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14. ABSTRACT

We have identified NuSAP as prognostic marker up-regulated in recurrent prostate tumors. Our grant aimed at identifying the role of NuSAP in promoting proliferation and invasion in Prostate Cancer and identify genes that upregulate NuSAP expression. To characterize the role and regulation of NuSAP in prostate cancer, we expanded our investigation into looking at critical regulatory elements within the NuSAP promoter region. We identified 2 non-canonical MYC binding sites within the 1000bp of NuSAP promoter. Luciferase expression studies using wildtype and deletion constructs suggested no direct response of MYC on NuSAP promoter region. We also carried out studies to understand whether NuSAP expression is regulated by RB1/E2F1 axis. We found NuSAP expression increased upon knockdown of RB1 and a further reduction of E2F1 resulted in reduction of NuSAP gene and protein suggesting that NuSAP is regulated by the RB1/E2F1 axis.

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Introduction:
We have identified NuSAP as prognostic marker upregulated in recurrent prostate tumors. Our grant aimed at identifying the role of NuSAP in promoting proliferation and invasion in Prostate Cancer and identify genes that upregulate NuSAP expression. Nucleolar and spindle-associated protein (NuSAP) is an essential microtubule- and chromatin-binding protein found in the proliferating cells. Its primary function is to induce extensive bundling and stabilization of spindle microtubules against depolymerization and cross-link large numbers of microtubules into aster-like structures and thick fiber networks during metaphase. Interestingly, both excessive amount and knockdown of NuSAP leads to disruption of cell division. Thus, NuSAP must be tightly controlled during cell cycle progression. However, how NuSAP protein is controlled and the precise role of NuSAP in regulation of cell cycle still remains unclear.

Specific Aim 1: profile the expression of NuSAP, C-Myc, RanGTP, NF-YA, c/EBPα and AR in Prostate Cancer Cell Lines. Investigate invasion and proliferation:
In the initial application we proposed to investigate the transcript level of NuSAP, c-MYC, NFYA, CEBPA and AR in prostate cancer cell lines. Based on the transcript profiles we found *NuSAP* expressed at relatively high levels in the prostate cancer cell lines LNCaP and PC3 (data not shown). These two cell lines hence became our model to investigate the role of NuSAP in prostate cancer. As stated in the initial application, knockdown of *NuSAP* transcript levels significantly decreased proliferation of PC3 cells *in vitro* compared with control cells (Figure 1a). In addition, knockdown of *NuSAP* transcript levels significantly decreased invasion to <5% compared with controls in which 40% of the cells invaded through the membrane (Figure 1b). Similarly, knockdown of *NuSAP* in LNCaP cells significantly decreased proliferation (not shown). However, because wild-type LNCaP cells were poorly invasive, we could not assess the effects of *NuSAP* knockdown on invasion in this cell line.

KEY RESEARCH ACCOMPLISHMENTS:

- Investigated the transcript levels of NuSAP, MYC, NFYA and AR in prostate cell lines.
- Demonstrated that upon knock down of NuSAP there is a significant decrease in the growth and invasion profile of in both PC3 and LNCaP cell lines.

Specific Aim 2: To understand the mechanisms of action of NuSAP by testing its effects on androgen receptor signaling and gene expression

So far, using the 1088bp NuSAP promoter we have not been able to identify a binding site for Androgen Receptor (AR). We have expanded our search to look at the larger part of the promoter region to look for AR binding regions or elements that could have a direct link to AR. There has been an AR enhancer region identified 100kb upstream of NuSAP transcription start site (Waltering et al., 2009). This enhancer region has been demonstrated to influence NuSAP transcript levels under varying concentration of Androgen. We would continue our efforts to experimentally validate this region and its influence on NuSAP gene expression.

KEY RESEARCH ACCOMPLISHMENTS:

- Analyzed extended NuSAP promoter region to identify an AR enhancer region which could have an influence on NuSAP expression.

Specific Aim 3: To define the important regulatory elements that influence NuSAP gene expression levels in prostate cancer cells.

To understand the underlying mechanisms of NuSAP over-expression in aggressive prostate cancers, we expanded our investigation into looking at critical regulatory elements within the NuSAP promoter region. Last year, we had reported identification of an E2F (-246/ -252) binding site within the promoter region. Since E2F1 is negatively regulated by RB1, and RB1 is frequently lost in aggressive prostate cancer, we hypothesized that NuSAP expression is regulated, at least in part, by the RB1/E2F1 axis. Furthermore, we found 2 non-canonical MYC binding sequence (-522/-528 and -802/-808) in the NuSAP promoter region. Over expression of MYC gene is also commonly observed in aggressive prostate cancer. Hence we designed experiments to demonstrate whether or not over expression of MYC could modulate NuSAP expression. We will summarize our findings on both these important regulating factors and their influence on NuSAP expression.

Regulation of NuSAP Expression by MYC Transcription Factor.

Using MATCH™ software (TRANSFAC), we investigated whether there might be other potential transcription factor binding sites in the 5'-upstream region of the NuSAP gene. We found, 2 non-canonical MYC (-522 (CACCTG) and -802 (CAGGTG)) binding sites instead of a putative binding site for MYC (CACGTG). However, previously it has been reported that MYC-MAX complexes are formed on both canonical as well as non-canonical binding sites (Blackwell et al., 1993). To start with, we purchased a longer (1088bp) human NuSAP promoter vector linked to a luciferase gene from Switchgear Genomics (Menlo Park, CA) and created 3 MYC deletion mutants (ND522, ND802 and DD (double deletion) mutating MYC binding site individually at -522 and -802 respectively and a construct with double deletion within the promoter region. We used 3 different cell line models to study the interaction between MYC and NuSAP. First, a human prostate cancer cell line PC3, second, RCC EC4, a mouse hepatic carcinoma cell line engineered to expresses human MYC under the influence of antibiotic tetracycline and third, a normal human Fibroblast cell line which stably expresses tamoxifen induced MYC protein. Both RCCEC4 and NHF-MYC-ER were a kind gifts from Dr. Dean Felsher of Stanford University). These cell lines were transiently transfected with either of wildtype promoter construct or MYC deletion constructs along with pBabe-Puro-MYC (PC3 only) containing MYC cDNA and luciferase activity was assayed. We did not observe any difference in the normalized luciferase expression obtained from either of wildtype NuSAP promoter and MYC deleted promoter constructs suggesting these non-canonical binding sites do not directly respond to MYC expression (Figure 1a,b&c).

We further investigated NuSAP-MYC interaction by transfecting PC3 cell line with either of NuSAP shRNA or MYC shRNA and looked at the expression profile of both NuSAP and MYC gene by quantitative PCR. We observed a concomitant down-regulation of both MYC and NuSAP with transfection of NuSAP shRNA and MYC shRNA respectively suggesting a possible indirect effect of both proteins on each other (Figure 1d).

KEY RESEARCH ACCOMPLISHMENTS:

- Non-canonical MYC binding site within 1088bp NuSAP promoter do not respond to MYC expression.

Regulation of NuSAP by RB1/E2F1 axis

We had previously identified that E2F1 directly bind to the promoter of NuSAP and enhance NuSAP expression (Gulzar et al., 2012). Since E2F1 is known to be negatively regulated by RB1, and RB1 is frequently lost in aggressive prostate cancer, we hypothesized that NuSAP expression is regulated, at least in part, by the RB1/E2F1 axis. To test this hypothesis, we designed 2 different experimental strategies to demonstrate that NuSAP expression is negatively correlated with RB1 and positively correlated with E2F1. Firstly, using SAM analysis, we probed 2 independent prostate cancer microarray datasets (Taylor et al., 2010; Sboner et al., 2010) to generate a list of genes whose expressions were significantly correlated with NuSAP gene expression. RB1 (negative correlation) and E2F1 (positive correlation) were both found significantly associated with NuSAP (Figure 2a&b). Secondly, we transduced PC3 and LNCaP with lentiviruses containing RB1 and E2F1 shRNA to create cell lines with a) RB1 knock down and b) both RB1 and E2F1 knock down. Then we evaluated NuSAP expression levels by RT-qPCR and western blot (Figure 2c&d). In agreement with our hypothesis, NuSAP expression increased upon knockdown of RB1 and a further reduction of E2F1 resulted in reduction of NuSAP gene and protein. All of these analyses support the notion that NuSAP is regulated by the RB1/E2F1 axis.

NuSAP upregulation is associated with increased invasion and metastasis

In order to further understand the role of NuSAP in prostate cancer progression we also examined the effects of NuSAP on cellular apoptosis, cell cycle stages, proliferation, invasion and metastasis. To examine how NuSAP affects apoptosis we knocked down or over expressed NuSAP in PC3 and LNCaP prostate cancer cell lines. These cell lines were stained with Propidium Iodide (PI) stain and analyzed by FACS flow cytometer. We observed that knockdown of NuSAP increases cell death compared to cells over expressing NuSAP which were found similar to control (Figure 3A). Similarly, knockdown of NuSAP resulted in an increase in number of cells in G2/M mitotic phase while cells over expressing NuSAP were found similar to control (Figure 3B).

We had previously reported that knockdown of NuSAP results in reduced proliferation and invasion (Gulzar et al., 2012). In order to determine if over expression of NuSAP would have an opposite effect, we repeated the experiments using PC3 and LNCaP cells over expressing NuSAP. In doing so we found that NuSAP over expression does not alter proliferation in LNCaP or PC3 cells (Figure 4A), however, significantly increases the number of invasive cells (Figure 4B).

Overexpression of NuSAP in recurrent prostate cancer

Since NuSAP overexpression is correlated with both recurrent prostate cancer and a more invasive phenotype, we hypothesized that NuSAP overexpression would also be associated with metastatic prostate cancer cells. To test this hypothesis we examined microarray datasets deposited in the Gene Expression Omnibus website, and found that higher levels of NuSAP was positively correlated with metastatic prostate cancer (Figure 5).

KEY RESEARCH ACCOMPLISHMENTS:

- Higher levels of NuSAP is positively correlated with metastatic prostate cancer cells
- NuSAP expression is negatively correlated with RB1 and positively correlated with E2F1 gene expression.
- Knockdown of NuSAP increases cell death and also results in more number of cells stuck in G2M phase.
- NuSAP overexpression does not alter proliferation in LNCaP or PC3 cells but significantly increases cellular invasion.

REPORTABLE OUTCOMES:

Overall:

We have made significant progress on all aims. In the third year of funding, we have planned to expand our effort into understanding the biological regulation of NuSAP especially by identifying binding partners that cooperate with NuSAP during various cell cycle stages and affect its expression. In this regards a potential hypothesis worth experimenting is looking at microtubules and its association with both AR and NuSAP. Microtubules have recently been demonstrated to shuttle androgen receptor to nucleus while higher levels of NuSAP, a protein linked to microtubule, have also been observed in cells resistant to Taxane chemotherapy (Mistry and Oh, 2013; Thadani-Mulero et al., 2012; Emanuele et al., 2011). Looking into these binding partners would also expand our understanding into role of AR and NuSAP as there might be some mediator such as microtubule helping both AR and NuSAP.

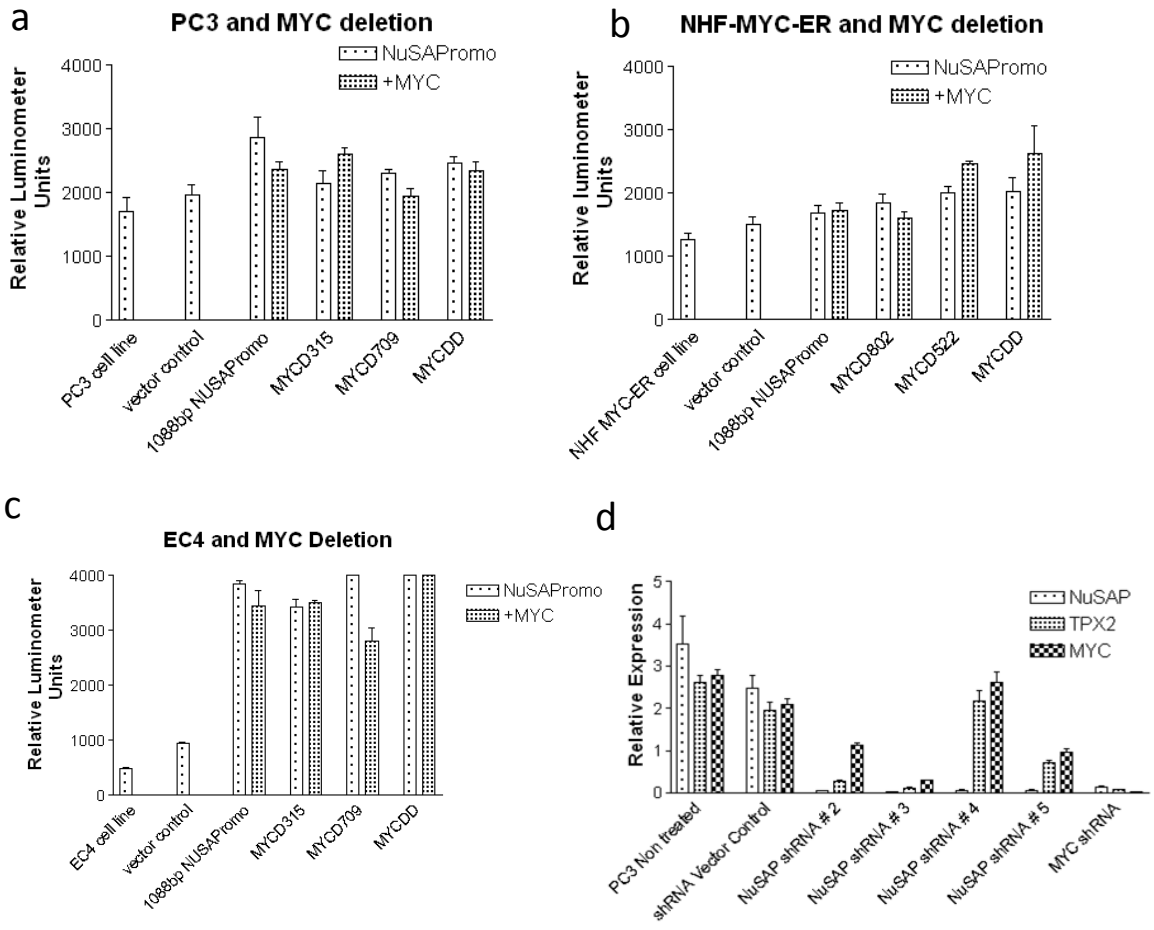

Figure 1. Luciferase assay of 1088bp human NuSAP promoter region. a) Baseline expression levels of the 1088bp human NuSAP gene promoter and 3 non canonical MYC binding site (-522, -802 and DD (double deletion) deletion constructs ligated to plightswitch-luciferase in PC3 cells. Results suggest no change in luciferase expression observed from either of wildtype NuSAP promoter and MYC deleted promoter constructs. b) Baseline expression for the promoter constructs in normal fibroblast NHF-ER-MYC cell line expressing tamoxifen induced human MYC shows identical regulation with no difference observed between wildtype and mutated vectors. c) Baseline expression for the promoter constructs in mouse RCC EC4 cell line expressing Tetracycline induced human MYC shows identical regulation with no difference observed between wildtype and mutated vectors. d) Quantitative PCR analysis of PC3 cells transfected with 5 NuSAP shRNA and a validated MYC shRNA (Felsher group;Stanford University). Data shows downregulation of both MYC and NuSAP when cells were transfected with either of NuSAP shRNA and MYC shRNA respectively. Data represents mean values from two separate experiments.

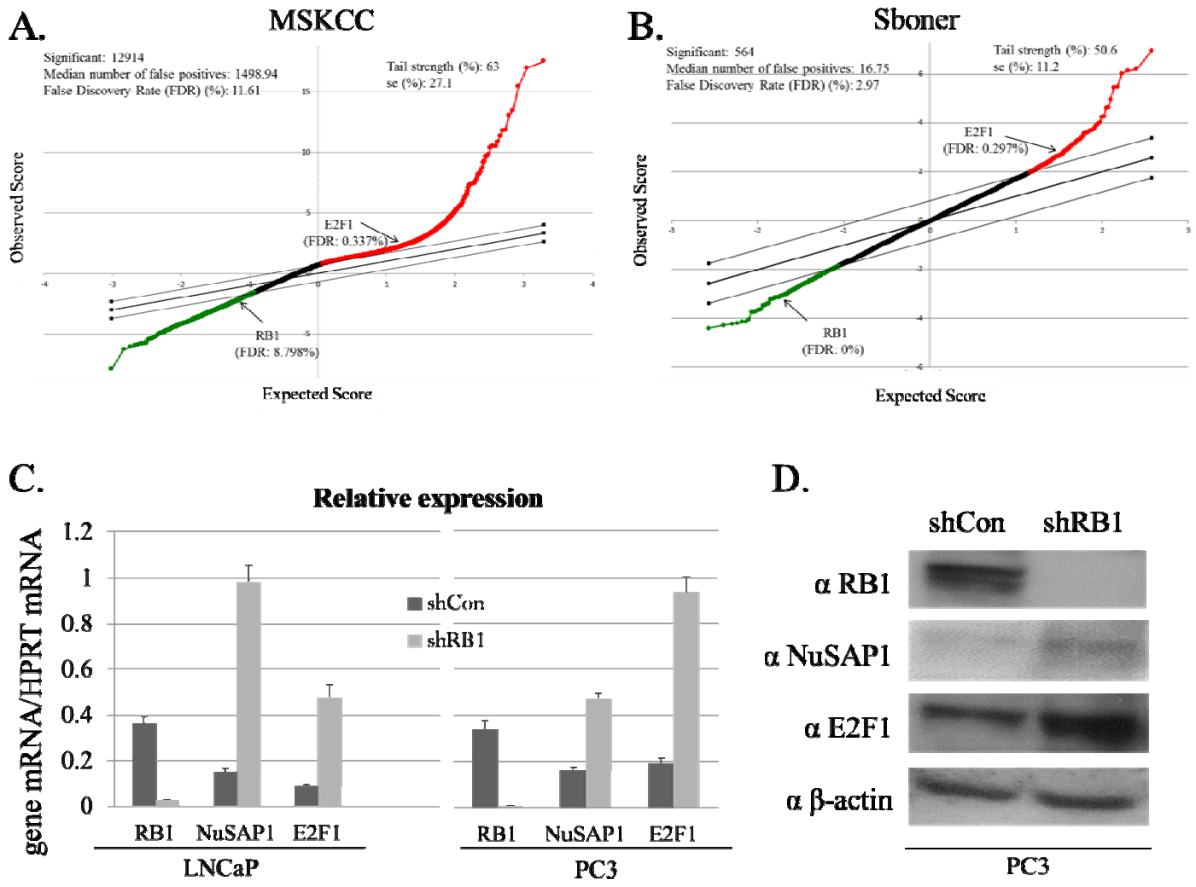

Figure 2. NuSAP is negatively correlated with RB1 and positively correlated with E2F1.Using Significance Analysis of Microarrays, we probed two prostate cancer microarray datasets (MSKCC and Sboner) for genes negatively and positively correlated with NuSAP. RB1 and E2F1 were found significantly associated with NuSAP gene expression. c&d) Knockdown of RB1 in PC3 and LNCaP results in increase of NuSAP mRNA and protein levels while a further knockdown of E2F1 results in reduced NuSAP mRNA and protein.

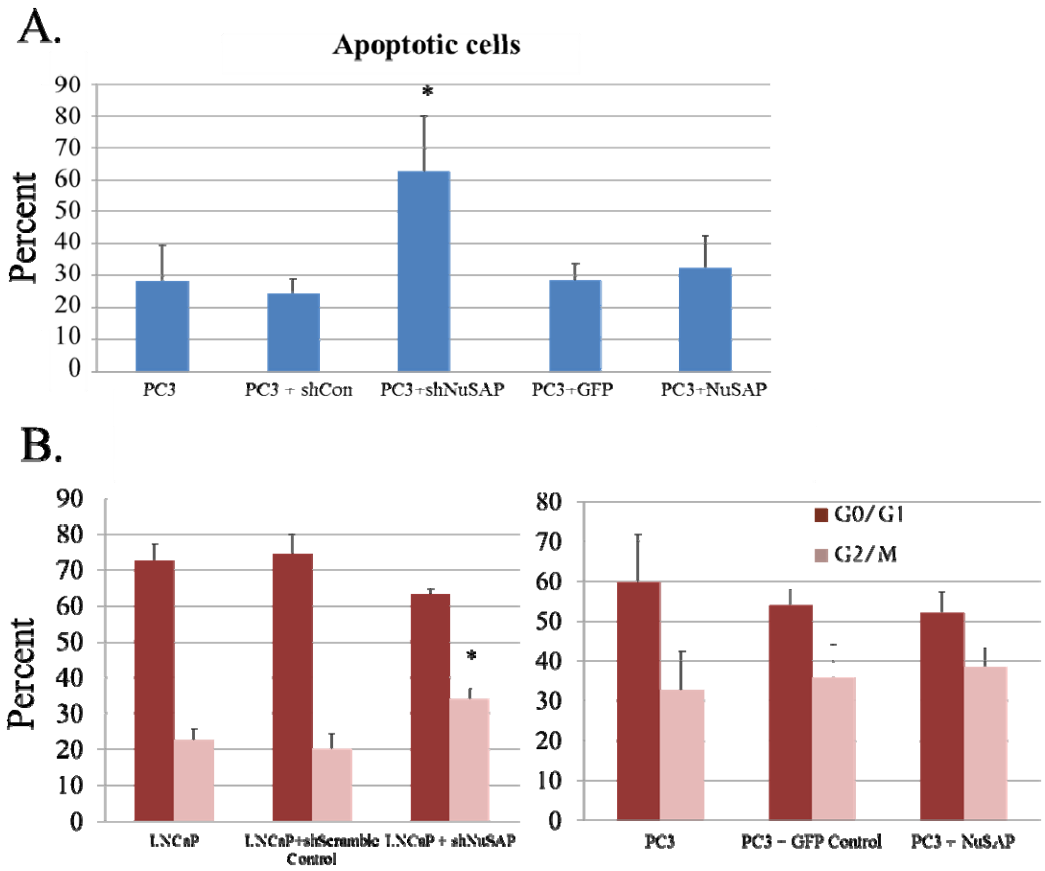

Figure 3. Knockdown of NuSAP increases the number of apoptotic cells and the number of cells in G2/M, but overexpression of NuSAP does not alter such parameters. Cell lines (2 x 10^5 PC3 cells and 5 x 10^5 LNCaP cells) were seeded per well in 6-well plates. The following day cells were infected with lentiviruses to knockdown or overexpress NuSAP or controls. a) Cells were stained with PI (propidium Iodide) and analyzed using FACS flow cytometry. Results show increase in the number of apoptotic cells in cells where NuSAP was knocked down while cells with over experssed NuSAP looked similar to control. Similarly, the cellular content analyzed from both knock down and over expressed cells suggested increase in the number of G2M mitotic stage observed in cells where NuSAP was knocked down compared to over expressed cells which were found similar to control. Error bars: SD, * P <0.05.

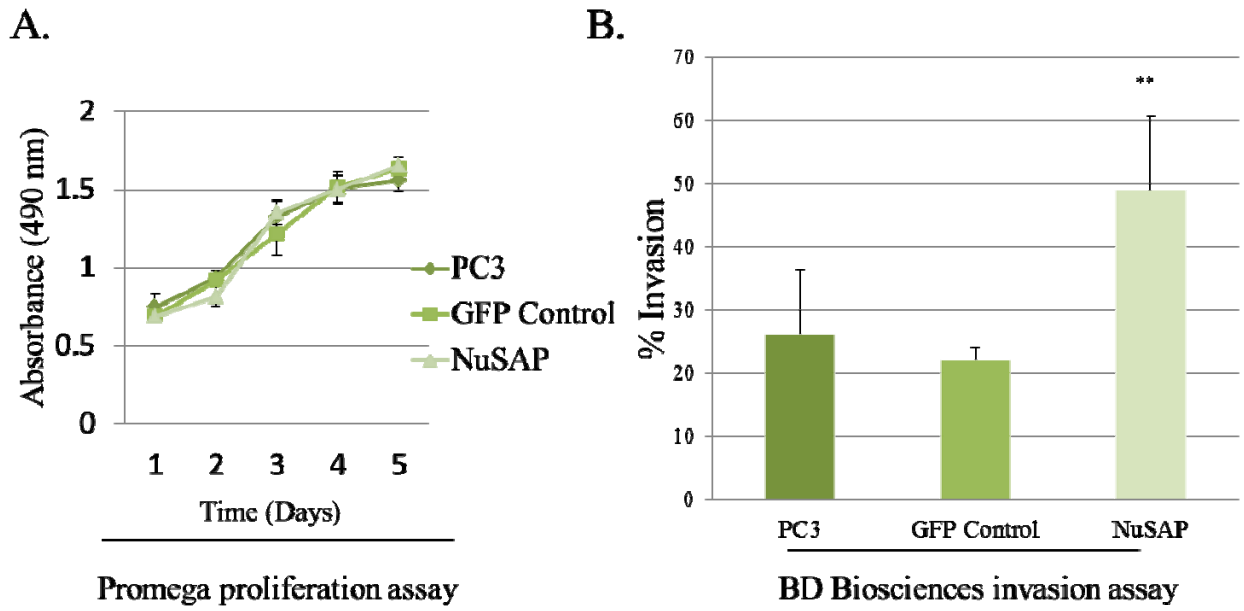

Figure 4. NuSAP overexpression does not alter proliferation of PC3 or LNCaP cells, but increases invasion of PC3 cells. (A) 2,000 NuSAP over expressing PC3 cells were plated in each well of a 96-well tissue culture plate. Using the Promega proliferation assay, cell proliferation was measured each day over a five day period. Error bars: SD. (B). 25,000 NuSAP overexpressing PC3 cells resuspended in serum-free DMEM were seeded per matrigel chamber per well of a 24-well plate containing DMEM with 10% FBS as a chemoattractant. The percentage of invading cells was determined by counting average number of cells crossing the matrigel layer (per five frames in triplicate) divided by the average number of cells of control inserts ** $P < 0.001$**.** Error bars: SD.

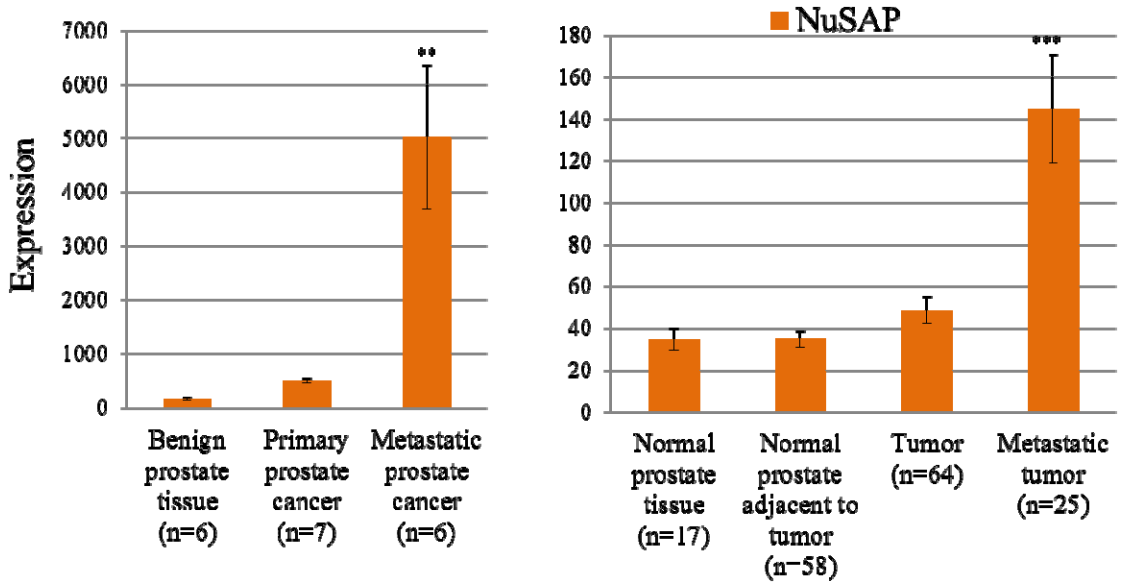

Figure 5. NuSAP expression is upregulated in metastatic prostate cancer. Prostate cancer microarray datasets deposited in the GEO were examined for NuSAP expression. Left:(Varambally et al., 2005), expression profiling by array, 19 samples., error bars: standard error of the mean (SEM), $p < .004$. Right: (Yu et al., 2004; Chandran et al., 2007), expression profiling by array, 164 samples, error bars: SEM, $p < 0.0001$.

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