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## SG2NA enhances cancer cell survival by stabilizing DJ-1 and thus activating Akt

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### ABSTRACT

SG2NA in association with striatin and zinedin forms a striatin family of WD-40 repeat proteins. This family of proteins functions as scaffold in different signal transduction pathways. They also act as a regulatory subunit of protein phosphatase 2A. We have shown that SG2NA which evolved first in the metazoan evolution among the striatin family members expresses different isoforms generated out of alternative splicing. We have also shown that SG2NA protects cells from oxidative stress by recruiting DJ-1 and Akt to mitochondria and membrane in the post-mitotic neuronal cells. DJ-1 is both cancer and Parkinson's disease related protein. In the present study we have shown that SG2NA protects DJ-1 from proteasomal degradation in cancer cells. Hence, downregulation of SG2NA reduces DJ-1/Akt colocalization in cancer cells resulting in the reduction of anchorage dependent and independent growth. Thus SG2NA enhances cancer cell survival. Reactive oxygen species enhances SG2NA, DJ-1 and Akt trimerization. Removal of the reactive oxygen species by N-acetyl-cysteine thus reduces cancer cell growth.

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### 1. Introduction

SG2NA was initially characterized as a nuclear localized tumor antigen whose expression is augmented during S to G2 phases of cell cycle [1,2]. Together with striatin and zinedin it forms striatin sub-family of WD-40 repeat superfamily [3]. Among these three members, SG2NA was the earliest to evolve [4]. Striatin family members act as the B'' subunit of the heterotrimeric protein phosphatase 2A complex [5,6]. We have reported earlier that SG2NA has several isoforms generated out of alternative splicing [7]. Expression profile of SG2NAs in tissues changes with stages of embryonic development and post-natal aging [7,8,9]. Expression of SG2NA is epigenetically regulated by Brg-1 [10]. Recently, we found that SG2NA recruits DJ-1 and Akt to plasma membrane and mitochondria to protect cells from oxidative stress [11].

Like SG2NA, DJ-1 was also discovered as an oncogene that in cooperation with ras transforms NIH3T3 cells [12]. Its expression

is augmented in prostate, lung, breast, renal, hepatocellular, ovarian, acute leukemia, cervical, papillary, thyroid cancer and squamous cell carcinomas [13,14]. It transforms NIH3T3 cells by directly interacting with the SV-40 large T antigen [15]. DJ-1 activates Akt signaling by direct interaction and inhibition of PTEN [16,17]. It also can sense increased generation of reactive oxygen species (ROS); thus it acts as redox-sensitive chaperone and scavenge excess ROS, enhancing resistance against oxidative stress [18].

Akt is activated by several extra cellular signals including growth factors and by oncogenic mutations [19]. Activated Akt plays crucial physiological functions in cell proliferation, metabolism and stress responses. Its activity is tightly regulated by different combinations of kinases and phosphatases [19]. It remains in an inactive state due to an intramolecular interaction between the PH and the kinase domain. Various growth signals activate Phosphatidylinositol-3-kinases (PI3Ks) that in turn phosphorylate phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-bisphosphate (PIP3) [20]. Akt simultaneously interacts with PIP3 and PDK-1, changing its conformation. PDK-1 then phosphorylates Akt at T308, inducing its activity [20]. However, for complete activation, it also needs to be phosphorylated at S473 though the mechanisms are not fully understood yet. A number of recent studies have shown

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PI3K independent activation of Akt, especially in cancer cells [20].

Cellular redox plays an essential role in maintaining the functions of the cell. Reactive oxygen species (ROS) like hydrogen peroxide and superoxide regulate many signaling pathways associated with cell growth, proliferation, survival, motility and transformation [21]. At a moderate level, ROS act as second messenger, while excessive generation is harmful as it leads to cell death.

Previously, we have shown that SG2NA acts as a scaffold for DJ-1-Akt interaction under moderate oxidative stress [11]. As cancer cells have enhanced generation of ROS [21], it is relevant to study whether SG2NA-DJ-1-Akt interaction plays a role in cancer cell survival.

## 2. Materials and methods

All the chemicals are purchased from Sigma–Aldrich, USA unless noted otherwise.

### 2.1. Cell culture

NIH3T3, H9c2, HEK293, HEK293T, DU145, HepG2 and HeLa cells were procured from NCCS, Pune. These cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-amphotericin cocktail at 37 °C in the presence of 5% CO<sub>2</sub>.

### 2.2. ShRNA constructs

The shRNA expression cassettes against SG2NA were constructed to pLKO.1 vector. HEK293T, DU145 and HepG2 cells were stably transfected with those shRNA constructs. The transfected cells were selected and maintained by adding 2.5 mg/ml and 0.5 mg/ml of puromycin to standard growth media.

### 2.3. Cell lysis and immunoblotting

The cells were treated as mentioned in the figures, lysed and western analysis was carried out as mentioned earlier [11].

### 2.4. Immunoprecipitation

Cells were lysed in buffer containing 50 mM Tris, pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, protease inhibitors and 1 mM PMSF on ice and centrifuged at 10,000 rpm for 10 min. One mg of the supernatant was pre-cleared with equilibrated protein A agarose beads by incubating for 2 h at 4 °C and then was incubated with 4 µg of primary antibody overnight at 4 °C in a rotator. Thirty µl of equilibrated protein A agarose beads (50/50 slurry) was added to lysate antibody complex and incubated for 3–5 h. The beads were washed with the lysis buffer for 3 times at 4 °C for 5 min each and then directly loaded on SDS-PAGE followed by immunoblotting.

### 2.5. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS were measured in different cells with the fluorescence probe DCFH-DA. Briefly, DCFH-DA was dissolved in dimethyl sulphoxide (DMSO) and the cells were incubated with 5 µM DCFH-DA at 37 °C for 20 min. Images were captured under fluorescence microscope (Nikon) with excitation wavelength at 488 nm and emission wavelength at 530 nm.

### 2.6. Immunofluorescence analysis

Cells were grown on cover slips. After treatment, the cells were then fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% triton-X-100 and blocked with 1% BSA prepared in 1× PBS for 1 h. The cells were incubated with primary antibody for 1 h followed by the incubation with secondary antibody for 1 h. Cover slips were mounted on slides over 10% glycerol solution, and sealed from the edges. The images were captured using confocal microscope of Olympus Fluoview 1000.

### 2.7. Proliferation assay

Cell proliferation assay was carried out HEK293T, HepG2 and DU145 cells stably transfected with shRNA against SG2NA and scrambled oligos.  $2 \times 10^4$  cells were plated into the 48 well plates. After 12, 24, 48 and 72 h of incubation in 5% CO<sub>2</sub> at 37 °C, the cells were trypsinized and viable cells were counted using Trypan Blue staining method. To study anchorage independent growth by colony forming assay with soft agar method,  $\sim 0.3 \times 10^6$  cells were plated in triplicate in 0.35% (w/v) agar in DMEM supplemented with 10% FBS on the top of 0.7% (w/v) agar in 6 well plate. Cells were incubated in 5% CO<sub>2</sub> at 37 °C for 14 days with refeeding every alternate day. Colonies were stained with 0.005% crystal violet and images were captured with a Nikon microscope.

### 2.8. Statistical analysis

The extent of co-localization was quantified using Image Pro Plus 6 software. Statistical analysis was carried out from the quantified data and expressed in terms of Mean  $\pm$  SEM and p value was calculated from student's t test with two tailed distribution considering two sample equal variance. Significance was considered as follows \* p < 0.05, \*\* p < 0.01 and \*\*\* p < 0.001.

## 3. Results

### 3.1. Association of Akt, DJ-1 and SG2NA is increased in cancer cell lines

Increased DJ-1 and Akt activities have been associated with cancer development [22]. In view of our recent observation that SG2NA modulates cell cycle progression (unpublished) and it recruits Akt and DJ-1 under oxidative stress [11]; we tested whether association of SG2NA, DJ-1 and Akt is also enhanced in cancer cells. A number of established cancer cell lines viz., A549, H1299, HEK293T, DU145, HepG2, HeLa, PC12 were immunostained for SG2NA, DJ-1 and Akt. As shown in Fig. 1, association of SG2NA-DJ-1, Akt-DJ-1 and SG2NA-Akt were significantly higher in those cell lines as compared to that in a number of immortalized untransformed cell lines viz., NIH3T3 and H9c2. Hence, increased co-localization of three proteins in those cells might be due to their recruitment by SG2NA.

### 3.2. Ablation of SG2NA disrupts DJ-1/Akt association

In cancer cells DJ-1 activates Akt by multiple mechanisms including inhibition of PTEN [16]. We thus tested whether SG2NA, the scaffold for DJ-1 and Akt interaction, plays any role in this process. Two well characterized cancer cell lines DU145 and HepG2 and an experimentally transformed cell HEK293T were stably transfected with shRNA against SG2NA. As shown in Fig. 2, down-regulation of SG2NA decreased the level of colocalization between

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