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Role of SNTA1 in Rac1 activation, modulation of ROS generation, and migratory potential of human breast cancer cells

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Background: Alpha-1-syntrophin (SNTA1) has been implicated in the activation of Rac1. However, the underlying mechanism has not yet been explored. Here, we show that a novel complex, involving SNTA1, P66shc, and Grb2 proteins, is involved in Rac1 activation.

Methods: Co-immunoprecipitation assays were used to show the complex formation, while siRNAs and shRNAs were used to downregulate expression of these proteins. Various Rac1 activation assays and functional assays, such as migration assays, *in vitro* wound healing assays, cell proliferation assays, and ROS generation assays, were also performed.

Results: The results showed a significant increase in activation of Rac1 when SNTA1 and P66shc were overexpressed, whereas depletion of SNTA1 and P66shc expression effectively reduced the levels of active Rac1. The results indicated a significant displacement of Sos1 protein from Grb2 when SNTA1 and P66shc are overexpressed in breast cancer cell lines, resulting in Sos1 predominantly forming a complex with Eps8 and E3b1. In addition, the SNTA1/P66shc-mediated Rac1 activation resulted in an increase in reactive oxygen species (ROS) production and migratory potential in human breast cancer cells.

Conclusion: Together, our results present a possible mechanism of Rac1 activation involving SNTA1 and emphasise its role in ROS generation, cell migration, and acquisition of malignancy.

The syntrophin family of proteins consists of the alpha-1- (α -1-) syntrophin, beta-1- (β -1-) syntrophin, beta-2- (β -2-) syntrophin, gamma-1- (γ -1-) syntrophin, and gamma-2- (γ -2-) syntrophin proteins (Ahn *et al*, 1994; Adams *et al*, 1995). The term 'syntrophin' was derived from the Greek word 'syntrophos', which means companion or associate (Froehner *et al*, 1997). These proteins function as scaffolds, orchestrating signal transduction complexes by clustering various signalling components, such as cellular proteins, neurotransmitters, receptors, glycoproteins, and lipids, into the appropriate subcellular compartments and regulating crucial intracellular signalling events (Ahn *et al*, 1994; Froehner *et al*, 1997).

Alpha-1-syntrophin (SNTA1) is mostly found as a peripheral cytoplasmic membrane protein associated with the dystrophin

glycoprotein complex (DGC) in muscle cells and other mammalian tissues (Hoffman *et al*, 1987; Butler *et al*, 1992; Kramarcy *et al*, 1994). The DGC complex is thought to be a signal transduction complex that serves as a link between the extracellular matrix, inner signalling pathways and cell cytoskeleton (Hoffman *et al*, 1987; Butler *et al*, 1992). SNTA1 is involved in the precise localisation and/or regulation of many transduction proteins, such as ion channels (Gee *et al*, 1998), ankyrin repeat-rich membrane spanning protein (ARMS) (Shuo *et al*, 2005), Grb2 (Oak *et al*, 2001), calmodulin (Newbell *et al*, 1997), G-proteins (Zhou *et al*, 2005), n-NOS (Hillier *et al*, 1999), ABCA1 (Youichi *et al*, 2004) and many other signalling proteins (Bhat *et al*, 2012), and SNTA1 was also recently identified as a substrate for stress-activated protein kinase-3 (SAPK-3) (Hasegawa *et al*, 1999). SNTA1 has

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been suspected of being involved in the regulation of cancer cell proliferation or involved in carcinogenesis. A study from the American Association for Cancer Research showed that syntrophin expression was correlated with colon cancer outcome (Wiseman *et al*, 2008). In another such study, SNTA1 was found to be a potential marker of metastases or tumour progression, which could potentially aid in the classification of pre-malignant lesions, in tongue squamous cell carcinomas (SCCs) (Carinci *et al*, 2005). In addition, in our previous work, we observed an increase in SNTA1 protein levels in human breast carcinoma tissue samples when compared with their respective adjacent controls, suggesting that it has a role in the development and progression of carcinogenesis (Bhat *et al*, 2010).

The interaction of SNTA1 with Grb2 through its proline-rich regions has served as the basis of its involvement in the activation of the important Rho family small GTPase Rac1 (Oak *et al*, 2001). A signalling pathway has been described that links the binding of matrix laminin on the outside of the sarcolemma to Grb2 binding to SNTA1 on the inside surface of the sarcolemma and by way of -Sos1-Rac1-PAK1-JNK ultimately results in the phosphorylation of c-jun on Ser65 (Oak *et al*, 2003). Previously, we showed that P66shc, an oxidative stress sensor protein, increased the Rac1-specific guanine nucleotide exchange factor (GEF) activity of Sos1 (Khanday *et al*, 2006b) by facilitating the formation of the Sos1-Eps8-E3b1 complex, which has intrinsic Rac1-specific activity. In addition, P66shc has been shown to bind both the SH3 and SH2 domains of Grb2 in its unphosphorylated and phosphorylated state, respectively (Khanday *et al*, 2006a). P66shc has been identified as an important cytoplasmic signal transducer that regulates the apoptotic response to oxidative stress (Migliaccio *et al*, 1999). The P66Shc isoform is downregulated and not required for the HER-2/neu signalling pathway in human breast cancer cell lines that overexpress HER-2/neu (Xie and Hung, 1996). However, increases in P66shc protein have been observed in breast cancer specimens with high metastatic potential, and a

similar phenomenon has been observed in lymph node-positive breast carcinomas (Jackson *et al*, 2000), as well as in proliferating thyroid tissues and in thyroid cell lines (Park *et al*, 2005), thus hinting at a role for P66shc in the metastatic process. Similarly, the levels of P66shc in primary tumours provide a unique and simple tool for stratifying stage IIA colon cancer patients by their risk of recurrence and disease-specific death, which may assist in determining the most efficacious treatment strategies for these patients (Grossman *et al*, 2007).

Rac1 has been shown to control multiple cellular processes, including actin/microtubule cytoskeleton organisation (Colley, 2000), reactive oxygen species (ROS) generation (Bishop and Hall, 2000; Whaley-connell *et al*, 2007), membrane trafficking (McDonald *et al*, 2007), hypoxia-stimulated breast cancer cell migration (Han *et al*, 2008; Vermeulen *et al*, 2010), cell adhesion and migration (Nobes and Hall, 1999; Kraynov *et al*, 2000; Ridley, 2001), and cell proliferation (Benitah *et al*, 2004; Choi *et al*, 2009). Because Rac1 is also a member of the NADPH oxidase, which generates ROS (Bokoch and Diebold, 2002), it regulates NADPH oxidase complex assembly in most cells (Diebold, *et al*, 2009), and it has been shown to be a critical determinant of intracellular redox status. Several lines of evidence support a link between Rac1 and carcinogenesis, and Rac1 activity has been implicated in cancer initiation, progression, invasion, and metastasis (Ellenbroek and Collard, 2007; Vega and Ridley, 2008). Overexpression of Rac1 has been reported in colorectal, pancreatic, and breast cancers as well as in various leukaemias (Fritz *et al*, 1999; Schnelzer *et al*, 2000; Wang *et al*, 2009; Wertheimer *et al*, 2012). Therefore, it is important to understand the involvement of SNTA1 in Rac1 activation and its effects on downstream signalling, such as ROS generation and cell migration. The complex relationships between these adaptor proteins and their involvement in Rac1 activation and/or carcinogenesis provide us with a chance to investigate the mechanistic involvement of these proteins in human breast cancer cell lines through their roles in Rac1 activation and downstream signalling.

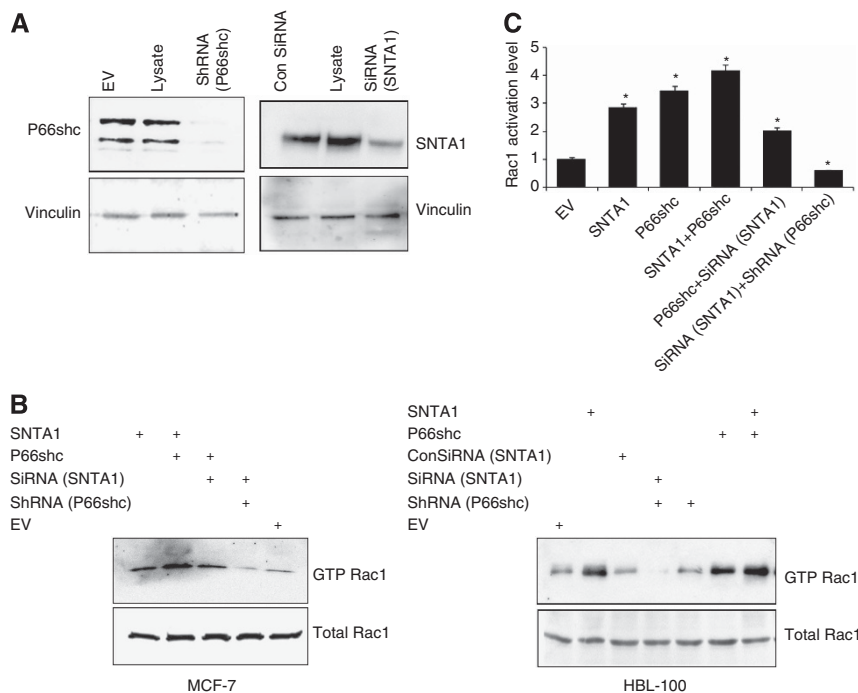


Figure 1. SNTA1 and P66shc enhance Rac1 activity. **(A)** Efficiency of the siRNA/shRNA against SNTA1 and P66shc; vinculin control blots are shown in the lower panels. **(B)** Comparison of the levels of active Rac1 in the immunoprecipitated lysates of MCF-7 (left panel) and HBL-100 (right panel) lysates transiently transfected with the empty vector (EV) or the indicated plasmid constructs. **(C)** The error bars depict the differences in Rac1 activity in cells transfected with the mentioned constructs compared with the empty vector control. The results are presented as the mean \pm s.e. from three separate experiments. *Differences were calculated against the EV and were considered significant at $P < 0.05$.

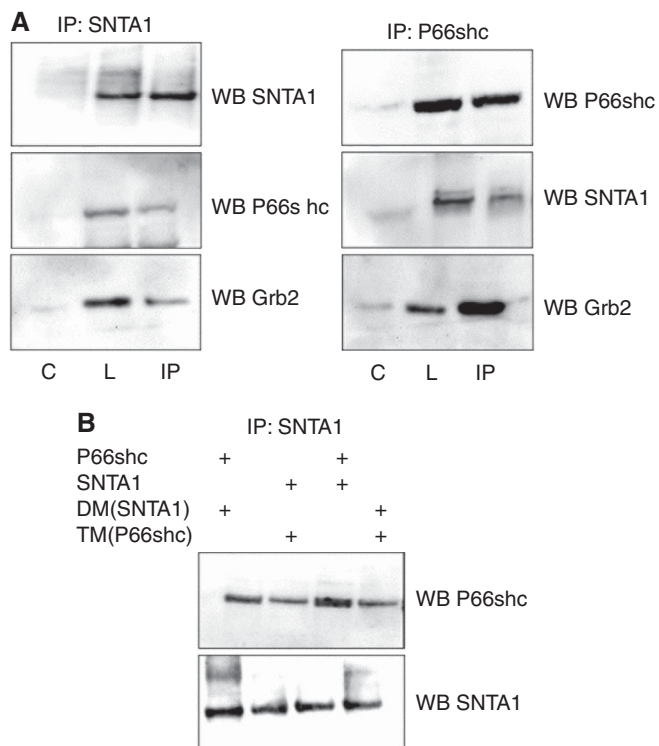


Figure 2. Syntrophin, Grb2, and P66shc form a trimeric complex. **(A)** Immunoprecipitations of HBL-100/MCF-7 cell lysates were performed using monoclonal antibodies against SNTA1 and P66shc that were bound to Protein A sepharose. After extensive washing, the bound proteins were eluted with SDS-PAGE sample buffer. After electrophoresis and electroblotting, the samples were probed with antibodies raised against the indicated proteins. The positions of the different proteins are indicated. The crude lysate (L) was also run on the same gel; C represents the IgG control, and IP represents the immunoprecipitated complex. **(B)** Cells transfected with the different constructs were examined for their effect on complex formation. DM(SNTA1) and TM(P66shc) represent the Y^{215,229} mutant of SNTA1 and the SYV⁴²⁶⁻⁴²⁸ mutant of P66shc, respectively.

MATERIALS AND METHODS

Plasmid constructs, reagents, and chemicals. Xpress-tagged P66shc WT cDNA in the pCDNA 3.1/His A vector was a kind gift from Dr Shaida Andrabi. The pCDNA 3.1, SNTA1 vectors were obtained from Addgene (Cambridge, MA, USA). The SNTA1 WT cDNA in the pQB125-myc plasmid and the SNTA1 WT pcis plasmid were kind gifts from Dr Gee SH and Dr Marvin Adams, respectively. The P66shc and SNTA1 mutants were generated by PCR using a site-directed mutagenesis kit (Quick Change XL, Stratagene, La Jolla, CA, USA), and sequencing confirmed the presence of the mutations.

Cell line maintenance and transfections. The MCF-7 and HBL-100 cell lines were obtained from NCCS and were maintained in DMEM supplemented with 10% heat inactivated FBS and 1 × antibiotic solution (100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin) at 37 °C in a 5% CO₂ atmosphere. Cells were transiently transfected with the desired plasmid constructs or siRNA (Sigma Inc., St Louis, MO, USA) specific for the gene of interest using Lipofectamine 2000 (Invitrogen, Carlsbad, CA USA) according to the protocol provided by the manufacturer.

Antibodies and immunoprecipitations. The anti-shcA (BD Biosciences, San Jose, CA, USA), anti-Shc^{P66} (Origene, San Jose, CA, USA), anti-SNTA1 (Life-span, US Biological, Salem, MA, USA), and anti-Rac1 (Sigma Inc.) antibodies were used in this study. Immunoprecipitations were typically carried out by incubating 2 µg of the antibody with ~1 mg of cellular protein lysate overnight; this mixture was then incubated with 50 µl of protein A-sepharose slurry (Invitrogen) for 4 h. After washing with cold PBS three times, the immunoprecipitates were boiled in 2 × loading buffer for 5 min, subjected to SDS-PAGE, transferred to a PVDF membrane, and probed with the indicated primary antibody and the appropriate peroxidase-conjugated secondary antibody. The blots were visualised by chemiluminescence using the Super Signal West Femto substrate (Pierce Chemical, Rockford, IL, USA) and were detected using the ChemiDoc system FlourChem-E (US Biological).

Rac1 activation assay. The amount of GTP-bound Rac1 was determined using a commercially available Rac1 activation assay kit (Upstate Biotechnology, Lake Placid, NY, USA) that affinity purifies GTP-Rac1 from cell lysates using agarose conjugated to the Rac1-binding domain of PAK. The assay was performed according to the protocol provided by the manufacturer, and the precipitates were then immunoblotted with the anti-Rac1 antibody provided with the kit.

ROS generation assay. The Amplex Ultra Red reagent (10 mM; Molecular Probes, Invitrogen) was used for the quantification of extracellular H₂O₂ in the cell media. For this assay, six-well plates were seeded at a density of 0.5 × 10⁶ cells per well and were transfected with the indicated plasmid or siRNA constructs. The fluorescence signal was detected using a spectrofluorophotometer (Shimadzu, RF-5301) with excitation at 568 nm and emission at 581 nm.

Cell proliferation assay. An MTT assay was used to measure cell proliferation in cells transfected with the desired plasmid constructs, siRNAs, and shRNAs. Acidic DMSO was used to dissolve the generated crystals, and the absorbance was measured at a wavelength of 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

In vitro wound healing assay. The standard protocol for an *in vitro* scratch assay was followed (Chun-Chi *et al*, 2007). The cells were incubated at 37 °C until the cells reached 100% confluence and formed a monolayer. A P-10 pipette tip was used to create a scratch in the monolayer. Pictures were taken using a camera fitted to a microscope. The gaps were measured, and the % wound closure was calculated.

Cell migration assay. Cell migration was assessed using 24-well transwell chambers (BD Biosciences) with an 8.0-µm pore size polycarbonate membrane. The cells that were transfected with the desired constructs were plated at a density of 5 × 10⁴ cells per well in 0.3 ml of starvation media (without FBS) in the upper well; the chamber was then placed into the lower well containing complete medium (10% FBS). After 24 h in a 37 °C, 5% CO₂ incubator, the experiment was stopped by removing the cells from the upper chamber using a cotton swab. The cells were fixed, stained, and de-stained using the cell stain and destain solutions provided with the kit. The absorbance was read at 565 nm.

Statistical analysis. The data were expressed as the mean ± s.e., and *P* < 0.05 was considered statistically significant. Representative experiments were replicated at least three times.

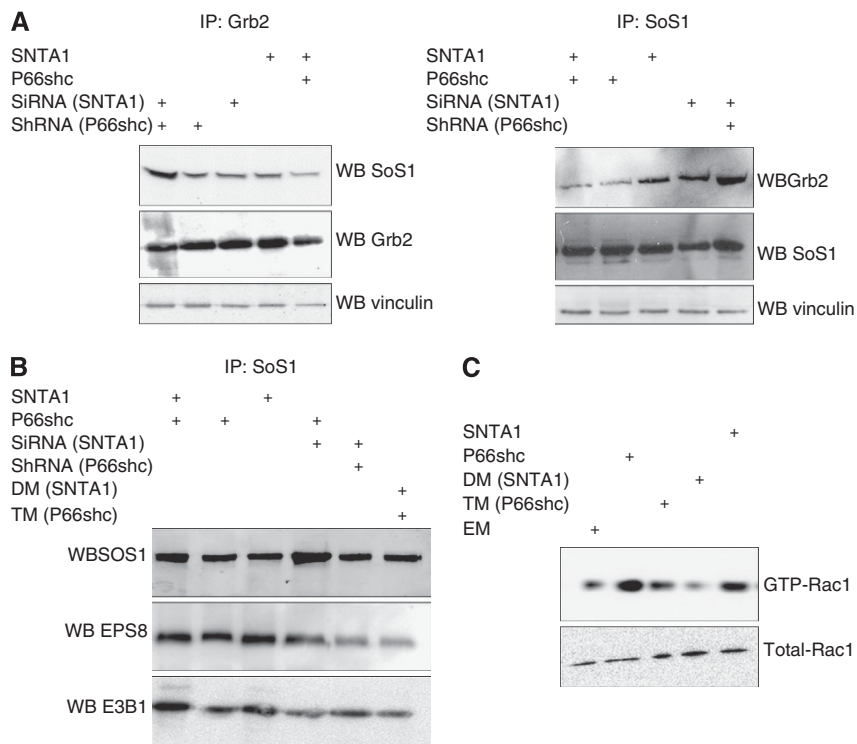


Figure 3. SNTA1/P66shc enhances the release of Sos1 from Grb2 and the formation of the Sos1-Eps8-E3b1 complex. **(A)** Cells were transfected with the indicated plasmid constructs, and immunoprecipitations were performed using monoclonal antibodies against Grb2 and Sos1 that were bound to Protein A sepharose. The resulting blots were then probed with antibodies against Sos1 and Grb2 to assess the formation of Sos1 complexes in these cells. **(B)** Cells were transfected with the indicated plasmid constructs, and immunoprecipitations were performed using a monoclonal antibody against Sos1 that was bound to Protein A sepharose. The resulting blots were probed with antibodies against Eps8 and E3b1 to assess the formation of the Sos1-Eps8-E3b1 complex in these cells. **(C)** Cells transfected with the SNTA1, P66shc or the mutant constructs were analysed using the Rac1 activation assay to investigate the effects of these motifs on Rac1 activation. DM(SNTA1) and TM(P66shc) represent the $\gamma^{215,229}$ mutant of SNTA1 and the SYV⁴²⁶⁻⁴²⁸ mutant of P66shc, respectively. Cells transfected with the wild-type SNTA1 and P66shc constructs showed an increase in Rac1 activity compared with the empty vector (EV).

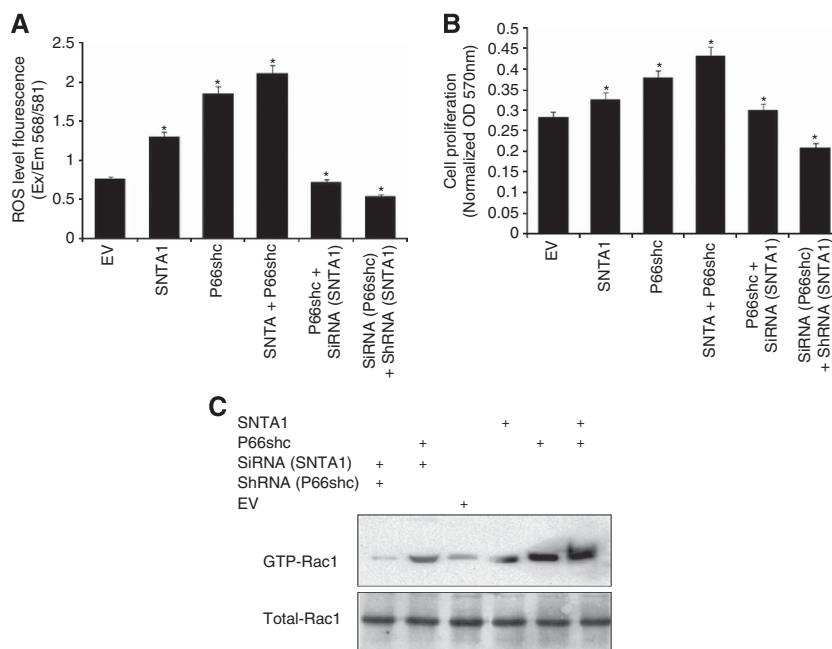


Figure 4. Rac1 activity correlates with intracellular ROS levels and proliferation in MCF-7 cells. **(A)** The ROS levels were evaluated in MCF-7 cells transiently transfected with the empty vector (EV) or the indicated plasmids and siRNAs. **(B)** Cell proliferation was analysed in MCF-7 cells using the MTT reagent after being transiently transfected with the empty vector (EV) or the indicated plasmids and siRNAs. The results are presented as the mean \pm s.e. from three separate experiments. *Differences were calculated against the EV (in **A**) or the control (in **B**) cells and were considered significant at $P < 0.05$. **(C)** Immunoblot showing the levels of total Rac1 and active Rac1 in MCF-7 cells transfected with the indicated plasmid constructs.

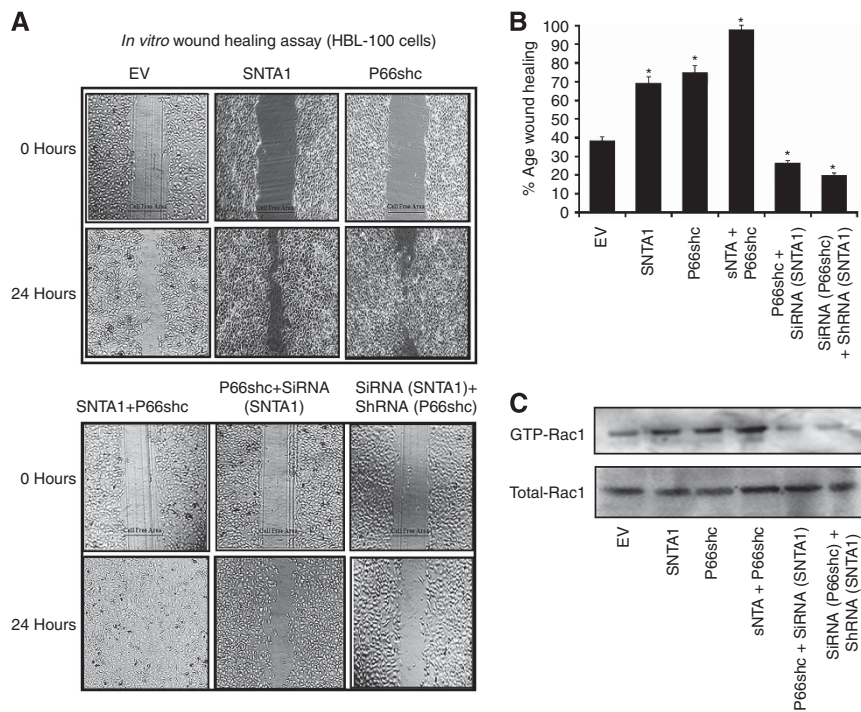


Figure 5. SNTA1/P66shc-mediated Rac1 activity correlates with cellular migration in HBL-100 cells. **(A)** HBL-100 cells transfected with the indicated plasmid constructs and siRNAs were used for wound healing assays as described in Material and Methods. The upper panels show the initial wound and cell-free area at 0 h, whereas the lower panels represent the observed wound healing after 24 h. **(B)** The error bars depict the % wound healing in cells transfected with the indicated constructs compared with the empty vector control (EV). The results are presented as the mean \pm s.e. from three separate experiments. *Differences were calculated against the EV and were considered significant at $P < 0.05$. **(C)** Immunoblot showing the levels of total Rac1 and active Rac1 in HBL-100 cells transfected with the indicated plasmid constructs.

RESULTS

Alpha-1-antitroponin and P66shc are involved in the activation of the Rho GTPase Rac1. Accumulating evidence has shown 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 of MCF-7 and HBL-100 cells transfected with full-length SNTA1 or P66shc plasmid constructs alone or in combination, along with the empty plasmid vehicle (EV) control. The Rac1 activation assay results (Figures 1B and C) showed that SNTA1 overexpression induced an approximately three-fold increase in Rac1 activation. In addition, the activation level of Rac1 protein reached maximal levels when SNTA1 was co-expressed with P66shc. Our results demonstrated that the Rac1 activation level was significantly decreased in cells transfected with siRNA targeting SNTA1. Furthermore, when P66shc was overexpressed in presence of siRNA against SNTA1, the Rac1 activation level was much lower compared with its level when cells were transfected with P66shc alone or when both P66shc and SNTA1 were expressed. In addition, the activation of Rac1 was found to be the lowest when both of these proteins were depleted (Figures 1B and C). This indicates that SNTA1 and P66shc cooperate and function in the activation of the Rho GTPase Rac1.

Alpha-1-syntrophin is co-immunoprecipitated along with P66shc and Grb2. The interaction between SNTA1, P66shc, and Grb2 was tested using co-immunoprecipitation assays. The results demonstrated that a multi-protein complex was responsible for the recruitment and activation of Rac1. We used an anti-SNTA1 monoclonal antibody to pull down the complex (Figure 2A). The presence of P66shc among the precipitated proteins was detected

by western blot analysis using a rabbit monoclonal antibody that specifically recognised human P66shc (Figure 2A). Conversely, the protein lysates from transfected cells were immunoprecipitated with a rabbit polyclonal antibody raised against P66shc, and the immunoprecipitated proteins were analysed by western blot analysis using a monoclonal anti-SNTA1 antibody and an anti-Grb2 antibody (Figure 2A). Our results indicate that SNTA1 is co-immunoprecipitated along with P66shc and Grb2. Thus, we hypothesise that a complex consisting of SNTA1, Grb2, and P66shc regulates Rac1 activation. To examine the role of the two conserved tyrosines (Y²¹⁵, Y²¹⁹) of SNTA1 (DM) and the SYV⁴²⁶⁻⁴²⁸ motif of P66shc (TM) in this primary complex formation, we transfected their mutant constructs into the cells and investigated for their effects on this complex formation. The results indicate that the introduced mutations significantly decreased the association of these proteins (Figure 2B).

Binding of SNTA1 and P66shc to Grb2 may enhance the release of Sos1 from Grb2 and the formation of the Rac1-activating Sos1-Eps8-E3b1 complex. The human guanine nucleotide releasing factor Son of sevenless (Sos) interacts with both SH3 domains of Grb2 (Yang *et al*, 1995). The interaction between Sos and Grb2 is thought to be important in determining the level of Rac1 activation in cells because Sos1 functions as a Rac1-specific GEF when it is part of the Sos1-Eps8-E3b1 complex (Migliaccio *et al*, 1997). We therefore examined the role of SNTA1 in regulating the formation of these Sos1-containing complexes. Because we could co-immunoprecipitate Grb2 and P66shc with SNTA1, we investigated whether SNTA1 and P66shc cooperate in displacing Sos1 from Grb2 to increase the potential binding of Sos1 to Eps8 and E3b1. Our pull-down results indicated that an increased amount of Sos1 was released from Grb2 when both SNTA1 and P66shc were overexpressed in HBL-100 cells, and the maximum release of Sos1

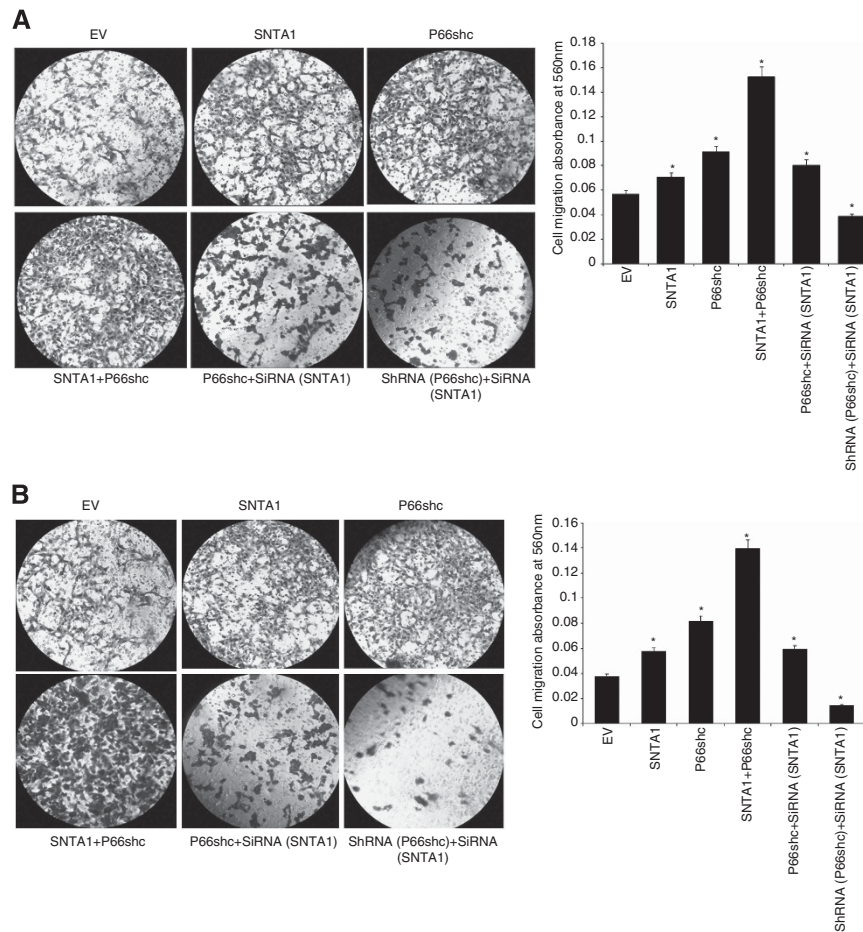


Figure 6. SNTA1/P66shc-mediated Rac1 activation increases the migratory capacity in MCF-7 and HBL-100 cells. EV, SNTA1, P66shc, and siRNAs were used to determine the migratory capacity of the cells, which was evaluated using the Boyden Transwell double chamber method as described in Material and Methods, in **(A)** HBL-100 cells and **(B)** MCF-7 cells. The error bars depict cell migration of cells transfected with the indicated constructs compared with the empty vector control (EV). The data are presented as the mean \pm s.e. from three separate experiments. *Differences were calculated against the EV cells and were considered significant at $P < 0.04$.

from Grb2 was observed when both constructs were transfected into the cells (Figure 3A). A consequent increase in the levels of the Sos1-Eps8-E3b1 complex was also observed under these conditions. In contrast, depletion of SNTA1 or P66shc expression using siRNAs resulted in a decrease in Sos1-Eps8-E3b1 complex formation and a more significant shift towards Sos1-Grb2 complex formation in these breast cancer cells (Figure 3B).

To elucidate the role of the conserved tyrosine residues in SNTA1 and P66shc in the formation of this trimeric complex and to examine any effects that the mutation of this motif has on Rac1 activity, we transfected cells with wild-type SNTA1 and P66shc or the tyrosine mutant (DM) SNTA1 and the SYV triple mutant (TM) P66shc and performed the Rac1 activation assay. Cells that were transfected with the wild-type SNTA1 and P66shc constructs showed increased Rac1 activity compared with the cells transfected with the empty vector (EV) (Figure 3C). Our results also showed a decrease in active Rac1 in the cells transfected with the SYV triple mutant of P66shc compared with the cells transfected with SNTA1 and wild-type P66shc, indicating a possible involvement of the SYV motif in the interaction between the proteins that compose the trimeric complex.

SNTA1/P66shc-mediated Rac1 activation increases intracellular ROS generation and cell proliferation. The Amplex Ultra-red reagent and MTT reagents were used to assess the effects of the overexpression or depletion of these proteins on extracellular ROS

generation and proliferation of HBL-100 cells, respectively. Expression of SNTA1 and wild-type P66shc in HBL-100 cells resulted in a significant increase in H_2O_2 , as well as an increase in the cell proliferation rate; combined expression of both these proteins resulted in maximal ROS generation and proliferation in these cell types (Figures 4A and B). Comparison of the P66shc (+) and P66shc (+)/SNTA1 (-) cells showed that the P66shc (+)/SNTA1 (-) cells had a significantly lower cellular proliferation rate and lower levels of H_2O_2 , which demonstrates that SNTA1 is involved in the complete activation of Rac1-mediated ROS generation or cell proliferation. In addition, the cells transfected with siRNA against SNTA1 or shRNA against P66shc showed significantly lower levels of ROS generation and cellular proliferation, as well as decreased expression of both these proteins. Cells that were treated with both SNTA1 siRNA and P66shc shRNA had a considerably larger decrease in cellular proliferation and H_2O_2 levels (Figures 4A and B). The Rac1 activation assay results (Figure 4C) were in agreement with these results.

SNTA1/P66shc facilitates cell migration as well as wound healing, in breast carcinoma cells. Our *in vitro* scratch assay indicated that overexpression of SNTA1 and P66shc facilitated the wound healing of HBL-100 cell monolayers. After 24 h, these cells had migrated into the wound, resulting in the complete closure of the scratch (Figure 5). To further confirm this, we reduced the endogenous expression of these proteins by transient

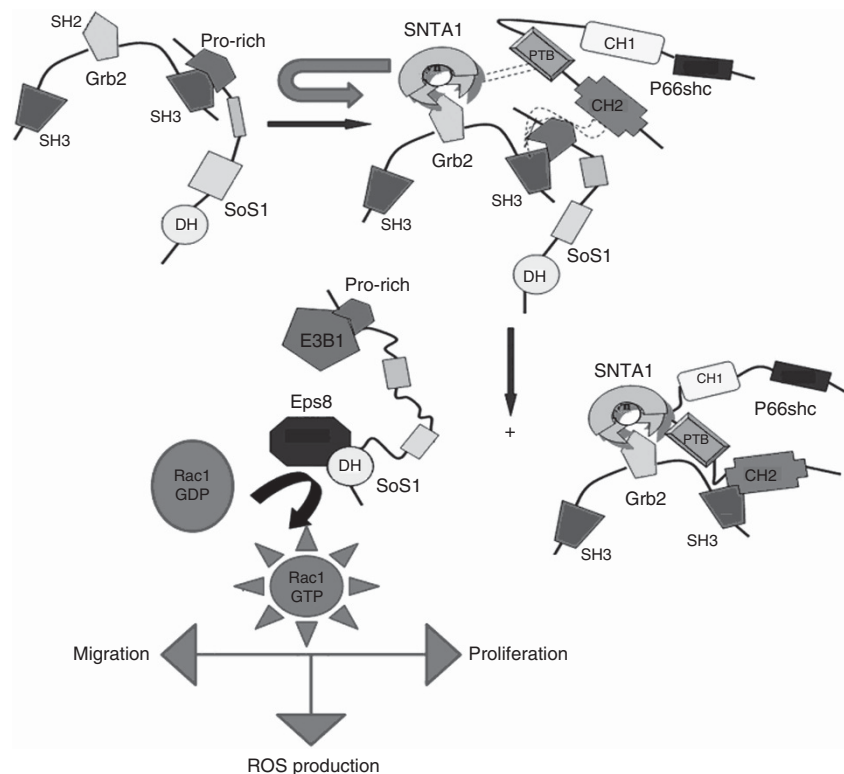


Figure 7. Proposed model for SNTA1-mediated Rac1 activation. Schematic representation of the proposed model for SNTA1-mediated Rac1 activation and downstream signalling.

transfection of siRNA or shRNA before performing the scratch assay. Figure 5 shows that, under these conditions, the cells remained at the edge of the wound. We also evaluated whether SNTA1-mediated Rac1 activity had a role in cellular migration. This was assayed in MCF-7 and HBL-100 mammalian cancer cells using the Boyden Transwell chamber method. As shown in Figure 6A, expression of SNTA1 and P66shc promoted the migration of these cells, which exhibited 2–3 times more migration capacity than the control EV cells. Alternatively, depleting cells of both these proteins using a siRNA targeting SNTA1 and an shRNA targeting P66shc resulted in a migration capacity ~33% lower than the EV cells. MCF-7 cells showed a similar pattern, and expression of SNTA1 or P66shc increased the migratory potential of these cells; expression of both SNTA1 and P66shc resulted in a four- to five-fold increase in cell migration, while depletion of these proteins considerably decreased cell migration (Figure 6B).

DISCUSSION

SNTA1, P66shc, and Grb2 have been shown to be upregulated in breast carcinomas and have roles in breast cancer development and/or progression (Jackson *et al*, 2000; Bhat *et al*, 2010). These proteins have also been implicated in the Rac1 protein activation pathway. The Rac1 protein itself has been implicated in several cancers, and Rac1 activity has also been shown to be increased in breast cancers (Schnelzer *et al*, 2000). In addition, SNTA1 and P66shc share many binding partners, such as Sos1, Grb2, that have also been implicated in the Rac1 activation pathway. Here, we showed that the transfection of SNTA1 or P66shc into human breast cancer cells resulted in a significant increase in Rac1 activation. The increase in active Rac1 was more

pronounced when SNTA1 was co-transfected with P66shc, indicating that this phenomenon is dependent on SNTA1 involvement and that both proteins function together in the Rac1 activation pathway. We also showed that SNTA1 and P66shc form a complex with Grb2 to activate Rac1; in this complex, SNTA1 acts as a signalling amplifier for Rac1 activation, resulting in an increase in ROS levels, cell proliferation and migration in breast cancer cells. Therefore, this study provides evidence for a novel role of SNTA1 in Rac1 activation. Our Rac1 activation assays and co-immunoprecipitation assays established that the interaction between SNTA1, Grb2, and P66shc is associated with an increase in Rac1 activation in these cells. Furthermore, knockdown of SNTA1 expression using small interfering RNAs, which were ~70–80% efficient, significantly decreased the active levels of Rac1 within the breast cancer cell lines. In addition, when P66shc was overexpressed while SNTA1 was depleted, Rac1 activation was much lower compared with its activation levels when cells were transfected with P66shc alone or with both P66shc and SNTA1. These findings lead us to hypothesise that the observed increase in Rac1 activation is a direct effect of interactions between the SNTA1, Grb2, and P66shc proteins.

Interestingly, distinct tyrosine/tyrosine phosphorylated regions of both syntrophin and P66shc can simultaneously bind the Grb2 molecule (Migliaccio *et al*, 1997; Oak *et al*, 2001). Furthermore, a short sequence motif (SYV⁴²⁶⁻⁴²⁸) is present in P66shc, and phosphorylation in this motif results in the specific, high affinity binding of the SH2 domain of Grb2. The two tyrosine residues (Y²¹⁵ and Y²²⁹) of SNTA1 that flanks its proline-rich motif are thought to be involved in its binding to Grb2 (Oak *et al*, 2001), while the SYV motif in several proteins has also been shown to bind to the PDZ domain of syntrophins (Bhat *et al*, 2012). Therefore, we generated a mutant form of SNTA1 (DM), in which both the tyrosine residues were mutated to phenylalanines, and a

mutant form of P66shc (TM), in which the SYV motif was mutated to ALA, using *in vitro* site-directed mutagenesis. A significant decrease in the association of SNTA1, Grb2, and P66shc was observed when these mutant forms were transfected into human breast cancer cells. Thus, we propose that the interactions between Grb2 and these two phospho-proteins are potentially mediated by the tyrosine/phosphotyrosine residues of SNTA1 and the SYV motif of P66Shc, respectively. Because both the N-terminal and C-terminal SH3 domains of Grb2 are known to interact with Sos1 (Yang *et al*, 1995), the binding of SNTA1 and P66shc would be expected to weaken the Sos1-Grb2 interaction, making Sos1 more available to interact with Eps8-E3b1, which is important for Rac1 activation. While the use of these mutants considerably decreased the formation of the Sos1-Eps8-E3b1 complex and the levels of active Rac1, the introduction of these mutants could not completely abrogate the formation of this Rac1 activating complex, and some active Rac1 remained, suggesting that the binding of SNTA1 and P66shc to Grb2 promotes the dissociation of Sos1 from Grb2 but is not solely responsible for this dissociation. Therefore, other residues may also be important for the interaction of SNTA1 and P66shc with Grb2.

Rac1 activity regulates mitogen-induced cytoskeletal changes and is a key component in actin reorganisation, the formation of cortical actin-containing membrane ruffles and lamellipodia, and the induction of gene expression programmes; Rac1 has also been shown to have a role in carcinogenesis and the progression of several human tumours (Fritz *et al*, 1999; Jaffe and Hall, 2005). Rac1 is a part of the NADPH oxidase, which is the ROS generating enzyme, and through its interaction with this complex, it participates in the control of ROS generation within cells (Whaley-Connell *et al*, 2007) and has been shown to induce lamellipodia extensions and membrane ruffling (Colley *et al*, 2000). Rac1 is also an upstream regulator of actin reorganisation and adhesive properties associated with cellular shape and motility (Nobes and Hall, 1999; Ridley *et al*, 2001). Similar to these functions, we observed increases in ROS production and cell migration due to SNTA1/P66shc-mediated Rac1 activation. A significant increase in the cell proliferation rate, cell migration, and ROS production was observed when these proteins were overexpressed in the breast cancer cell lines; similarly, a decrease in all these functions was observed when these proteins were depleted using siRNA. Therefore, it may be reasonable to consider that the SNTA1/Grb2/P66shc complex-induced Rac1 activation has downstream effects on ROS generation, cell proliferation, and cell migration in these breast cancer cell lines.

Taken together, our results and other data available allowed us to reasonably hypothesise a model for the activation of Rac1 through the formation of the SNTA1-Grb2-P66shc complex interactions and through the increased formation of the Sos1-Eps8-E3b1 complex (Figure 7). This model suggests a novel mechanism for Rac1 activation and predicts that the formation of the SNTA1-Grb2-P66shc complex determines the amount of cellular Sos1 that is bound to Grb2 or E3b1-Eps8; in this way, SNTA1 and P66shc modulate Rac1 activation and its downstream cellular functions. Thus, our model integrates previous studies with our new findings and suggests a mechanism in which syntrophin provides the core for the formation of a large signalling complex involved in Rac1 activation and downstream signalling in breast cancer cells. However, because SNTA1, P66shc, and Grb2 are all crucial multifunctional adaptor proteins involved in several other transduction pathways, we cannot rule out the involvement of other possible interacting partners and mechanisms that influence how SNTA1 regulates the activation of Rac1. While a more detailed understanding of Rac1 signal transduction will be uncovered by the characterisation of these interactions, the present study may be of major clinical importance and may provide new insights into Rac1 activation.

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