric complex formation thereby. We first mutated the SYV motif of P66shc with AFA residues to generate the required triple mutant (TM). Further the interaction between SNTA1 and Grb2 has been implicated to be mediated by the tyrosine flanked -PXXP- motif protein spanning the amino acids 215 to 229 of SNTA1. To check for the effect of this tyrosine flanking motif on the trimeric complex formation between SNTA1, P66shc and Grb2 proteins, later on we also created a tyrosine (**Y^{229}**) single mutant plasmid construct and a tyrosine double mutant (DM) i.e. (**$Y^{215,229}$**) plasmid construct of SNTA1 protein.

All these mutants were constructed by a PCR based method using Quick Change TM (Stratagene Inc. USA). 10 μL of the PCR reaction mixture after DpnI digestion (which cleaves methylated parental plasmid DNA) was run on a 1% Agarose gel, to confirm amplification of the plasmid DNA with possible mutations. 5 μL of the mixture was transformed into chemically competent XL1-Blue cells supplied with kit by heat shock method, plated on ampicillin LB-agar. Ampicilin resistant colonies (5 in number) were picked at random and plasmid DNA isolated (Figure 1.25). Mutagenesis was confirmed by sequence analysis using services of Scigenom Labs Pvt. Ltd (Kerala,India). Mutagenesis effeciency was recorded at above 90%.

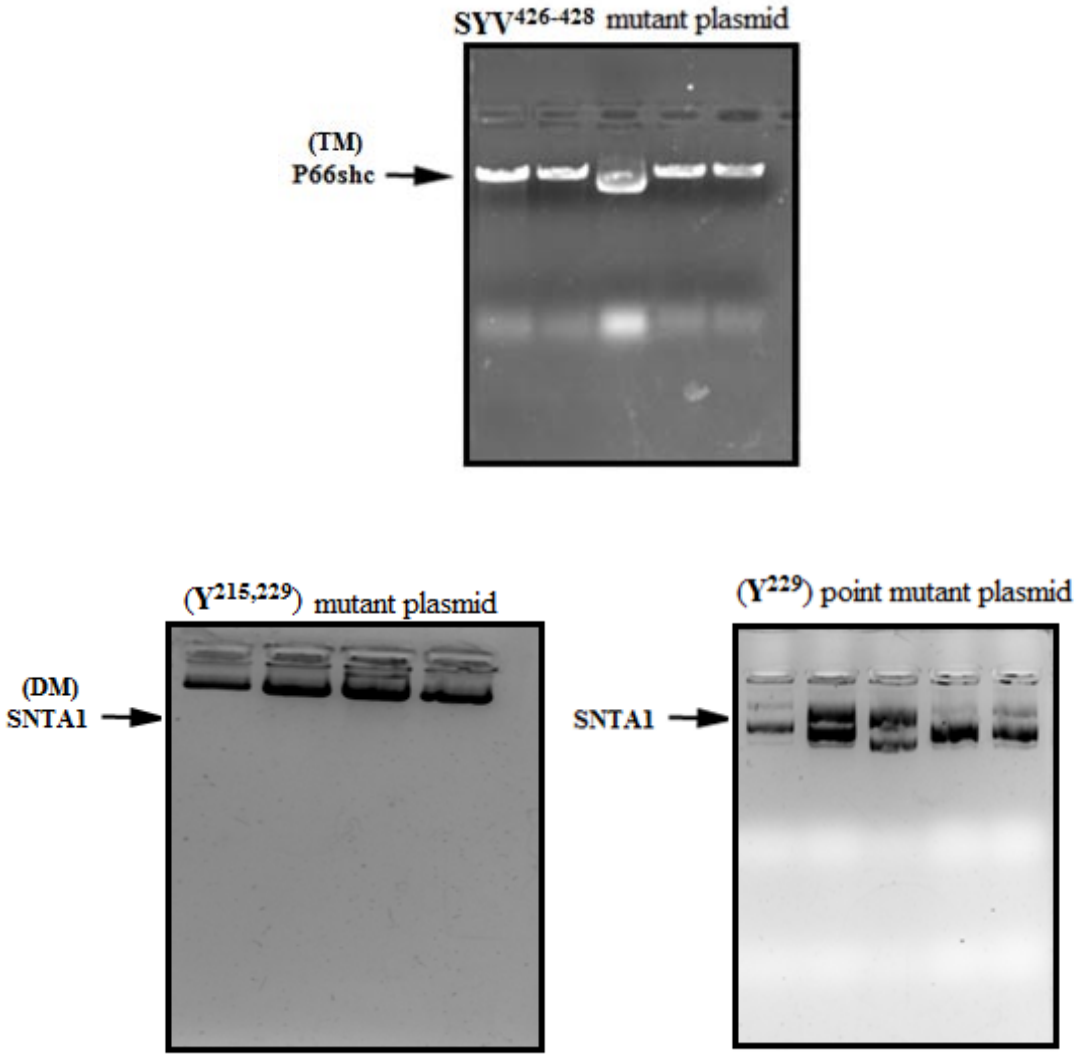


Figure 1.25: Agarose gel picture showing mutagenised P66shc SYV$^{426-428}$ mutant (TM) and SNTA1 Y^{215,229} (DM) and SNTA1 Y^{229} mutants: *Mutagenesis was carried out using Quick change TM kit and 5µL of the amplified product was run on a 1% Agarose gel and mutagenesis confirmed by sequence analysis.*

1Y SNTA1 Mutant:

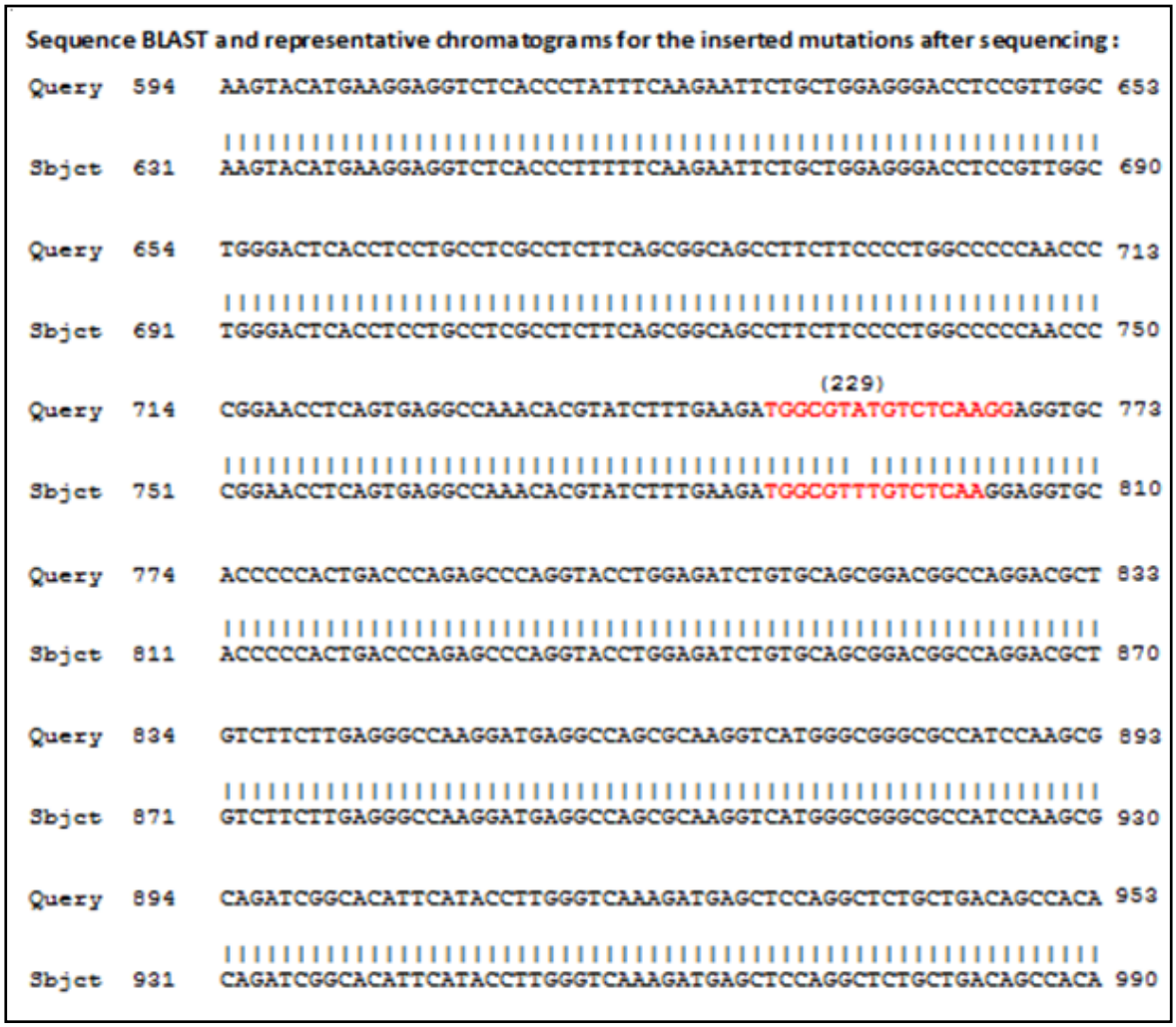


Figure 1.26: Sequence BLAST for the inserted mutations: *The BLAST sequence shows the inserted mutation at position 229 (in red) confirming the inserted mutation.*

1Y SNTA1 Mutant:

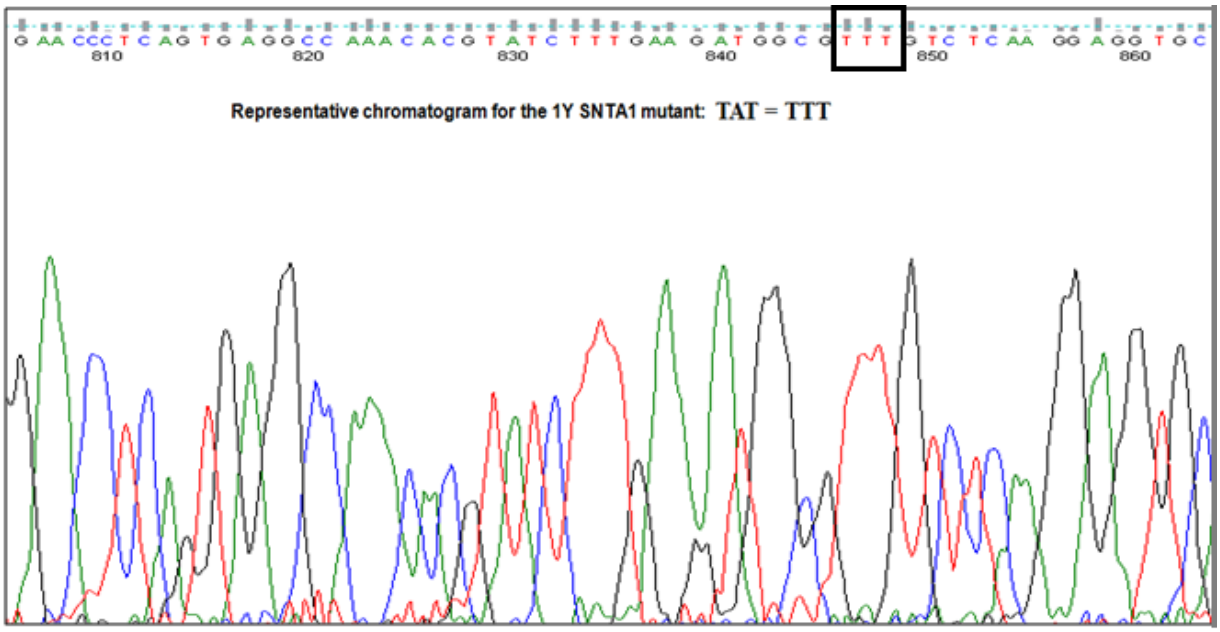


Figure 1.27: Representative chromatogram for the SNTA1 mutant: *Chromatogram showing TAT to TTT mutation replacing Tyrosine with Phenylalanine at position 229.*

2Y SNTA1 Mutant (DM):

Figure 1.28: Sequence BLAST for the inserted mutations: *The BLAST sequence shows the inserted mutation at position 215 and 229 (in red) confirming the inserted mutation.*

2Y SNTA1 Mutant (DM):

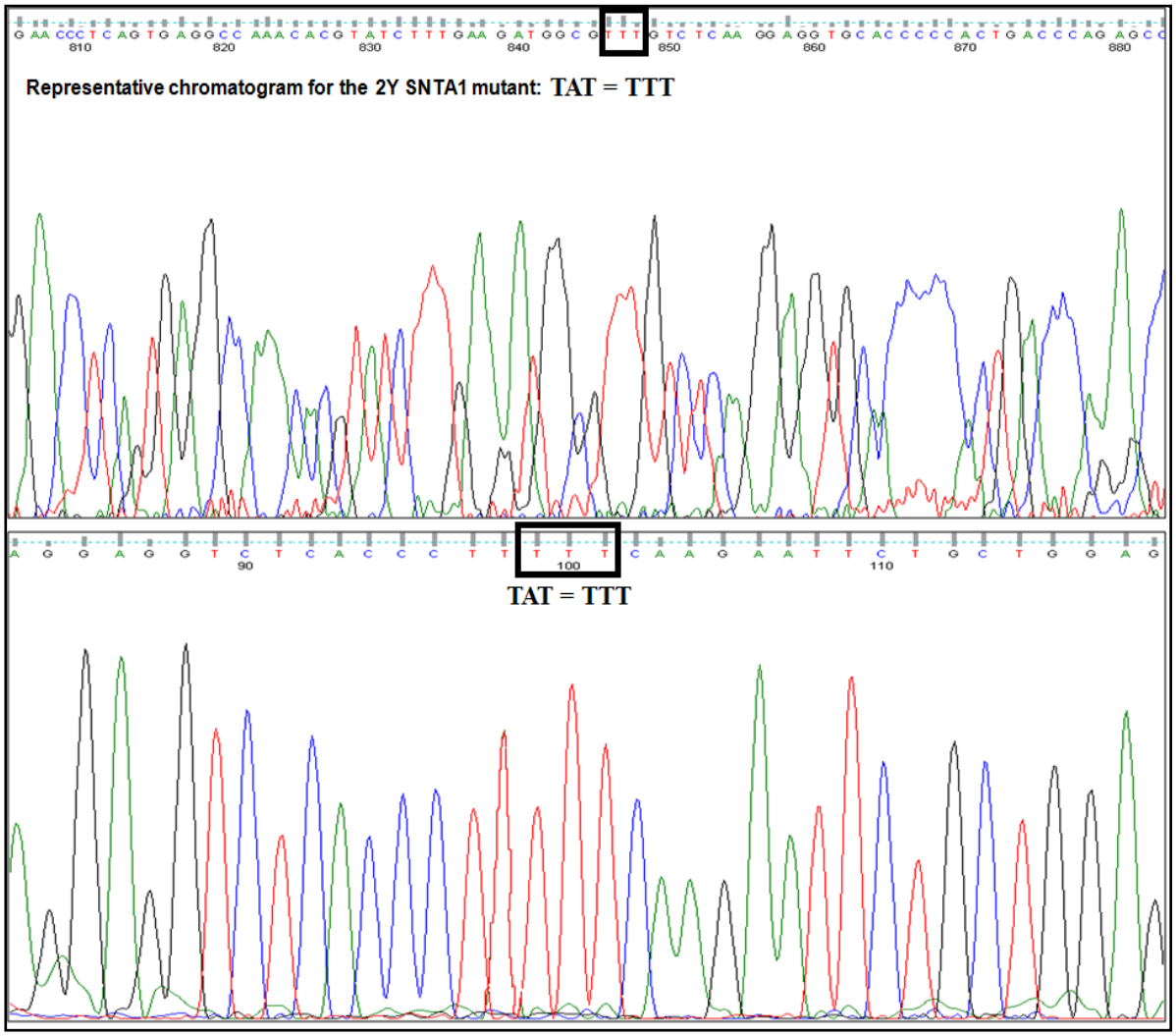


Figure 1.29: Representative chromatogram for the SNTA1 double mutant (DM): *Chromatogram showing TAT to TTT mutation replacing Tyrosine with Phenylalanine at position 215 and 219 in SNTA1.*

P66shc TM:

Figure 1.30: Sequence BLAST for the inserted mutations: *The BLAST sequence shows the inserted mutation at position 426 to 428 (in red) confirming the inserted mutation in P66shc.*

P66shc TM:

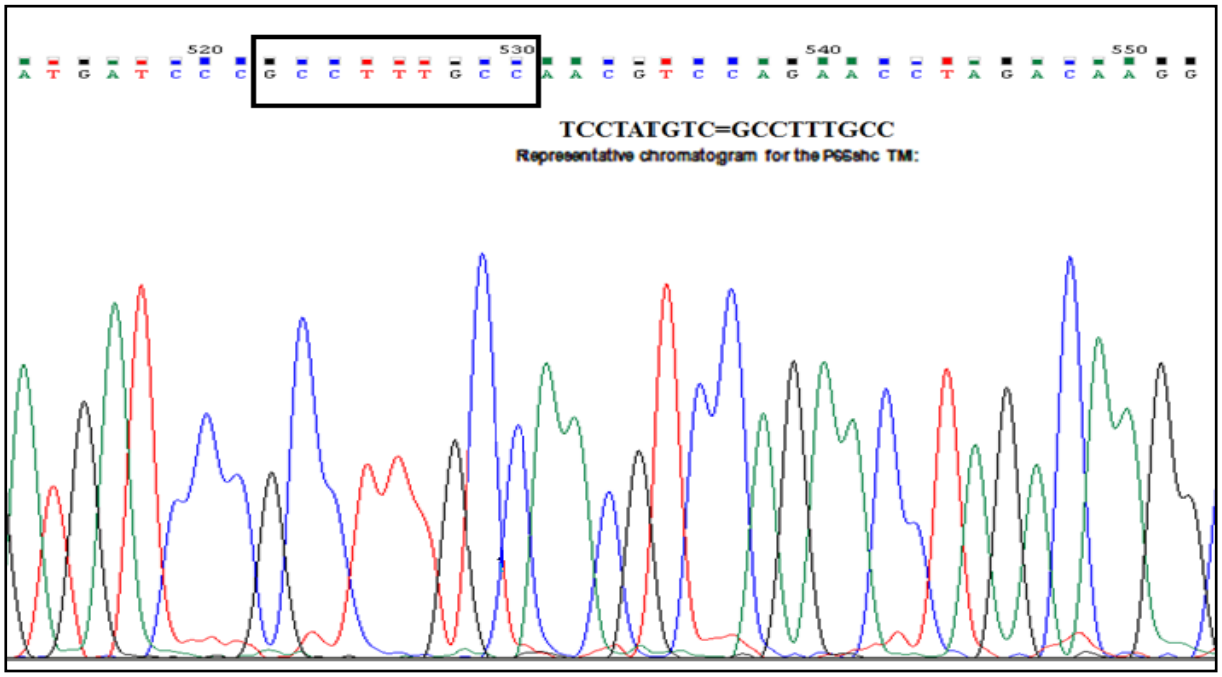


Figure 1.31: Representative chromatogram for the SYV$^{426\text{-}428}$ mutant of P66shc (TM): *Chromatogram showing TCCTATGTC to GCCTTTGCC mutation replacing Serine, Tyrosine and Valine with Alanine, Phenylalanine and Alanine respectively at positions 426 to 428 in P66shc.*

4.6. SYV motif of P66shc and Tyrosine residues of SNTA1 play a role in the trimeric complex formation:

It has been shown that the PDZ domain of SNTA1 binds to many proteins via their C-terminal SYV motif. P66shc adaptor protein contains one such motif spaning residues 426 to 428. Once the required SYV dominant negative mutant of P66shc was generated, we tried to look into the effect of this mutation on the complex formation between SNTA1, P66shc and Grb2 proteins. We surmised that interaction between SNTA1 and P66shc to be through this motif. We transfected the HBL-100 cell lines with the wild type SNTA1, wild type P66shc and SYV mutated construct of P66shc and anti-myc (SNTA1) monoclonal antibody was used to carry out the immune-precipitation assay. Our results are indicative that the mutation of SYV motif drastically affects the interaction between these proteins (Figure 1.32, 1.33).

Next we tried to elucidate the role of Tyrosine residues within SNTA1 that are thought to be important in its binding to Grb2 protein. The two tyrosine residues (229 and 215 position) flank the proline rich sequence in SNTA1. We transfected the HBL-100 cell lines with the wild type SNTA1, wild type P66shc and the double mutant for these two tyrosine residues i.e DM(SNTA1) mutated construct and anti-SNTA1 monoclonal antibody was used to carry out the immune-precipitation assay. Our results are indicative that these tyrosine residues are important for this trimeric complex formation and that the mutations in these residues drastically affect the interaction between these proteins (Figure 1.32, 1.33).

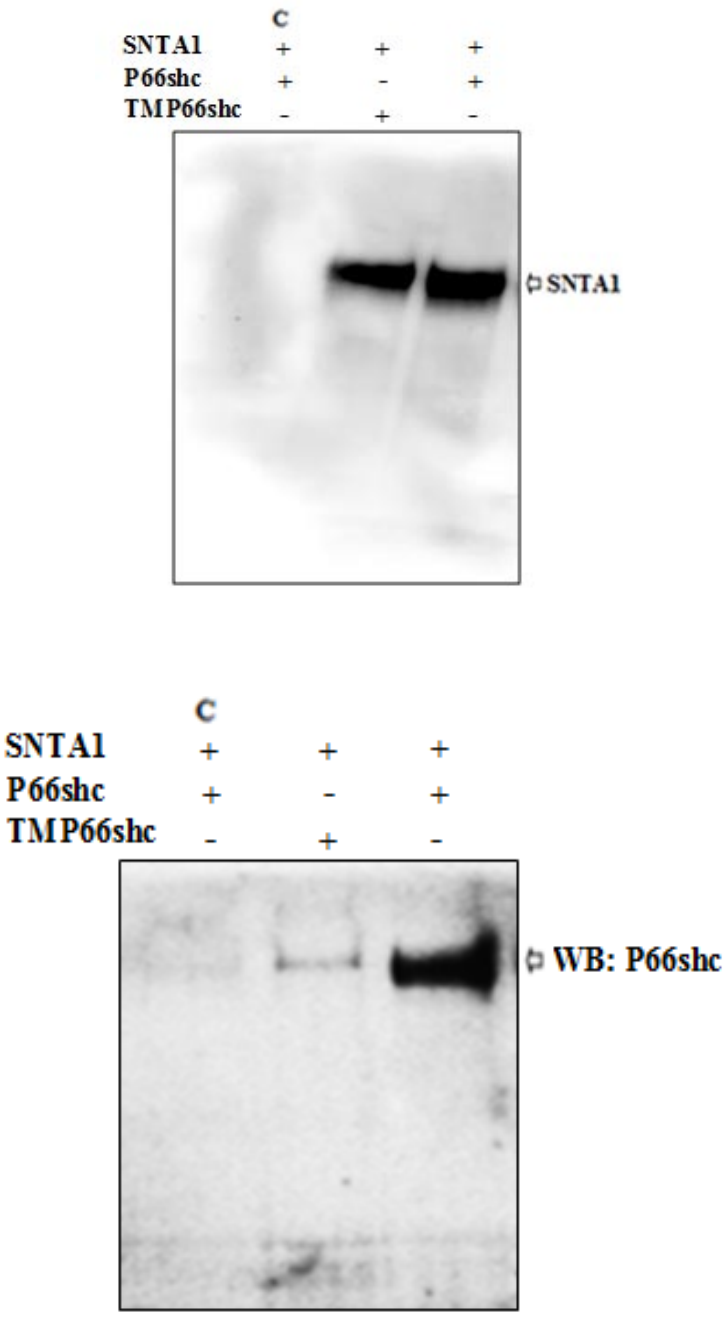


Figure 1.32: SYV motif of P66shc is involved in interaction with SNTA1: *Comparison of SNTA1 levels in immune-precipitated lysates from HBL-100 lysates transiently transfected with SNTA1, P66shc or triple mutant (TM) construct of P66shc. The cells transfected with the mutant form of P66shc showed a significant decrease in interaction between the two proteins.*

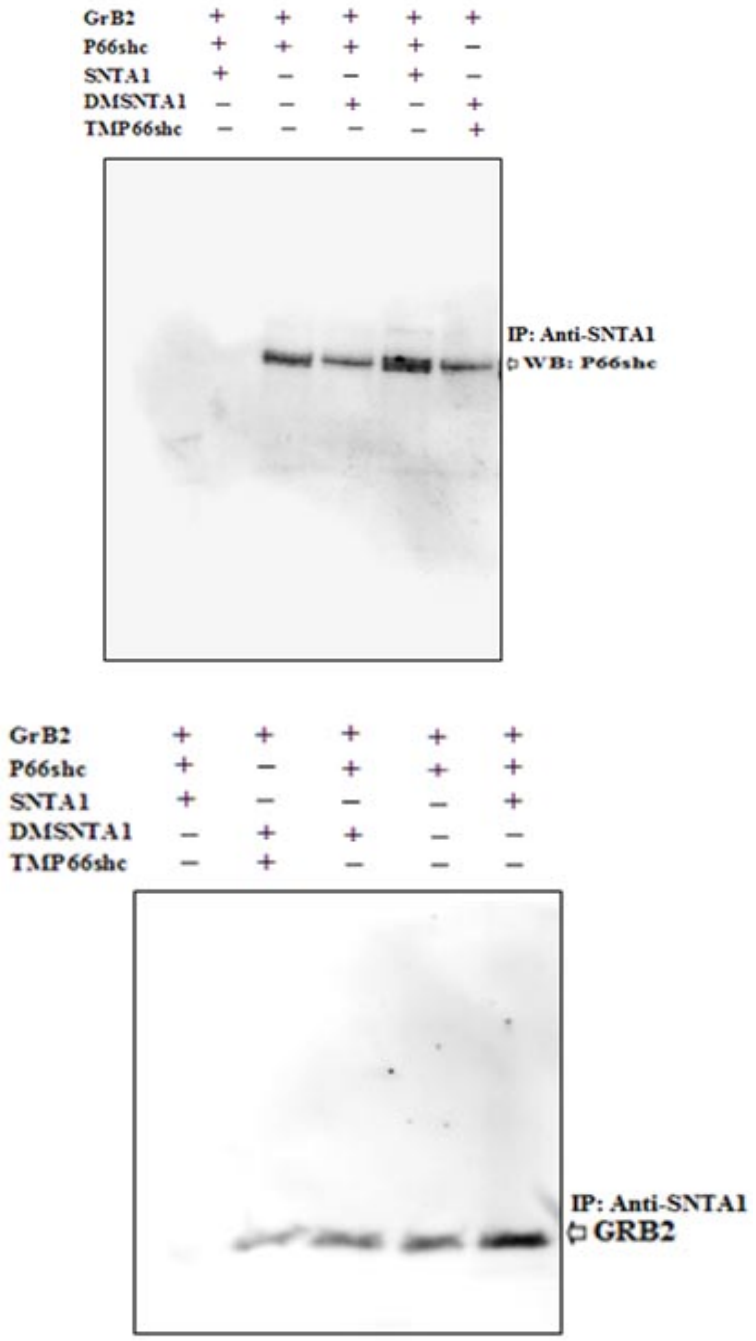


Figure 1.33: Tyrosine residues of SNTA1 and SYV motif of P66shc are both involved in the trimeric complex formation: *Comparison of SNTA1 and Grb2 protein levels in immune-precipitated lysates from HBL-100 lysates transiently transfected with SNTA1, P66shc or triple mutant (TM) construct of P66shc, double mutant construct of SNTA1 (DM). The cells transfected with the mutant form of P66shc and SNTA1 showed a significant decrease in interaction between these proteins.*

4.7. Binding of SNTA1 and P66shc to Grb2 enhances release of Sos1 from Grb2 and formation of the Rac1 activating Sos1-Eps8-E3b1 complex:

Next we tried to look into the mechanism involved in SNTA1 mediated Rac1 activation. The human guanine nucleotide releasing factor Son of sevenless (Sos) is one protein that interacts with both the SH3 domains of Grb2. The interaction between Sos and Grb2 is thought to be important in determining the Rac1 activation levels in cell. Since Sos1 functions as a Rac1-specific GEF when it is part of the sos1-eps8-e3b1 complex. We therefore tried to examine the role of SNTA1 in regulating the formation of these sos1-containing complexes. In previous work we showed that P66shc promotes oxidative stress and increases the Rac1-specific GEF activity of sos1 by binding to Grb2 and increases the formation of the sos1-eps8-e3b1 tri complex. Since we could co-immunoprecipitate Grb2, P66shc, proteins along with SNTA1 protein, we were tempted to check if SNTA1 and P66shc cooperate in displacing SoS1 from Grb2 and function to increase its availability for the formation of a complex with eps8/e3b1 proteins. We transfected cells with the different plasmid containing SNTA1, P66shc and SiRNA's etc to look into their effect on the two Sos1 containing complexes i.e Sos1-Grb2 and Sos1-Eps8-E3b1 (Figure 1.34). Our pull-down assays results indicate an increase in the release of Sos1 from Grb2 when both SNTA1, P66shc were over expressed in HBL-100 cells and a maximum release of Sos1 from Grb2 was observed when both were co-transfected (Figure 1.34 (A)). A consequent increase in the levels of Sos1-eps8-e3b1 complex formation under these conditions respectively was also observed, while an induced decrease in the expression of SNTA1, P66shc, using SiRNA, reflected a decrease in Sos1-eps8-e3b1 complex formation and a more significant shift towards Sos1-Grb2 complex formation in these breast cancer cells (Figure 1.34 (B)). SNTA1 and P66shc may thus share a common pathway in stimulating the Rac1-specific GEF activity of Sos1.

To elucidate the role of tyrosine residues in both SNTA1 and P66shc in this trimeric complex formation and to look for any effect of mutating this motif on Rac1 activity, we transfected cells with SNTA1, P66shc or Tyrosine mutant (DM) SNTA1 and SYV triple mutant (TM) P66shc and performed the Rac1 activation assay. Cells transfected with the wild type constructs of SNTA1 and P66shc show an increase in the Rac1 activity as

compared to the empty vector (EV) (Figure 1.35). Our results indicate a decrease in the active Rac1 in cells transfected with SYV triple mutant of P66shc as compared to cells transfected with SNTA1 and wild type P66shc indicating a possible involvement of SYV motif and Tyrosine residues of SNTA1 in the trimeric complex formation.

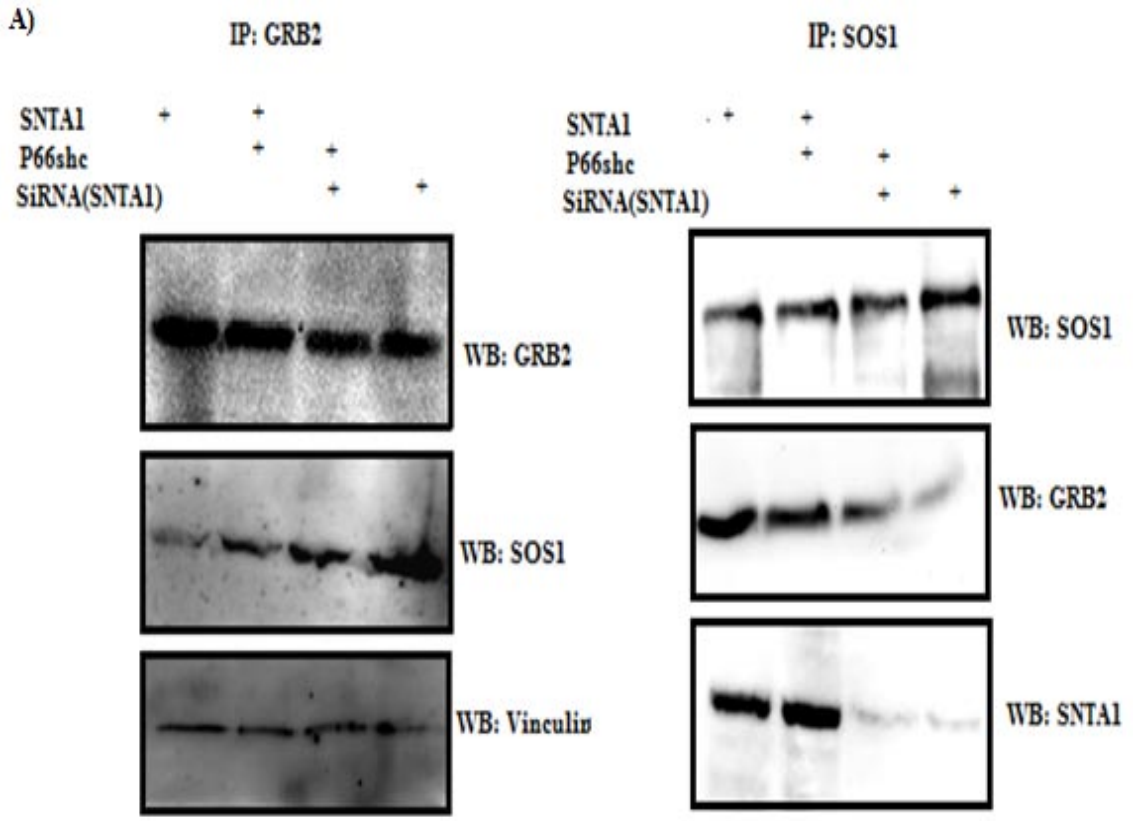
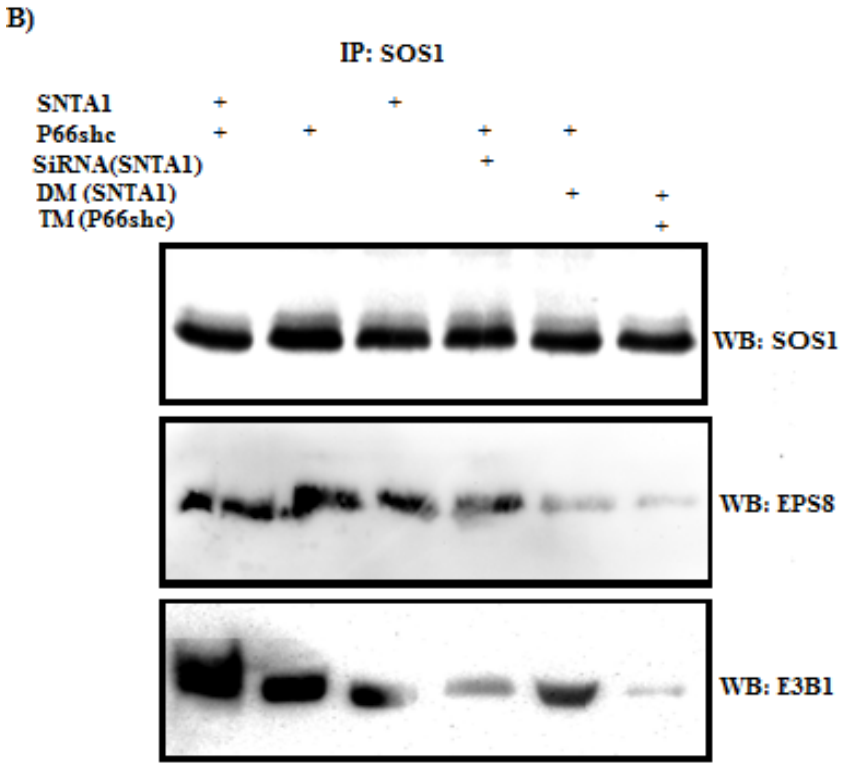


Figure 1.34: SNTA1/P66shc enhances release of Sos1 from Grb2 and formation of Sos1-Eps8-E3b1 complex: (*A) HBL-100 cells were transfected with the plasmid constructs as indicated and Immuno-precipitations of cell lysates was done using Protein A Sepharose bound monoclonal antibody against Grb2 and Sos1 and were probed with antibodies against Sos1 and Grb2 respectively to assess the formation of Sos1-Grb2 complex levels in cells under these conditions.*



(B): *HBL-100 cells were transfected with the plasmid constructs as indicated and Immuno-precipitations of these cell lysates were was done using Protein A Sepharose bound monoclonal antibody against Sos1 and were probed with antibodies against Eps8 and E3b1 to assess the formation of Sos1-Eps8-E3b1 complex in cells under these conditions.*

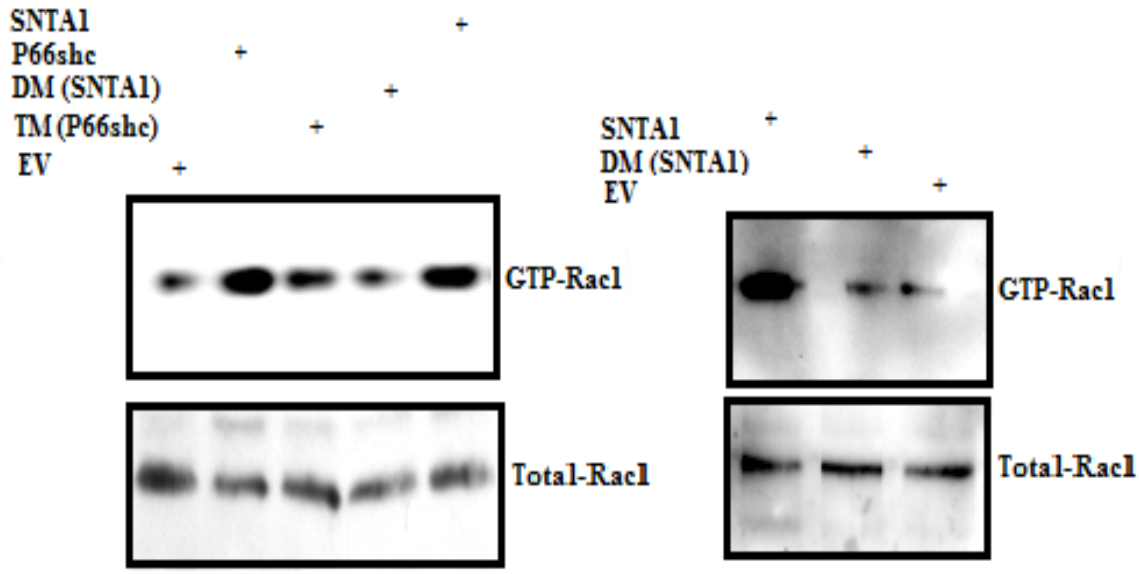


Figure 1.35: P66shc and SNTA1 mutants show decreased Rac1-GTP levels in HBL-100 cells as compared to wild type P66shc , SNTA1 constructs: *Cells transfected with the SNTA1, P66SHC plasmids or the mutant forms of these constructs were used to perform Rac1 activation assay to look into the effect of these motifs in Rac1 activation. DM(SNTA1) and TM(P66shc) represent the* $Y^{215,229}$ *mutant of SNTA1 and* $SYV^{426-428}$ *mutant of P66shc respectively. Cells transfected with the wild type constructs of SNTA1 and P66shc show an increase in the Rac1 activity as compared to the empty vector (EV) while the mutant forms show decreased levels of active Rac1 as compared to the wild type constructs.*

4.8. SNTA1/P66shc mediated Rac1 activation increases intracellular ROS generation and Cell proliferation:

We were intrigued by the possibility that the SNTA1 mediated Rac1 activity may also determine its ability to regulate the downstream effects of Rac1 activity such as ROS generation or regulation of cellular oxidative stress and cell proliferation. We therefore tried to assess the effect of SNTA1 and P66shc on intracellular ROS generation as well as cell proliferation. Amplex Ultra-red reagent and MTT reagents were used to assess the effect of over-expressing or down regulating these proteins on the levels of extracellular ROS generation and proliferation in H.B.L-100 cells respectively. Expression of SNTA1, P66shcWT in H.B.L-100 cells resulted in a significant increase in H_2O_2 as well as cell proliferation, while expression of both these vectors together showed a maximum ROS generation, proliferation in these cell types (Figure 1.36, 1.37). Comparison of cellular proliferation or H_2O_2 levels between P66shc (+) and P66shc (+)/SNTA1 (−) cells showed significantly lower levels in the latter, which shows the involvement of SNTA1 in complete activation of Rac1 mediated ROS generation or cell proliferation. In addition, cells transfected with SiRNA(SNTA1) or ShRNA(P66shc) showed significantly lower levels of ROS generation and cellular proliferation and down regulating expression of both these proteins [SiRNA(SNTA1)+ShRNA(P66shc)] decreased cellular proliferation and H_2O_2 levels to a considerably larger degree in these cells (Figure 1.36,1.37). Rac1 activation assay results (Figure 1.36 lower panel) were in consensus with these results. Collectively, these findings suggest that SNTA1/P66shc dependant Rac1 activation plays a crucial role in regulating H_2O_2 production and proliferation in human breast cancer cells.

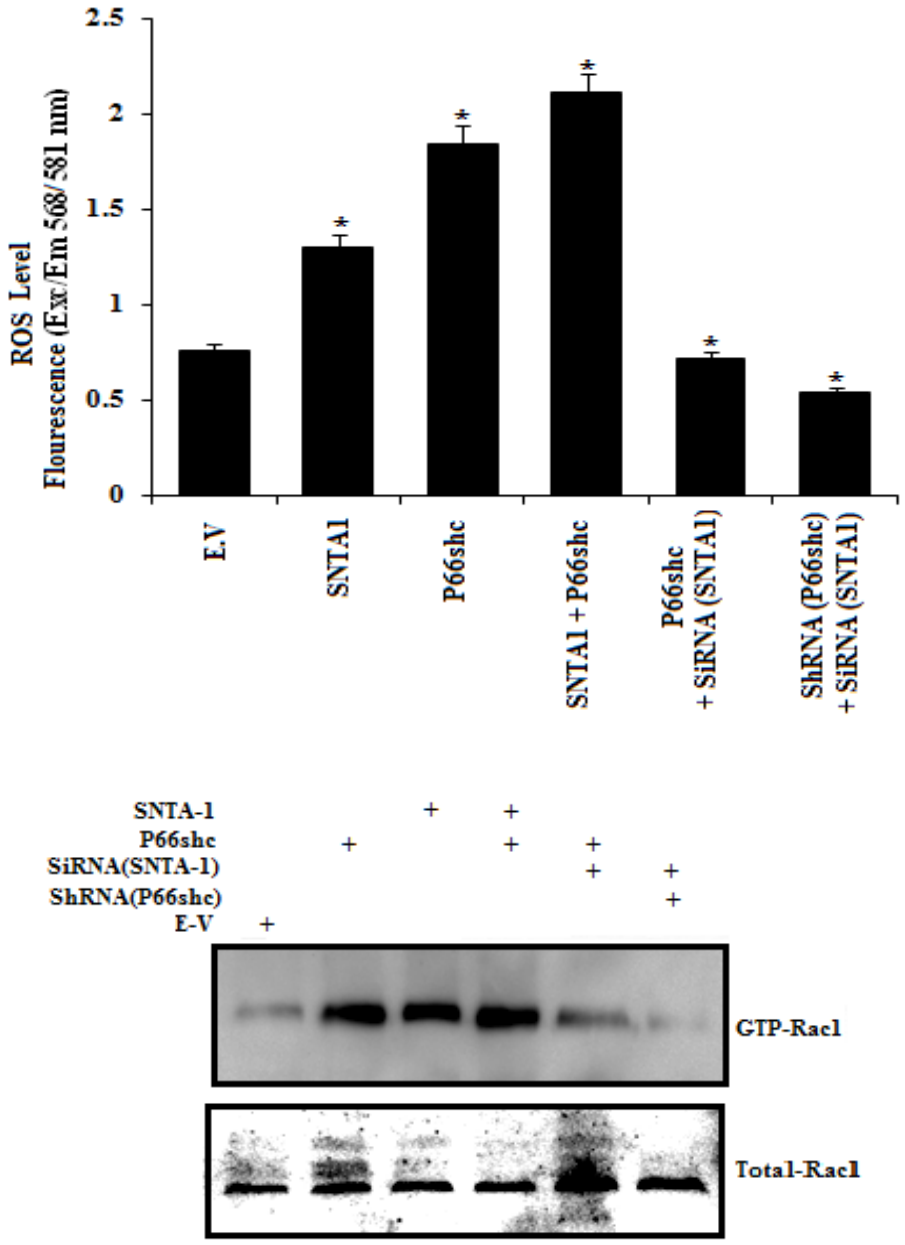


Figure 1.36: Rac1 activity correlates with intracellular ROS level in MCF-7 cells: *Extracellular ROS was evaluated in MCF-7 cells transiently transfected with the Empty-Vector (EV), and the indicated plasmid constructs, SiRNA's respectively using amplex ultra red reagent. Results are mean values ± SE from three separate experiments. *Differences were calculated against EV (in a) or control (in b) cells and considered significant at $p < 0.05$. The Lower panel shows immunoblot for total Rac1 and active Rac1 in HBL-100 cells transfected with the mentioned plasmid constructs.*

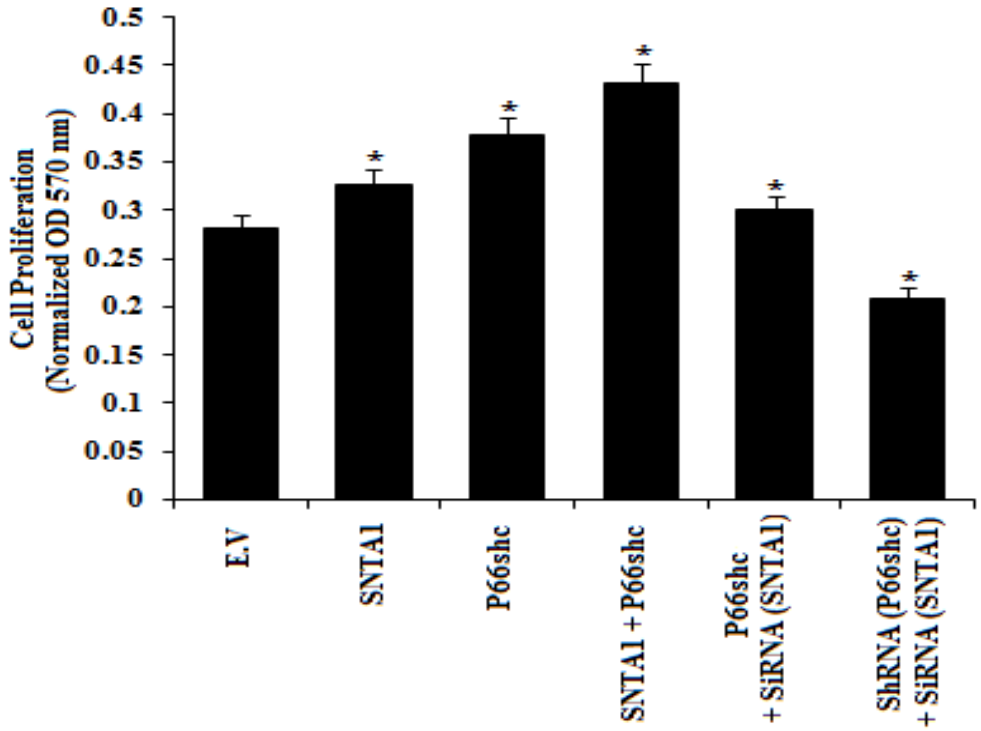
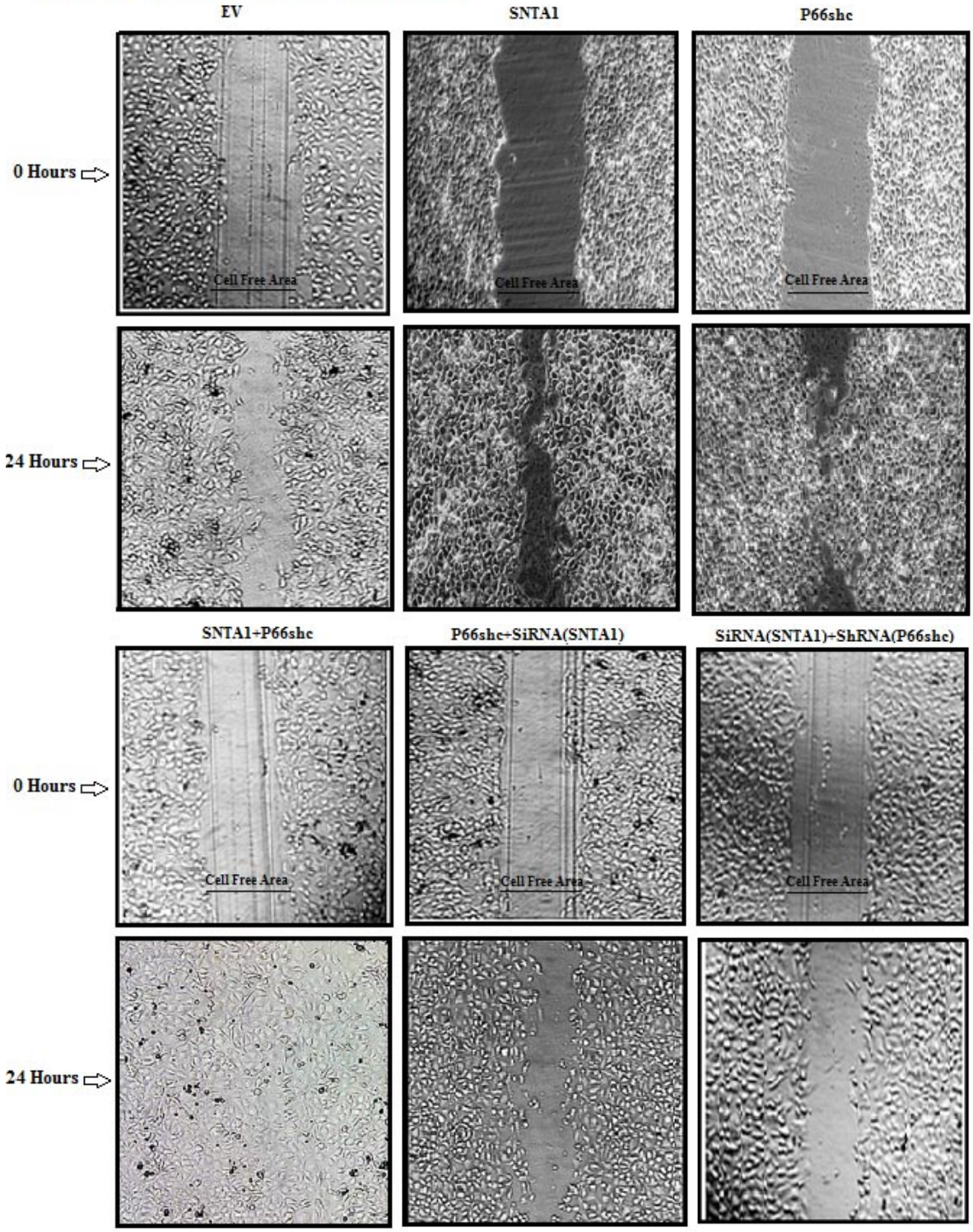


Figure 1.37: Rac1 activity correlates with cellular proliferation level in MCF-7 cells: *Cellular proliferation was detected in MCF-7 cells using MTT reagent after being transiently transfected with the Empty-Vector (EV), and the different plasmids, SiRNA's respectively as shown above. Cells transfected with both SNTA1 and P66shc showed maximum levels of proliferation while downregulating the expression of these proteins using SiRNA/ShRNA against them showed to decrease the Rac1 activity as well as proliferation of these cells.*

4.9. SNTA1/P66shc mediated Rac1 activation facilitates wound healing of breast cancer cell monolayers:

The Rho GTPase Rac1 controls cell adhesion and motility and its activity is increased in migrating cells [149]. We therefore asked whether the SNTA1 influences the migratory potential of these cells. This was tested by expressing SNTA1 and P66shc in HBL-100 cells that were subsequently induced to migrate in a 'scratch' assay. While cells transfected with SNTA1 and P66shc showed a significant increase in the percent wound closure of the induced scratch in these cells, co-expression of these proteins showed a maximum wound healing. SNTA1 and P66shc over-expression facilitated Wound Healing of HBL-100 cell monolayers as determined after 24 h following the wound, these cells were found to be migrating into the wound and caused complete closure of the scratch (Figure 1.38). To further confirm this, we reduced endogenous expression of these proteins by transient transfection of siRNA/ShRNA, before performing scratch assay. Figure 1.38 shows that under these conditions cells are at the edge of a wound and the healing was found to be minimum in these cells.

(A) In-vitro Wound Healing Assay (HBL-100 Cells):



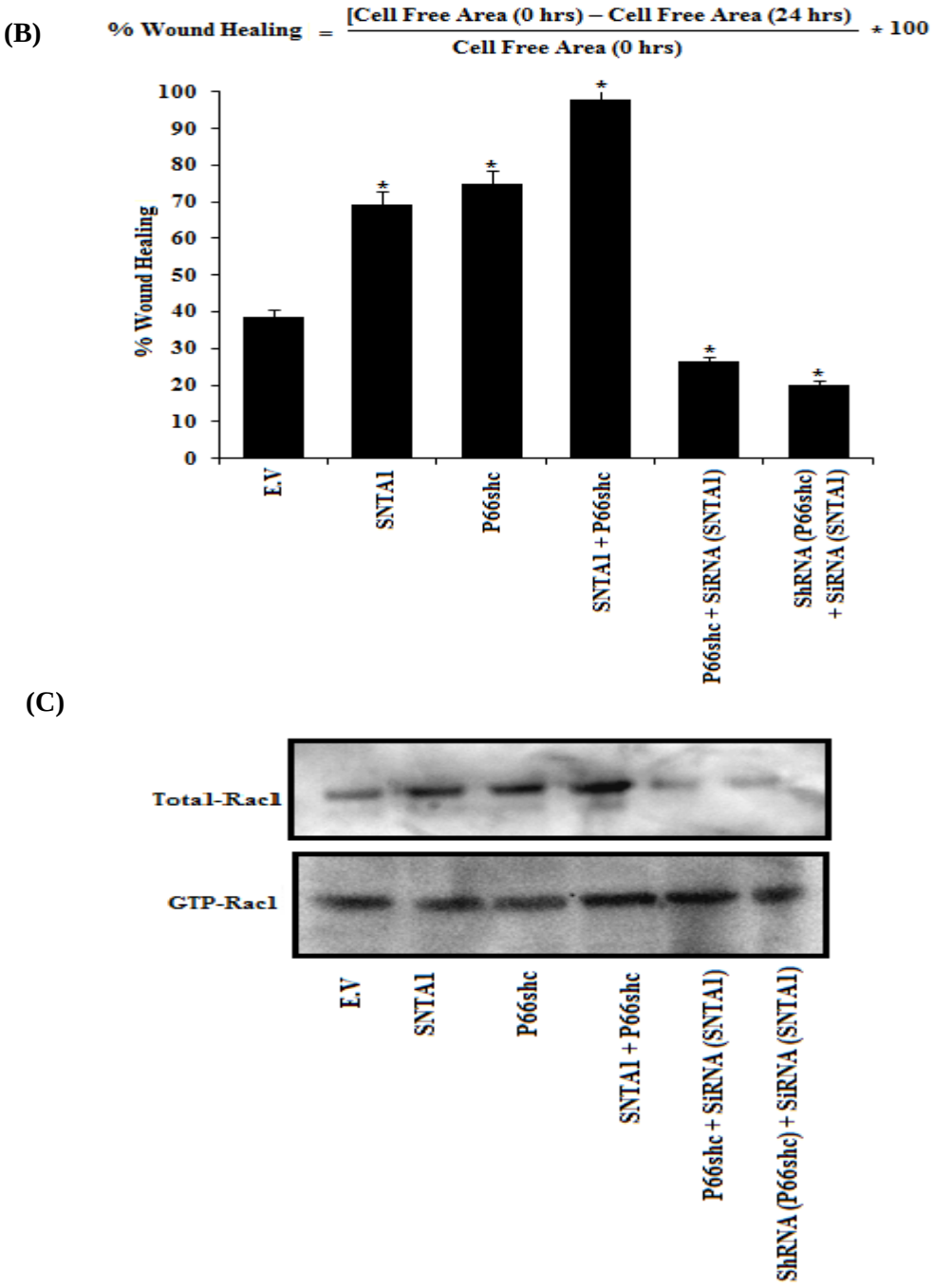
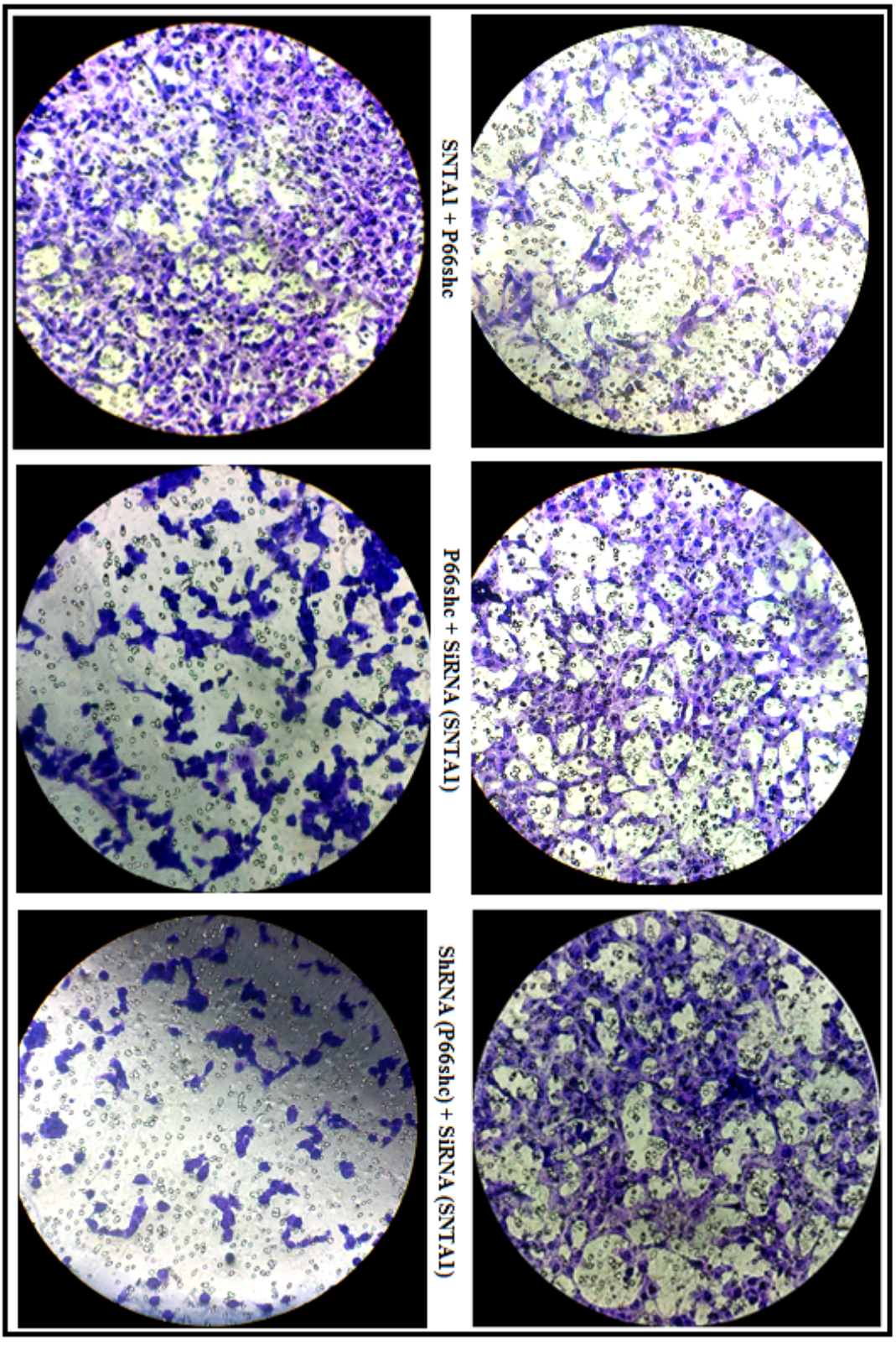


Figure 1.38: SNTA1/P66shc mediated Rac1 activation facilitates wound healing in HBL-100 cells. (A) *Representative pictures for EV, SNTA1, P66shc, and SiRNA's transfected HBL-100 cells after 0 and 24 hours of wounding. (B) Error bars depicting the percentage wound healing in cells transfected with the mentioned constructs as compared to the empty vector control [EV] have been shown. Data are mean values ± SE from three separate experiments. *Differences from EV cells were considered significant at $p < 0.04$. (C) Immunoblot for total Rac1 and active Rac1 in HBL-100 cells transfected with the mentioned plasmid constructs.*

4.10. SNTA1/P66shc mediated Rac1 activation facilitates cell migration in human breast cancer cells:

We also evaluated whether this SNTA1 mediated Rac1 activity plays a role in the acquisition of cellular migration/invasion. This was assayed in M.C.F-7 and HBL-100 mammalian cancer cells using the Boyden Transwell double chamber method. While SNTA1 and P66shc transfection significantly increased the migration of HBL-100 cells, SNTA1 and P66shc together promoted invasive migration of these cells and exhibit 3-4 times more migration capacity than that of the control cells i.e transfected with the empty vector (EV). Down-regulating expression of both these proteins in HBL-100 using SiRNA/ShRNA against the same [SiRNA(SNTA1)+ShRNA(P66shc)] display one third of the migration capacity of EV cells (Figure 1.39). M.C.F-7 cells showed a similar pattern were SNTA1 as well as P66shc transfection increased the migratory potential of these cells, co-expression showed an almost 4-5 times increase in the cell migration while down-regulation of these proteins decreased the cell migration considerably (Figure 1.40).

(A)


E.V
SNTA1
P66shc
SNTA1 + P66shc
P66shc + SiRNA (SNTA1)
ShRNA (P66shc) + SiRNA (SNTA1)

(B)

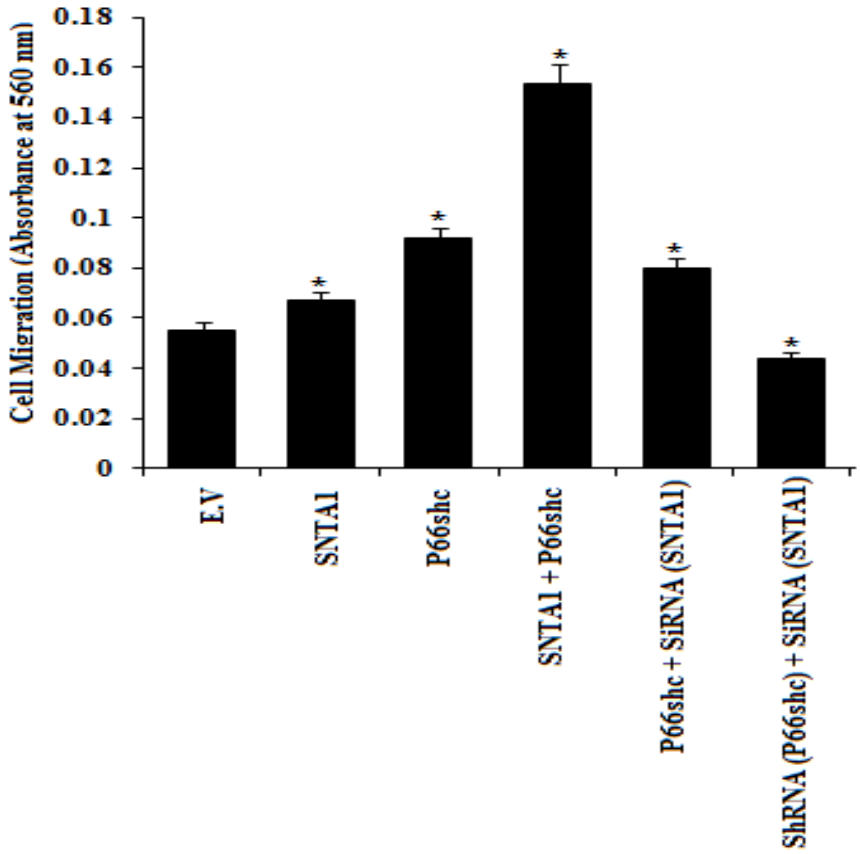
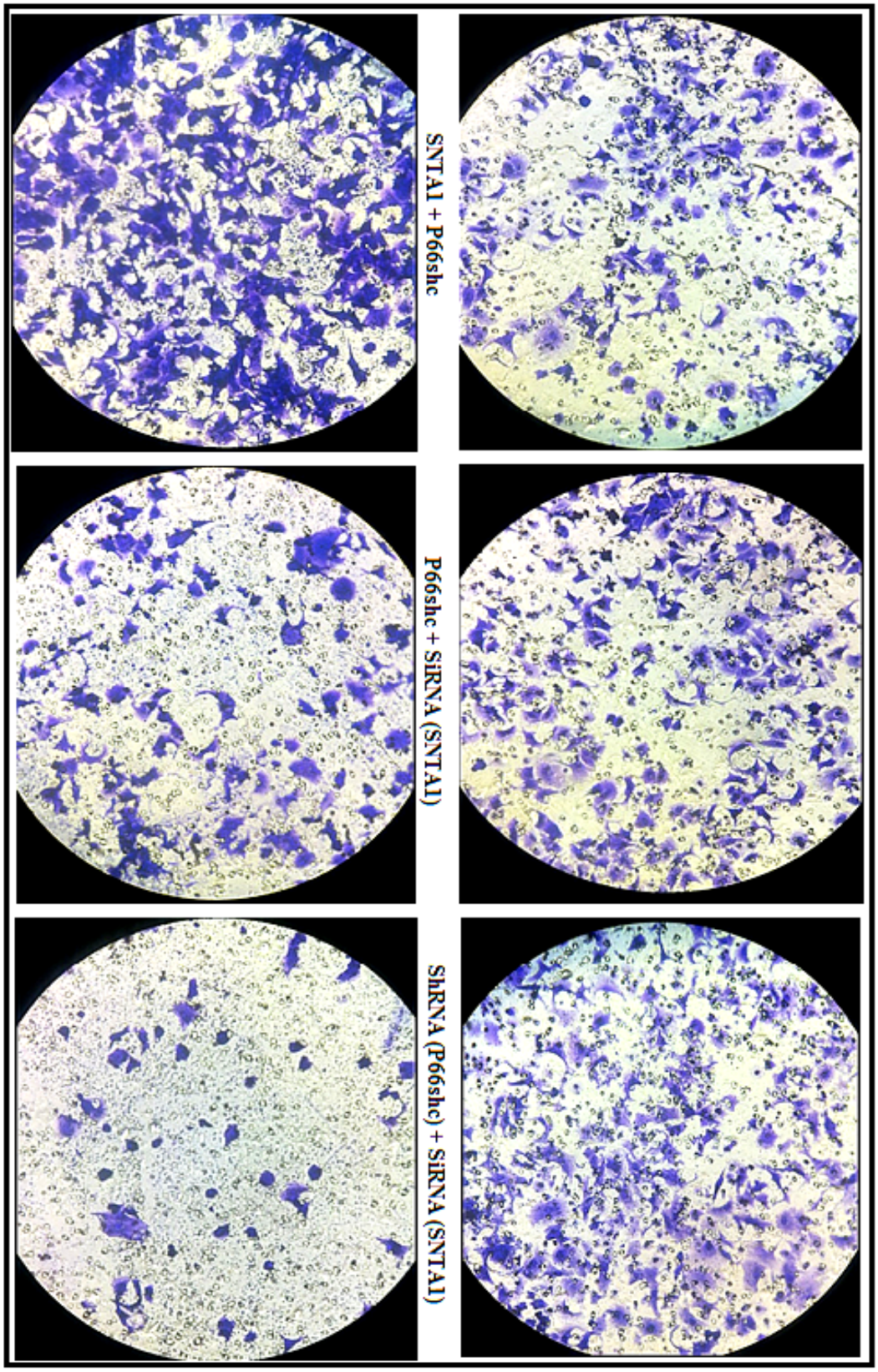


Figure 1.39: SNTA1/P66shc mediated Rac1 activation increases the migratory capacity in HBL-100 cells: (A) *EV, SNTA1, P66shc, and ShRNA, SiRNA's were used to determine migratory capacity, evaluated using the Boyden Transwell double chamber method described in material and methods in HBL-100 cells. (B) Error bars depicting the cell migration in cells transfected with the mentioned constructs as compared to the empty vector control [EV] have been shown. Data are mean values ± SE from three separate experiments. *Differences from EV cells were considered significant at $p < 0.05$.*

(C)


E.V
SNTA1
P66shc
SNTA1 + P66shc
P66shc + SiRNA (SNTA1)
ShRNA (P66shc) + SiRNA (SNTA1)

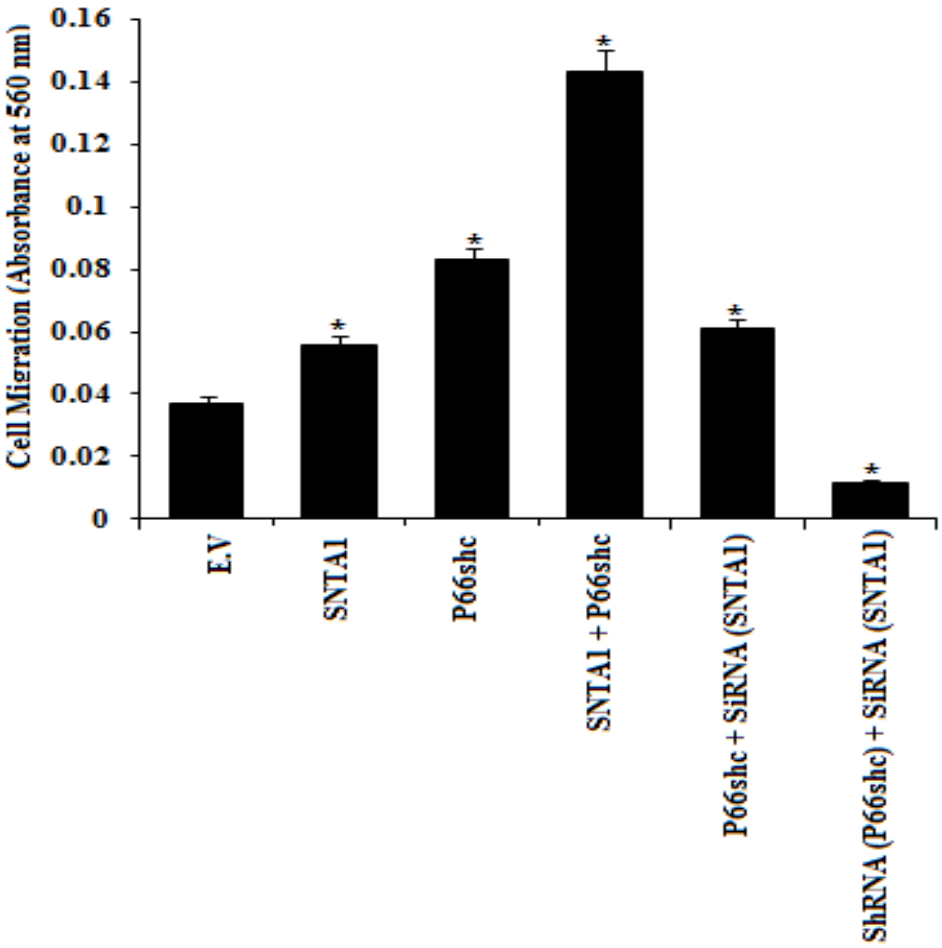


Figure 1.40: SNTA1/P66shc mediated Rac1 activation increases the migratory capacity in MCF-7 cells: (A) *EV, SNTA1, P66shc, and SiRNA, ShRNA's were used to determine migratory capacity, evaluated using the Boyden Transwell double chamber method described in material and methods in MCF-7 cells. (B) Error bars depicting the cell migration in cells transfected with the mentioned constructs as compared to the empty vector control [EV] have been shown. Data are mean values ± SE from three separate experiments. *Differences from EV cells were considered significant at $p < 0.05$.*

Discussion & Conclusion

5. Discussion and Conclusion:

The exact mechanism for Rac1 activation has not yet been clearly understood. Alpha-1-syntrophin and P66shc have been implicated in Rac1 activation and both have been shown to form a stable complex with Grb2. Here, we show that SNTA1 and P66shc associate into a complex with Grb2 for the activation of Rac1 were SNTA1 acts as a signalling amplifier for its activation and this result in an increase in the ROS levels, proliferation and/or migratory properties of these cells. Our results emphasize the importance of SNTA1 in intracellular ROS generation as well as its role in cellular migration.

In the present study, we show that transfection of SNTA1 into human breast cancer cell, results in a significant increase in the activation of Rac1. The increase in active Rac1 was more pronounced when SNTA1 was co-transfected with P66shc, which indicates that this phenomenon is dependent of SNTA1 involvement and that both these proteins function together in the Rac1 activation pathway. Further, SNTA1 as well as P66shc has been shown to be up regulated in breast carcinoma [20, 155] while Rac1 activity has also been reported to be increased in breast cancers [156]. A further support for our assumption is that both these proteins share many of their binding partners that are already implicated in Rac1 activation. The study thus provides an evidence for a novel role of SNTA1 protein in Rac1 activation. Our functional assays and co-immunoprecipitation assays establish that interaction between SNTA1, Grb2 and P66shc is associated with Rac1 activation. Knockdown of SNTA1 expression using small interfering RNA, which was approximately 70-80% efficient, decreased significantly the active levels of Rac1 within breast cancer cell lines. Also, when P66shc was over-expressed in presence of SiRNA against SNTA1, activation level of Rac1 was found to be much lower as compared to its activation levels when transfected with P66shc alone or when P66shc and SNTA1 were co-expressed. These findings lead us to hypothesize that the observed increase in Rac1 activation is a direct effect of a trimeric complex formation between SNTA1, Grb2 and P66shc proteins. Taken together, our observation that Grb2 interacts with both P66Shc and SNTA1 suggests that the functional consequences of these interactions could be numerous and it is likely that these functions include the stimulation of Rho GTP'ase

activity and the downstream signalling mediated by Rac1 activation within human breast cancer cells.

Our results suggest that the interactions between Grb2 and these two proteins are possibly mediated by the binding of the SH2/SH3 domain of Grb2 to tyrosines/phosphotyrosines in SNTA1 and p66Shc respectively. Since both the N- and C-SH3 domains of Grb2 are known to interact with Sos1 [157], binding of the SNTA1 and P66shc to Grb2 would be expected to weaken the Sos1-Grb2 interaction, making Sos1 more available for interaction with EPS8-E3B1 pool within the cell. Sos1-EPS8-E3B1 is a tri-molecular Rac-guanine nucleotide exchange factor complex, which is important for passing the signal from Ras and PI3K to Rac. Rac regulates mitogen-induced cytoskeletal changes and is a key component in the actin reorganization as well as associated with carcinogenesis and progression of several human tumors. Interestingly, distinct tyrosine phosphorylated regions of both SNTA1 and P66shc can simultaneously bind the Grb2 molecule [127, 140]. Furthermore a short sequence motif (SYV) is present in P66Shc, which on phosphorylation has been shown to specifically bind the SH2 domain of Grb2 with high affinity. Use of the mutants thought to be involved in these interactions (Tyrosine double mutant of SNTA1/ Tyrosine mutant of P66shc) although considerably decreases the formation of sos1-eps8-e3b1 complex or that of active Rac1 levels, yet it does not completely abrogate the formation of this Rac1 activating complex and we were able to retain some active Rac1 suggesting that SNTA1/P6shc binding to Grb2 promote dissociation of Sos1 from Grb2 but is not solely responsible for it, thus suggesting that other residues may also be important for their interaction with Grb2.

Rac1 is a part of the structure of NADPH oxidase i.e the ROS generating enzyme whereby it participates in the control of the ROS generation within cells [158-159] and has been shown to induce lamellipodia extensions and membrane ruffling [160-161]. Rac1 also constitutes an upstream regulator of actin reorganization and adhesive properties associated with cellular shape and motility [147, 150]. Similar to these functions of Rac1, according to our findings we assume that the observed increase in ROS is likely due to the SNTA1 mediated Rac1 activation signalling events. This explanation is supported by the finding that breast cancer cells transfected with SNTA1/P66shc show increased extracellular ROS. Therefore, it may be reasonable to consider that the trimeric complex induced Rac1 activation stimulated ROS generation in

breast cancer cell lines. Our results also show that the migratory capacity of these breast cancer cells is sensitive to SNTA1 down-regulation and intracellular ROS generation. Taken together all our work and other available data, we could reasonably hypothesize a model for the activation of Rac1 and the downstream signalling via SNTA1/Grb2/P66shc complex formation and increased sos1-eps8-e3b1 complex formation (Figure 1.41).

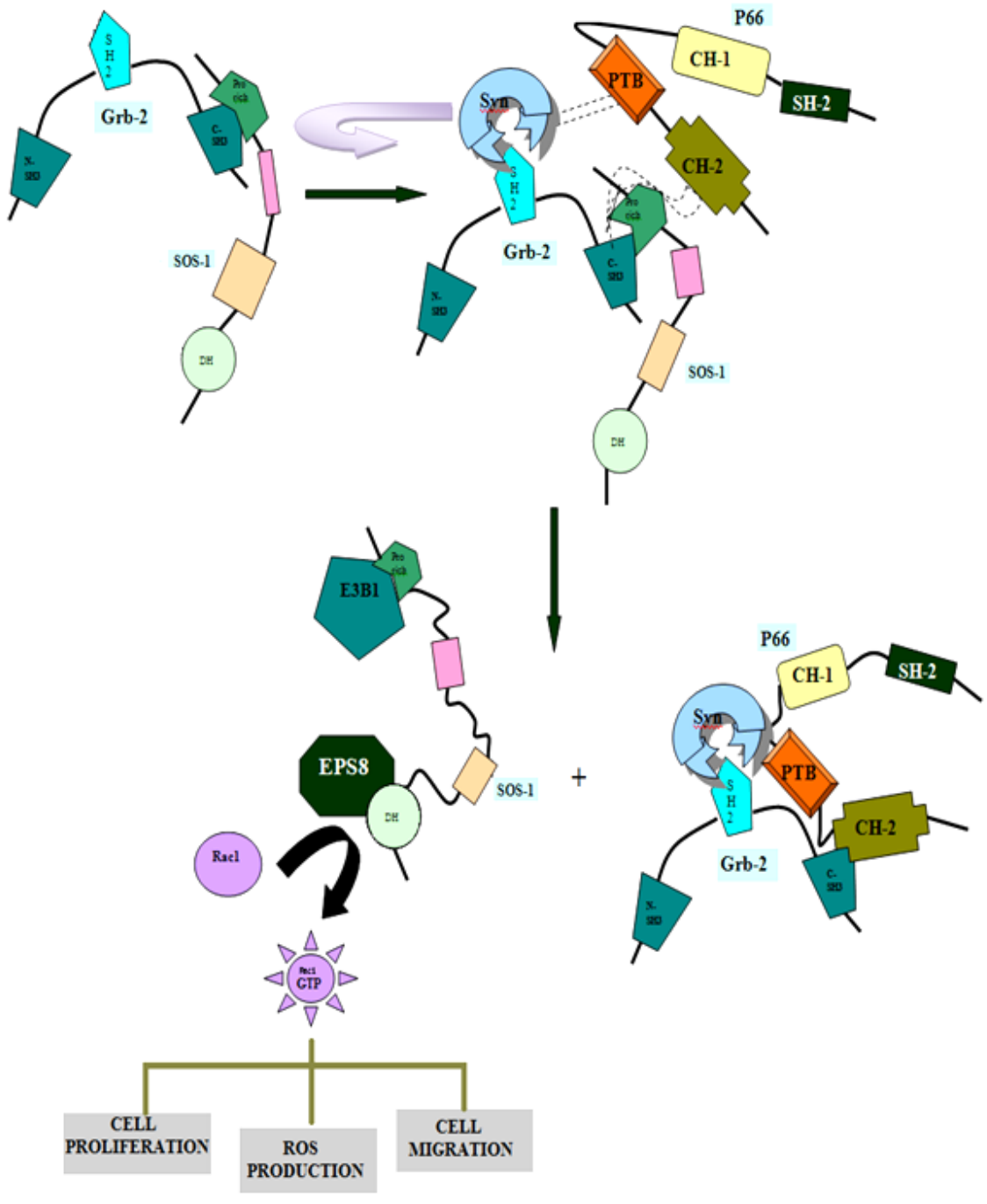


Figure 1.41: Proposed model for the activation of Rac1 protein and the downstream pathway: *SNTA1/Grb2/P66shc tri-complex formation and increased sos1-eps8-e3b1 complex formation leads to the activation of Rac1, which in turn increases the ROS production, proliferation and the migratory potential of human breast cancer cells.*

In conclusion, this study highlighted the role of SNTA1 in the activation of Rac1 pathway in human breast cancer cells. Our results present a novel mechanism for the Rho GTP'ase Rac1 activation. We propose a model (Figure 1.41) whereby formation of multiple protein-protein interactions between Grb2, SNTA1 and P66shc and Sos1, may play a crucial role in determining the levels of active Rac1 and the control of downstream signalling i.e generation of reactive oxygen species (ROS) and/or cell migration in human breast cancer cells. This model provides a novel mechanism for the activation of Rac1 and predicts that the formation of SNTA1-Grb2-P66shc complex determines the fraction of cellular Sos1 bound to Grb2, or E3B1-EPS8 and thereby the levels of these two Sos1 bound complexes within the cell which in turn modulates the activation of Rac1 and the cellular functions that are governed thereby. Thus our model integrated the earlier works with our new findings and suggests for a mechanism whereby syntrophin could provide the core for the formation of a large signalling complex involved in Rac1 activation and the downstream signalling in breast cancer cells. However since SNTA1, P66shc and Grb2 are all crucial multifunctional adaptor proteins involved in several other transduction pathways as well, we cannot not rule out the involvement of or any other possible interacting partners/mechanisms by which SNTA1 also regulates the activation of Rac1. Although a more detailed understanding of the Rac1 signal transduction awaits further characterization of these interactions, however the present work may be of major clinical importance and may provide new insights for the Rac1 activation signalling related studies.

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List of Publications:

- **Bhat HF**, Adams ME, Khanday FA. Syntrophin proteins as the Santa Clause: role(s) in cell signal transduction. *Cell Mol Life Sci.* Early online Dec 21st (2012).

- **Bhat HF**, Baba RA, Bashir M, Saied S, Kirmani D, Wani MM, Wani NA, Wani KA, Khanday FA. Biomarkers. Alpha-1-syntrophin protein is differentially expressed in human cancers. *Biomarkers.* 24: 1-6. (2011).

  Publication was also highlighted in the Target Intelligence Service (TIS), a database used by pharmaceutical companies worldwide to identify ground breaking research of relevance to their own work on potential drug targets. A link to this report is given below:

  https://www.targetintelligenceservice.com/main/public/case.jsp?report_id=43671.

Other Publications:

- Kirmani D, **Bhat HF**, Bashir M, Zargar MA, Khanday FA. P66Shc-rac1 pathway-mediated ROS production and cell migration is downregulated by ascorbic acid. *J Recept Signal Transduct Res. Early Online: 1–7.* (2013).

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