

## ORIGINAL ARTICLE

Increased expression of *NuSAP* in recurrent prostate cancer is mediated by *E2F1*ZG Gulzar<sup>1</sup>, JK McKenney<sup>2</sup> and JD Brooks<sup>1</sup>

Increasing evidence suggests that prostate cancer is overdiagnosed and overtreated, and prognostic biomarkers would aid in treatment selection. To define prognostic biomarkers for aggressive prostate cancer, we carried out gene-expression profiling of 98 prostate tumors and 52 benign adjacent prostate tissue samples with detailed clinical annotation. We identified 28 transcripts significantly associated with recurrence after radical prostatectomy including *NuSAP*, a protein that binds DNA to the mitotic spindle. Elevated *NuSAP* transcript levels were associated with poor outcome in two independent prostate cancer gene-expression datasets. To characterize the role and regulation of *NuSAP* in prostate cancer, we studied the expression of *NuSAP* in the LNCaP and PC3 human prostate cancer cell lines. Posttranscriptional silencing of the *NuSAP* gene severely hampered the ability of PC3 to invade and proliferate *in vitro*. The promoter region of the *NuSAP* gene contains two CCAAT boxes and binding sites for E2F. Transient transfection of an *E2F1* cDNA and 431 bp of the *NuSAP* promoter demonstrated *E2F1* as an important regulator of expression. Deletion of the E2F-binding site at nucleotide –246 negated the effects of *E2F1* on *NuSAP* expression. Electrophoretic mobility shift assays demonstrated that nuclear extracts of cells overexpressing *E2F1* bound directly to the E2F-binding site in the *NuSAP* promoter region. Finally, immunohistochemistry showed a strong correlation between *E2F1* and *NuSAP* expression in human prostate cancer samples. *NuSAP* is a novel biomarker for prostate cancer recurrence after surgery and its overexpression appears to be driven in part by *E2F1* activation.

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**Keywords:** prostate cancer; gene-expression profiling; *NuSAP*; *E2F1*; recurrence

## INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in the western world. An estimated 648 400 men will be diagnosed with prostate cancer this year and 136 500 will die of their disease.<sup>1</sup> The disparity between the number of men with prostate cancer and the number of men who die of their disease is largely due to the wide variation in the behavior of prostate cancer. Only a fraction of patients have tumors capable of metastasizing and causing death. One of the most important challenges in treating prostate cancer is in identifying patients with potentially lethal tumors needing aggressive treatment and those with indolent tumors that can be safely watched. The European Randomized Study of Screening for Prostate Cancer trial estimated that the number of prostate-specific antigen (PSA)-detected prostate cancers treated to save one man's life was 48.<sup>2</sup> A recent analysis that was restricted to the men who were actually screened suggested a smaller ratio of treated cancers at 30 to save one man's life at 10 years.<sup>3</sup>

Many investigators have documented significant molecular diversity in human prostate cancer and this heterogeneity undoubtedly contributes to the spectrum of clinical behavior. Several groups have shown that comprehensive gene-expression profiling can capture molecular features that distinguish normal from cancerous prostate, and a few have identified genes associated with prognosis.<sup>2,4–11</sup> However, many of these studies have been small in size and on samples with minimal clinical annotation, limiting their ability to identify prognostic markers or provide insights into the biology of prostate cancer. To identify gene-expression signatures associated with recurrence after

definitive prostate cancer surgery, we performed gene-expression profiling of 98 primary prostate tumor samples from 86 patients with detailed clinical annotation and clinical follow-up. From this analysis, we identified overexpression of the nucleolar and spindle-associated protein (*NuSAP*) gene as an important marker of prognosis in prostate cancer. Functional studies confirm the potential biological importance of the *NuSAP* gene in prostate cancer and identify *NuSAP* as a potential end-target of *E2F1*.

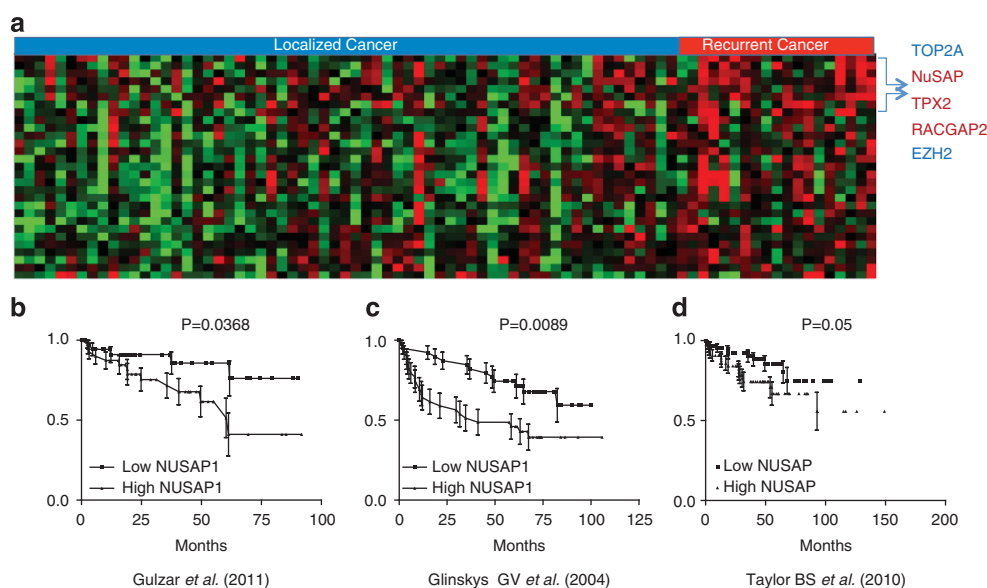
## RESULTS

We performed gene-expression profiling on 98 tumors from 86 individuals using HEEBO (human exonic evidence-based oligonucleotide) spotted microarrays containing 44 544 70-mer probes. The prostate tissue samples were harvested from men who underwent radical retropubic prostatectomy for clinically localized prostate cancer at Stanford University by a single surgeon (JDB) between 1998 and 2007. Detailed clinical data, including follow-up and recurrence status, were available in 90 patients (92%). Mean patient age, preoperative serum PSA levels, clinical stage and pathological Gleason grade were compatible with the risk profiles of contemporary patients undergoing surgery for prostate cancer (Supplementary Table S1).

Screen-detected prostate cancers found in contemporary surgical series are rarely lethal even after a decade of follow-up. Recurrence after surgery has been associated with a more aggressive clinical course and lethality, and has therefore been used as a surrogate endpoint of prostate cancer aggressiveness in many studies.<sup>12</sup> To identify genes that are associated with

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**Figure 1.** (a) Two-class SAM survival analysis comparing 19 recurrent and 63 nonrecurrent prostate cancer samples. A false discovery rate of 4% resulted in 28 gene transcripts differentially expressed between the two groups. Each tumor sample is represented in a column and individual transcripts are displayed in rows. Red indicates relative increased expression level of transcripts relative to the median level across the samples, whereas green represents relative decrease in expression levels, and the degree of color saturation corresponds to the degree of change. (b) Kaplan–Meier survival analysis of *NuSAP* gene expression performed in our dataset. Tumor samples were divided into two groups based on whether the *NuSAP* gene expression value was above or below the median value. (c) Increased expression of *NuSAP* is prognostic in that dataset from Glinisky GV *et al.*<sup>34</sup> and (d) Taylor BS *et al.*<sup>35</sup> prostate datasets. *P*-values calculated using the log-rank test.

biochemical recurrence following radical prostatectomy, we performed a statistical analysis of microarray (SAM) survival analysis using all 83 of the tumor samples that had associated clinical follow-up. SAM survival analysis uses Cox modeling to identify genes whose expression levels are significantly associated with time to biochemical recurrence. Because enrichment for highly variable genes improves the performance of SAM, we selected 1600 genes that varied by at least five-fold across the entire dataset and used these to perform SAM survival analysis. From this, 28 transcripts were identified that were significantly associated with recurrence after radical prostatectomy at FDR of 4% (Figure 1 and Supplementary Table S2). Among the 28 genes, there were several (*KIAA0101*, *TOP2*, *EZH2*, *IGFBP3*, *RAC2*, *RCS1*, *CYP2D6*, *MCM6*, *Versican*, *HGF* and *ETV5*) that have been reported to be prognostic in a variety of malignancies including prostate cancer.<sup>13–32</sup> More than half of the transcripts have not been previously implicated as prognostic and included *NuSAP*, *TPX2*, *RACGAP1*, *CP*, *SIPA1L2*, *CF1*, *LOC391426*, *EST\_AA496936*, *THBS2*, *SCUBE2*, *CERK*, *CRABP2*, *ENO1*, *AK2*, *CYP2D6*, *LOC285296*, *BCR/TCR\_IGKV1/OR-2* and *APOC1*.

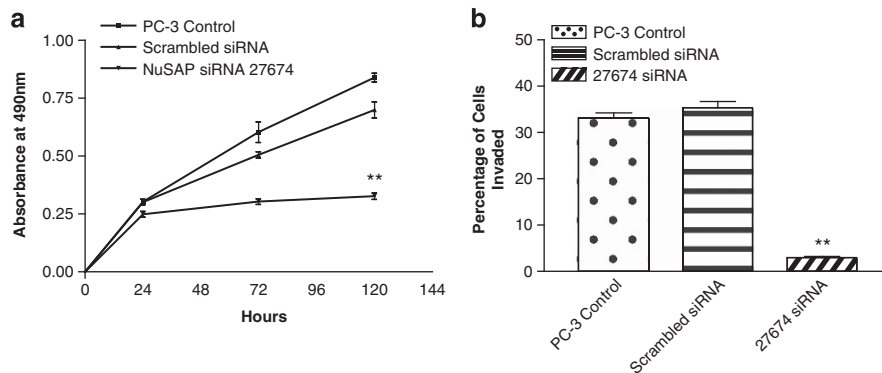
Interestingly, *TPX2*, *RACGAP1* and *NuSAP* are important members of the microtubule and mitotic spindle regulation pathway and were found upregulated in recurrent samples compared with nonrecurrent samples. Both *NuSAP* and *TPX2* are indispensable proteins required for microtubule stabilization and cross-linking in response to local generation of RanGTP and its regulator *RACGAP1*. *NuSAP* has been associated with poor prognosis in human melanoma<sup>33</sup> but has never been linked to outcomes in other solid tumors. Expression levels of *NuSAP* in the prostate samples were validated by qPCR on 20 (4 adjacent normal, 8 nonrecurrent and 8 recurrent) prostate samples and excellent concordance was found with the microarray data (Supplementary Figure S1).

We hypothesized that *NuSAP* might have an important role in prostate cancer progression and aggressiveness. Not surprisingly, when cancers in our dataset were segregated into groups with *NuSAP*-expression levels above and below the median value, tumors with higher *NuSAP* levels had a significantly increased risk

of biochemical recurrence after surgery ( $P < 0.01$  by log-rank test; Figure 1b). To validate this observation, we investigated the relationship between *NuSAP*-expression levels and outcomes in two independent prostate cancer microarray datasets. Both of these datasets have used a large cohort of patients with well-characterized prostate tumor samples and associated clinical follow-up.<sup>34,35</sup> Increased *NuSAP* expression levels were significantly associated with recurrence after radical prostatectomy ( $P < 0.01$  and  $P = 0.05$ , log rank test; Figures 1c and d).

*NuSAP* is expressed at relatively high levels in the prostate cancer cell lines LNCaP and PC3. To evaluate the possible effects of *NuSAP* overexpression in prostate cancer we knocked down expression levels of *NuSAP* in the prostate cancer cell line PC3. Cells were transiently transfected with either the *NuSAP* siRNA; Silencer Select 27674 (Invitrogen–Life Technologies, Grand Island, NY, USA) or the scrambled siRNA control, and *NuSAP* transcript levels were evaluated by qPCR (Figure 2b and Supplementary Figure S2). Knockdown of *NuSAP* transcript levels significantly decreased proliferation of PC3 cells *in vitro* compared with control cells (Figure 2a). 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 2b). 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.

To understand the underlying mechanisms of *NuSAP* overexpression in aggressive prostate cancers, we investigated the promoter sequences of the *NuSAP* gene. Previously, *NFYA* and *MYC* have been implicated as transcriptional regulators of *NuSAP*.<sup>36,37</sup> Using MATCH Software-Biobase Biological Databases (Beverly, MA, USA) (TRANSFAC), we investigated whether there might be other potential transcription factor-binding sites in the 5'-upstream region of the *NuSAP* gene. As expected, two putative *NFYA*-binding sites (–139/–144 and –310/–315) were identified, although no *MYC*-binding sites were observed. Interestingly, one



**Figure 2.** *NuSAP* knockdown reduces proliferation and invasion in PC3 cells. (a) Cells transfected with *NuSAP* siRNA; Silencer Select 27674 show significantly decreased cell viability/proliferation, measured by the MTS assay. (b) Cells transfected with *NuSAP* siRNA; Silencer Select 27674 display significantly decreased cell invasion through Matrigel. \*\* $P < 0.001$  compared with scrambled vector.

E2F-binding site (−246/−252) also was identified (Figure 3a). We cloned 431 bp of the human *NuSAP* promoter region and created five deletion mutants containing the putative *NFYA* and E2F regulatory elements. The cloned *NuSAP* promoter constructs were ligated into pGL4.11–luciferase vector and the promoter-reporter constructs were verified by direct sequencing. LNCaP and PC3 prostate cancer cell lines were transiently transfected with 431 bp promoter region and the five deletion constructs, and luciferase activity was assayed. In both PC3 and LNCaP cell lines, constructs containing −123 to −431 of the *NuSAP* promoter showed comparable luciferase activity, which essentially decreased to background levels in the constructs lacking those sequences, suggesting this region harbors critical regulatory elements (Figures 3b and c).

To further investigate the regulatory elements within the −431 to −123 region, we co-transfected the 431-bp *NuSAP* promoter luciferase reporter in tandem with cMyc or *NFYA* cDNAs into LNCaP and PC3 cell lines. As anticipated, *MYC* and *NFYA* resulted in six- and four-fold, respectively, higher luciferase activity above baseline activity (Figures 3d and e).

Increased expression of the *E2F1* transcription factor has previously been suggested to be prognostic for prostate cancer<sup>38,39</sup> but has not been implicated as a regulator of *NuSAP* gene expression. When *E2F1* and the *NuSAP* promoter-reporter construct were co-transfected into LNCaP and PC3 cell lines, luciferase activity increased three-fold above baseline (Figures 4a and b). Targeted mutation of the E2F-binding sequence (5′-TTTGGCGC-3′ to 5′-TTTGATAC-3′) ablated *E2F1*-enhanced expression.

To demonstrate whether *E2F1* directly interacts with *NuSAP* gene promoter sequences, we prepared nuclear extracts from *E2F1*-transfected LNCaP and PC3 cell lines and ran a mobility shift assay using a 24-bp biotin-labeled oligonucleotide probe corresponding to the −237 to −261 of *NuSAP* promoter region containing the E2F-binding site (Figure 5). Nuclear extracts from both LNCaP and PC-3 bound the *NuSAP* E2F promoter sequences, suggesting a direct interaction between *E2F1* and the *NuSAP* promoter. We were able to compete away binding with unlabeled probe and eliminate binding using an anti-*E2F1* antibody (BD-Pharmingen, San Diego, CA, USA).

To further test whether *E2F1* and *NuSAP* show coordinated expression *in vivo*, we performed immunohistochemical staining with *NuSAP* and *E2F1* antibodies of a tissue microarray containing 121 primary prostate cancers (Figures 6a and b). Each core was evaluated for nuclear overexpression of *E2F1* and *NuSAP*. An individual core was regarded as ‘positive’ if the neoplastic cells showed unambiguous (2+ or greater) nuclear staining intensity on a 0–3 scale. The number of neoplastic cells showing positive nuclear reactivity was also counted in each core for each antibody. In all, 35 cancer samples showed focal nuclear immunoreactivity for both proteins, 20 cancers were positive for *E2F1* alone, 10 were

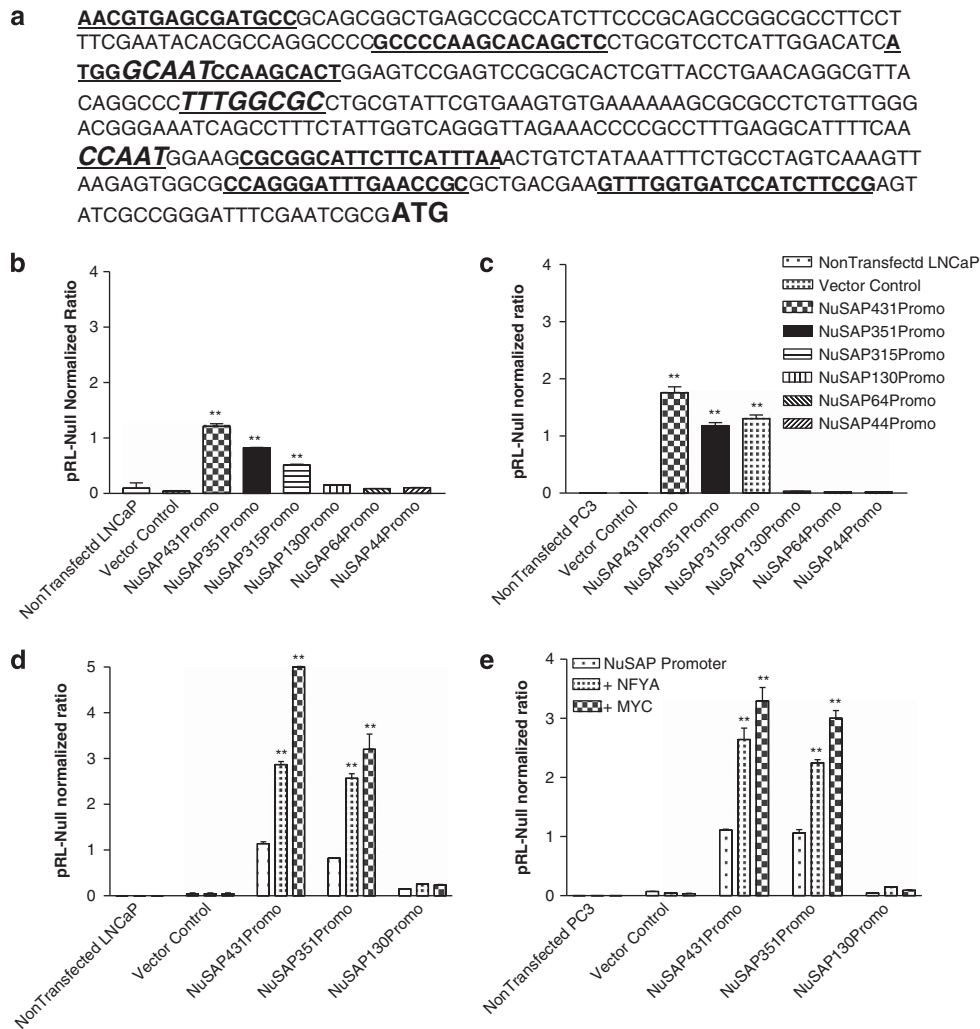
positive for *NuSAP* alone and 47 were negative for both proteins ( $\chi^2 = 20.8$ ,  $P = 0.000$ ) (Figure 6c). Because Gleason grade is a powerful predictor of prostate cancer aggressiveness, we also looked at the association between Gleason grading of the TMA core and the staining profile of both antibodies. Tumors with higher Gleason grades showed a significant increase in numbers of cells positive for *E2F1* ( $P < 0.000$ ). Interestingly, the number of cells positive for *NuSAP* staining did not correlate with Gleason grade ( $P = 0.8$ ) (Figure 6d).

## DISCUSSION

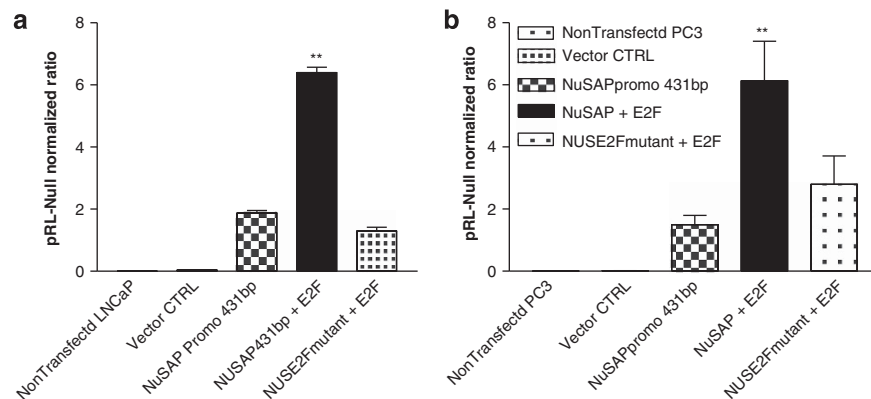
Through gene-expression profiling, we identify *NuSAP* and a candidate biomarker for recurrence after radical prostatectomy. Overexpression of *NuSAP* is associated with recurrence of prostate cancer and this finding was validated in two independent datasets. It is particularly notable that out of the 28 transcripts associated with biochemical recurrence after surgery, 3 of them (*NuSAP*, *TPX2* and *RACGAP1*) are members of the microtubule-associated protein family that regulate mitotic spindle organization. *NuSAP* appears to be critical for mitotic spindle assembly and for binding of DNA to the microtubules. Our data suggest that *NuSAP* has an important functional role in some aggressive prostate cancers.

One obvious explanation for the role of *NuSAP* in prostate cancer is that it is a marker for proliferation. Increased expression of proliferation biomarkers has been shown to be prognostic in many cancer types.<sup>40–42</sup> A recent analysis of gene sets shown to be prognostic in breast cancer suggests that they all reflect increased proliferation in aggressive cancers.<sup>40</sup> Several studies have demonstrated that biomarkers of increased proliferation connote poor prognosis in prostate cancer and that measurement of several markers of prognosis simultaneously improves outcome prediction.<sup>39</sup> Therefore, *NuSAP* represents a promising candidate biomarker to add to an immunohistochemical panel of prognostic biomarkers.

Because *NuSAP* is correlated with more aggressive prostate cancers, we sought to understand the mechanisms by which it becomes overexpressed in some prostate cancers. *NuSAP* is located on chromosome 15q15.1, a region not implicated in copy number alterations or other structural alterations in human prostate cancers.<sup>5,43</sup> Whole-genome sequencing of several prostate cancers has not identified mutations within or near the *NuSAP* gene.<sup>44</sup> We therefore investigated the 5′-regulatory elements of the *NuSAP* gene. Copy number gains of chromosome 8q in the region of the *Myc* gene are relatively common in prostate cancer. We confirmed that overexpression of *Myc* in prostate cells *in vitro* is associated with increased expression of a *NuSAP* promoter construct, despite the absence of canonical *Myc*-binding



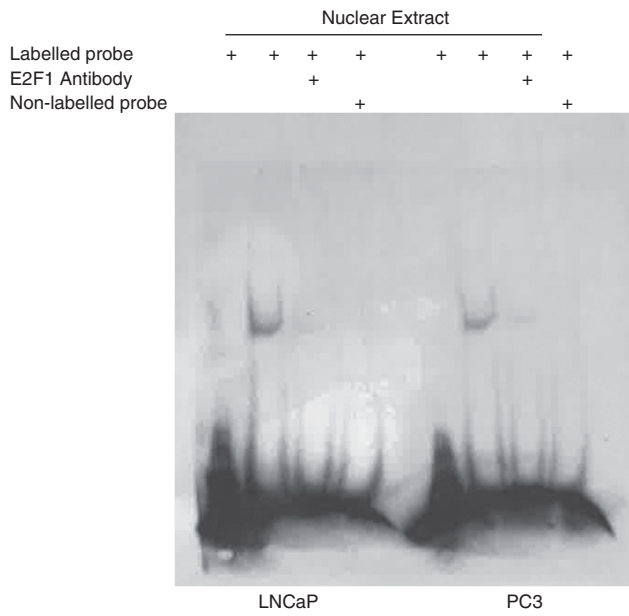
**Figure 3.** Promoter analysis of the human *NuSAP* gene. **(a)** The 431 bp human *NuSAP* promoter sequence. Bold underlines represent primer sequences for amplifying *NuSAP* promoter deletion constructs. Bold italics represent transcription factor-binding sequences for *NFYA* (GCAAT and CCAAT) and *E2F* (TTTGGGCGC). **(b)** Baseline expression levels of the 431-bp human *NuSAP* gene promoter and five deletion constructs ligated to pGL4.11-luciferase in LNCaP cells. The -123 to -431-bp region of the *NuSAP* promoter induces expression of *NuSAP* in LNCaP cells. **(c)** Baseline expression for the promoter constructs in PC3 cells shows identical regulation. **(d)** Co-transfection of LNCaP cells with *NFYA* and *MYC* increases *NuSAP* expression above baseline in LNCaP cells in the -123 to -431-bp region. **(e)** Similar regulation by *MYC* and *NFYA* in PC3 cells. Data represent relative luciferase activity (firefly luciferase activity versus Renilla luciferase activity) that was calculated in each cell line and are the mean values from three separate experiments.  $**P < 0.001$  compared with vector controls.



**Figure 4.** *E2F1* enhances *NuSAP* expression. Co-transfection of 431 bp *NuSAP* promoter and *E2F1* results in significantly increased *NuSAP* reporter expression in **(a)** LNCaP and **(b)** PC3 cell lines. Mutation of *E2F*-binding sequence in *NuSAP* promoter negates the effects of *E2F1*. Data represent relative luciferase activity (firefly luciferase activity versus Renilla luciferase activity) that was calculated in each cell line. Data are the mean values from three separate experiments.  $**P < 0.001$  compared with cells not transfected with *E2F1*.



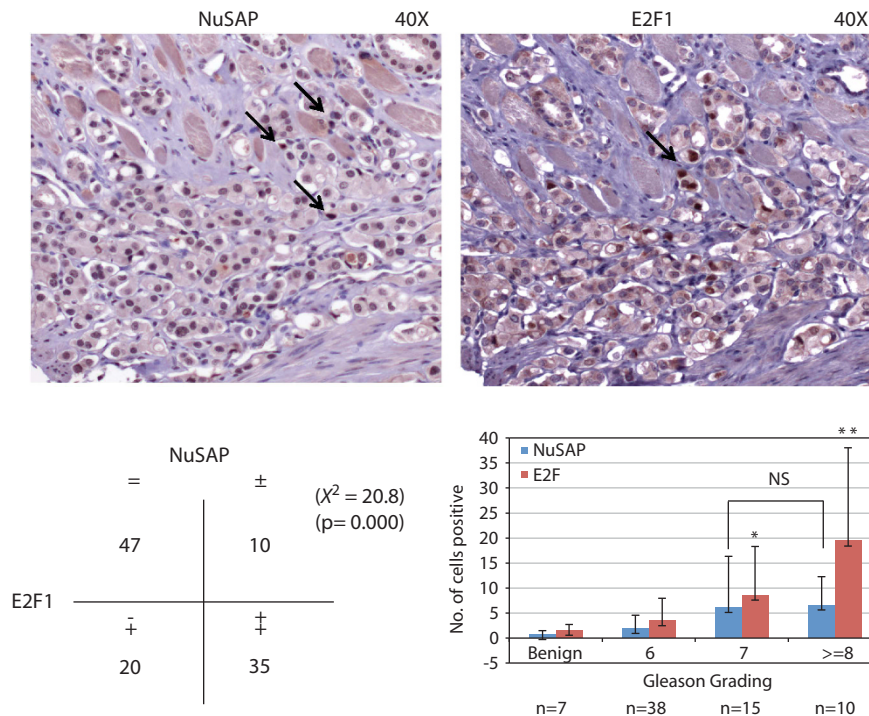
sequences in this region. Although *NFYA* also increased expression of the *NuSAP* promoter-reporter construct, *NFYA* is an unlikely regulator of *NuSAP* in prostate cancer because it is expressed



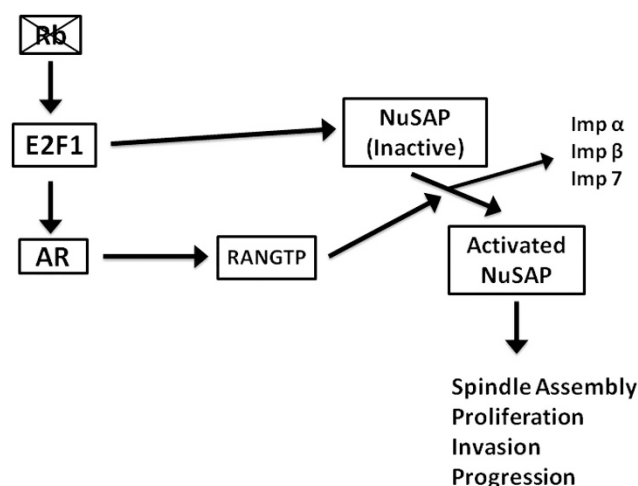
**Figure 5.** *E2F1* directly binds to *NuSAP* promoter. Nuclear extracts from both LNCaP and PC3 cell lines were incubated with a biotin-labeled oligonucleotide probe corresponding to the E2F-binding site in the *NuSAP* promoter at  $-246/-254$  bp (lanes 2 and 6). Binding could be competed away by coinubation with an anti-*E2F1* antibody (lanes 3 and 7) and with 100-fold molar excess of unlabeled probe (lanes 4 and 8).

constitutively and *NuSAP* and *NFYA* transcript levels were not correlated in our dataset.<sup>36</sup> The *NuSAP* promoter does harbor an E2F-binding site and we were able to document that *E2F1* overexpression results in increased expression of a *NuSAP* promoter-reporter construct. In addition, deletion of this binding site abrogates expression, and *E2F1* physically interacts with the binding sequence in the *NuSAP* gene promoter. Because *E2F1* has been correlated with aggressive prostate cancer, it is a promising regulator of increased *NuSAP* expression in aggressive prostate cancers.

Identification of *E2F1* as a regulator of *NuSAP* has potential implications in understanding prostate cancer progression. E2F controls cell division by regulating the transcription of genes that are essential for DNA synthesis and cell cycle progression. Aberrant expression of *E2F1* has been documented in large number of malignancies including prostate cancer. Overexpression of *E2F1* has been found in invasive ductal breast carcinomas and non-small-cell lung carcinomas, where high levels of *E2F1* were associated with advanced disease and poor prognosis.<sup>38</sup> Increased expression of E2F transcript levels occurs as part of a proliferation cluster in a prostate cancer gene-expression dataset that we have reported previously. Expression of *E2F1* protein, along with proliferation markers Ki67 and TOP2A, is associated with an increased risk of recurrence after surgery in men with prostate cancer, independent of clinical stage, pretreatment serum PSA levels and tumor Gleason grade.<sup>39</sup> The retinoblastoma protein negatively regulates E2F and deletions of the Rb gene are relatively common events in prostate cancer. Recently, Sharma *et al.*<sup>45</sup> have suggested that the progression to castration-resistant prostate cancer is dependent on disruption of Rb that produces increased expression of the androgen receptor through the direct action of *E2F1*. Therefore, like androgen receptor, *NuSAP* could represent another important effector protein in the Rb-E2F regulatory pathway in prostate cancer. In addition, the activation and release of *NuSAP* from Imp  $\alpha$ , Imp  $\beta$  and Imp  $\gamma$  is mediated by



**Figure 6.** Immunostaining of prostate TMA with *NuSAP* and *E2F1*. (a) Nuclear staining of *NuSAP* observed in isolated prostate cancer nuclei (arrows). (b) Nuclear staining of *E2F1* in an adjacent section of prostate cancer. (c) Correlation between *NuSAP* and *E2F1* staining in 121 prostate cancer specimens on a tissue microarray. (d) Number of nuclei with positive staining per 1 mm core of prostate cancer tissue on the tissue microarray separated by Gleason grade of the core. \* $P < 0.05$ , \*\* $P < 0.001$  compared with benign tissue.



**Figure 7.** Schematic representation of possible role of *NuSAP* in the Rb-E2F signaling pathway including possible interactions with androgen receptor signaling.

*RANGTP*, an androgen receptor-regulated gene.<sup>46</sup> Therefore, *NuSAP* overexpression could cooperate to androgen receptor signaling in prostate cancer progression (Figure 7).

The functional consequences of *NuSAP* overexpression are somewhat unclear. *NuSAP* is indispensable to cell division and is selectively expressed in the proliferating cells. Its expression peaks during G2-mitosis phase and declines rapidly following cell division. *NuSAP* expression is highly correlated with cell proliferation during embryogenesis and adult life, and *NuSAP* deficiency in mice leads to early embryonic lethality.<sup>47</sup> In agreement with this finding, we found that knockdown of *NuSAP* in LNCaP and PC3 prostate cancer cell lines essentially stopped cell growth and significantly inhibited invasion of matrigel. *NuSAP* overexpression appears to be an end product of a regulatory pathway important in prostate cancer and might represent a critical effector protein in this pathway in its effects on the microtubules. Selective inhibition of *NuSAP*-mitotic spindle complex results in mitotic arrest, abnormal chromatin condensation, apoptosis and cell death. Based on its critical role in cell division, *NuSAP* could represent an important candidate target protein for therapy. For example, in acute myelogenous leukemia, some patients who undergo ablative chemotherapy and stem cell transplant subsequently develop autoantibodies against *NuSAP* and these antibodies are produced by the stem cell graft. Patients with autoantibodies against *NuSAP* show improved cancer remission rates and it is hypothesized that *NuSAP* is the direct target of a graft-versus-leukemia response.<sup>44</sup> Therefore, *NuSAP*, as an important effector protein in proliferation, could represent a novel therapeutic target in prostate cancer, melanoma and other malignancies.

## MATERIALS AND METHODS

### Sample collection

All prostate samples used for this study were collected at the Stanford University Medical Center between 1999 and 2007 with patient's informed consent under an Institutional Review Board (IRB)-approved protocol. Multiple tissue samples were harvested from each prostate, flash-frozen and stored at  $-80^{\circ}\text{C}$ . Frozen sections of each prostate sample were performed and evaluated by a genitourinary pathologist (JKM). The tumor and nontumor areas were marked and contaminating tissues were trimmed away from the block as described previously.<sup>48</sup> Tumor samples in which at least 90% of the epithelial cells were cancerous were selected for extraction of DNA and RNA. In total, we selected 98 tumors from 86 patients that met these criteria. Associated clinical data were collected and

included preoperative PSA levels, clinical stage, pathological stage, tumor Gleason grade and clinical follow-up. Recurrence was defined as a measurable serum PSA ( $>0.1$  ng/ml on two consecutive measurements) after surgery.

### DNA/RNA extraction

The tumor and normal prostate tissue samples ( $\leq 100$  mg) were homogenized for 1 min using P-2100 Polytron homogenizer (Polytron Homogenizer Capitol Scientific, Austin, TX, USA). The homogenates were centrifuged at 9000 r.p.m for 3 min and the supernatants were passed through a 21 g-needle four to five times before proceeding for DNA/RNA extraction. DNA, RNA and microRNA were isolated from each tissue sample using Qiagen AllPrep DNA/RNA mini kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. RNA quality was assessed by the integrity of rRNA bands following gel electrophoresis.

### Gene-expression profiling

Gene-expression profiling was performed as reported.<sup>48</sup> Briefly, Cy5-labeled cDNA was prepared by using  $50\mu\text{g}$  of total RNA from prostate samples and Cy3-labeled cDNA was prepared by using common reference mRNA (Stratagene-Agilent Technologies, Inc, Santa Clara, CA, USA), pooled from 11 established human cell lines. For each experimental sample, Cy5- and Cy3-labeled samples were cohybridized to HEEBO spotted microarrays.<sup>49</sup> HEEBO microarrays were manufactured in the Stanford Functional Genomics Facility at Stanford University and contained 44 544 70-mer probes. After hybridization, microarrays were imaged using an Axon GenePix 4000 scanner (Axon Instruments-Molecular Devices, LLC, Sunnyvale, CA, USA). Fluorescence ratios for array elements were extracted using GENEPiX software and uploaded into the Stanford Microarray Database for subsequent analysis.<sup>50</sup> Fluorescence ratios were normalized by mean centering genes for each array. Ratios were then mean-centered for each gene across all arrays within each of the four different array print runs used, to minimize potential print run-specific bias. Gene expression data have been deposited in GEO (ID no. pending).

### Plasmid construction and mutagenesis

The human *NuSAP* promoter along with promoter deletion constructs were amplified from normal human genomic DNA and ligated to *Kpn1/HindIII* restriction site of the pGL4.11 luciferase reporter plasmid. Following primers were used to amplify the *NuSAP* promoter and its deletion constructs: (positions  $-1$  to  $-431$ ) Forward  $5'$ -GGTACCAACGTGAGC GATGCC- $3'$ , Reverse  $5'$ -AAGCTTCGCGATTGAAATCCC- $3'$ , (positions  $-1$  to  $-352$ ) Forward  $5'$ -GGTACCGCCCCAAGCACAGCTC- $3'$ , (positions  $-1$  to  $-316$ ) Forward  $5'$ -GGTACCATGGGCAATCCAAGCACT- $3'$ , (positions  $-1$  to  $-130$ ) Forward  $5'$ -GGTACCGCGGATTTGATTTAA- $3'$ , (positions  $-1$  to  $-71$ ) Forward  $5'$ -GGTACCGCGGATTTGATTTAA- $3'$ , (positions  $-1$  to  $-45$ ) Forward  $5'$ -GGTACCGTTGGTGATCCATCTTCCG- $3'$ . The E2F-binding site within the *NuSAP* promoter was mutated using the QuikChange multi site-directed mutagenesis kit (Stratagene) based on the manufacturer's recommendations. The primers used for mutagenesis were Forward  $5'$ -AGGCGTTACAGGCCCTTTGATACCTGCGTATTGCGTGAAGTG- $3'$  and Reverse  $3'$ -TCCGCAATGTCCGGGAACTATGGACGCATAAGCACTTAC- $5'$ . Human *E2F1*, *c-MYC* and *NFYA/NFDN* (dominant negative) were kind gifts from Drs Julien Sage, Dean Felsner (Stanford University) and Roberto Mantovani (University of Milan).

### Cell culture and transfection

LNCaP and PC-3 prostate cancer cell lines were grown in T-Medium and DMEM, respectively, supplemented with 10% fetal bovine serum. A total of  $2 \times 10^5$  cells were transfected with  $1.8\mu\text{g}$  of firefly reporter plasmid (pGL4.11-Luc) containing the *NuSAP* gene promoter constructs and 200 ng of Renilla luciferase reporter plasmid (pRL-Null) using lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's recommendations. After 48 h, cells were harvested and both firefly and Renilla luciferase activities in the cell extracts were determined by Dual Luciferase Assay kit (Promega, Madison, WI, USA). Co-Transfections of cells with either of *E2F1*,

NF-YA, NFDN, c-MYC and NuSAP promoter plasmids were done using the same protocol stated above keeping the ratio of Firefly to Renilla constant (1:0.1).

### Cell proliferation and invasion assays

Cell proliferation was quantified using the MTS assay (Promega), a colorimetric assay based on detection of MTS tetrazolium compound (Owen's reagent) by metabolically active cells. Cell invasion was measured by a Boyden chamber assay (BD Biosciences, Franklin Lakes, NJ, USA). In all, 5000 PC3 cells per 24-well insert were seeded onto precoated filters (8 µm pore size, Matrigel 100 µg/cm<sup>2</sup>), using a 0.5–10% fetal bovine serum gradient. After 24 h, cells traversing the filter were fixed with 10% buffered formalin, stained with crystal violet and manually counted. All the above assays were done in triplicate and all experiments were replicated at least once.

### Nuclear extract and electrophoretic mobility shift assay

Nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Nuclear extracts (10 µg) from E2F1-transfected LNCaP and PC3 cell lines were incubated for 15 min at room temperature with 20 nM of a biotin-labeled oligonucleotide probe containing a putative E2F-binding sequence from the NuSAP promoter in a 20 µl binding reaction containing 5 × binding buffer (final concentration 20 mM HEPES pH 8.0, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM MgCl<sub>2</sub> and 5% glycerol) and 1 mg/ml Poly (dl-dC). The oligonucleotide sequences used were Forward 5'-CAGGCCCTTTGGCGCCTGCGTATT-3' and Reverse 5'-GTCCGGGAAACCGCGGACGCATAA-3'. In order to demonstrate the specificity of the reaction, the extracts were also incubated with either of E2F1 antibody (1 µg) (sc-22820, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or (2 µM) non-labeled probe for 15 min at room temperature prior to adding the labeled oligonucleotide. The probe-bound nuclear extracts were separated from the free probe in a 6% DNA retardation gel (Invitrogen) and the biotin-labeled probe was detected using the Phototope Star kit (New England Biolabs, Ipswich, MA, USA).

### Immunohistochemistry

A standard two-layer streptavidin-biotin method was used to stain a prostate tissue microarray containing 121 cases. For NuSAP protein detection, sections were heated to 95 °C after adding Tris-EDTA solution (10 mM Tris-HCl, 1 mM EDTA, pH 9). E2F1 antigen retrieval was carried out using 1 mM EDTA pH 9.0 and the samples were microwaved for 20 min. Endogenous peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol. Nonspecific binding was blocked with protein block serum-free reagent (Dako, Glostrup, Denmark). Sections were incubated overnight with rabbit anti-human NuSAP polyclonal antibody (Proteintech Group) (1:400) or mouse anti-E2F1 monoclonal antibody (1:50; BD-Pharmingen, San Diego, CA, USA). Antibody binding was visualized with the Dako Envision System (Dako). The stained sections were reviewed and scored by a genitourinary pathologist (JKM). Only luminal epithelial cells displaying nuclear expression of NuSAP or E2F1 were scored as positive.

### Data analysis

For analysis of gene expression data, we included only well-measured genes whose expression varied, as determined<sup>1</sup> by signal intensity over background > 1.5-fold in both test and reference channels in at least 75% of samples,<sup>2</sup> and 2-fold ratio variation from the mean in at least two samples. Transcripts associated with recurrence after surgery were identified using the SAM survival method.<sup>51</sup> Hierarchical clustering was performed and displayed using Cluster and TreeView software (Eisen Lab). Kaplan-Meier analysis and the log-rank test were performed using Prism Software version 2.01 (GraphPad Prism Software, Inc., La Jolla, CA, USA) to test the association between NuSAP expression levels and clinical outcome. The association between NuSAP and E2F1 protein expression levels was carried out using Chi-square analysis. Comparison of the expression levels in the transfection assays was done by two-tailed Student's t-test using SPSS (IBM SPSS, Armonk, NY, USA).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**Author Contributions:** ZGG, JDB and JKM agree with the manuscript's results and conclusions, analyzed the data, prepared tissue samples and contributed to the writing of the paper. ZGG and JDB designed the experiments/study. ZGG and JKM collected the data/did experiments for the study. ZGG wrote the first draft of the paper.

### REFERENCES

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
- Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V et al. Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med* 2009; **360**: 1320–1328.
- Roobol MJ, Kerkhof M, Schroder FH, Cuzick J, Sasieni P, Hakama M et al. Prostate cancer mortality reduction by prostate-specific antigen-based screening adjusted for nonattendance and contamination in the European Randomised Study of Screening for Prostate Cancer (ERSPC). *Eur Urol* 2009; **56**: 584–591.
- Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K et al. Delineation of prognostic biomarkers in prostate cancer. *Nature* 2001; **412**: 822–826.
- Lapointe J, Li C, Giacomini CP, Salari K, Huang S, Wang P et al. Genomic profiling reveals alternative genetic pathways of prostate tumorigenesis. *Cancer Res* 2007; **67**: 8504–8510.
- LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V et al. Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. *Cancer Res* 2002; **62**: 4499–4506.
- Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML et al. Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. *Cancer Res* 2001; **61**: 4683–4688.
- Luo JH, Yu YP, Cieply K, Lin F, DeFlavia P, Dhir R et al. Gene expression analysis of prostate cancers. *Mol Carcinog* 2002; **33**: 25–35.
- Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C et al. Gene expression correlates of clinical prostate cancer behavior. *Cancer Cell* 2002; **1**: 203–209.
- Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 2002; **419**: 624–629.
- Welsh JB, Sapinoso LM, Su AI, Kern SG, Wang-Rodriguez J, Moskaluk CA et al. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. *Cancer Res* 2001; **61**: 5974–5978.
- Freedland SJ, Humphreys EB, Mangold LA, Eisenberger M, Dorey FJ, Walsh PC et al. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. *JAMA* 2005; **294**: 433–439.
- Abraham JE, Maranian MJ, Driver KE, Platte R, Kalmayraev B, Baynes C et al. CYP2D6 gene variants: association with breast cancer specific survival in a cohort of breast cancer patients from the United Kingdom treated with adjuvant tamoxifen. *Breast Cancer Res* 2010; **12**: R64.
- Amemiya H, Menolascino F, Pena A. Role of the expression of c-Met receptor in the progression of gastric cancer. *Invest Clin* 2010; **51**: 369–380.
- Aune G, Lian AM, Tingstad S, Torp SH, Forsmo S, Reseland JE et al. Increased circulating hepatocyte growth factor (HGF): a marker of epithelial ovarian cancer and an indicator of poor prognosis. *Gynecol Oncol* 2011; **121**: 402–406.
- Bryant RJ, Cross NA, Eaton CL, Hamdy FC, Cunliffe VT. EZH2 promotes proliferation and invasiveness of prostate cancer cells. *Prostate* 2007; **67**: 547–556.
- Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M et al. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. *J Natl Cancer Inst* 2002; **94**: 1099–1106.
- Chotteau-Lelievre A, Revillion F, Lhotellier V, Hornez L, Desbiens X, Cabaret V et al. Prognostic value of ERM gene expression in human primary breast cancers. *Clin Cancer Res* 2004; **10**: 7297–7303.
- Engers R, Ziegler S, Mueller M, Walter A, Willers R, Gabbert HE. Prognostic relevance of increased Rac GTPase expression in prostate carcinomas. *Endocr Relat Cancer* 2007; **14**: 245–256.
- Gorter A, Zijlmans HJ, van Gent H, Trimbos JB, Fleuren GJ, Jordanova ES. Versican expression is associated with tumor-infiltrating CD8-positive T cells and infiltration depth in cervical cancer. *Mod Pathol* 2010; **23**: 1605–1615.



- 21 Helfenstein A, Frahm SO, Krams M, Drescher W, Parwaresch R, Hassenpflug J. Minichromosome maintenance protein (MCM6) in low-grade chondrosarcoma: distinction from enchondroma and identification of progressive tumors. *Am J Clin Pathol* 2004; **122**: 912–918.
- 22 Kosari F, Munz JM, Savci-Heijink CD, Spiro C, Klee EW, Kube DM *et al*. Identification of prognostic biomarkers for prostate cancer. *Clin Cancer Res* 2008; **14**: 1734–1743.
- 23 Lammers LA, Mathijssen RH, van Gelder T, Bijl MJ, de Graan AJ, Seynaeve C *et al*. The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. *Br J Cancer* 2010; **103**: 765–771.
- 24 Miyata Y, Sakai H, Kanda S, Igawa T, Hayashi T, Kanetake H. Expression of insulin-like growth factor binding protein-3 before and after neoadjuvant hormonal therapy in human prostate cancer tissues: correlation with histopathologic effects and biochemical recurrence. *Urology* 2004; **63**: 1184–1190.
- 25 Monge M, Colas E, Doll A, Gil-Moreno A, Castelli J, Diaz B *et al*. Proteomic approach to ETV5 during endometrial carcinoma invasion reveals a link to oxidative stress. *Carcinogenesis* 2009; **30**: 1288–1297.
- 26 Murphy AJ, Hughes CA, Barrett C, Magee H, Loftus B, O'Leary JJ *et al*. Low-level TOP2A amplification in prostate cancer is associated with HER2 duplication, androgen resistance, and decreased survival. *Cancer Res* 2007; **67**: 2893–2898.
- 27 Mustjoki S, Hernesniemi S, Rauhala A, Kahkonen M, Almqvist A, Lundan T *et al*. A novel dasatinib-sensitive RCD1-ABL1 fusion transcript in chemotherapy-refractory adult pre-B lymphoblastic leukemia with t(1;9)(q24;q34). *Haematologica* 2009; **94**: 1469–1471.
- 28 Schrader C, Janssen D, Klapper W, Siebmann JU, Meusers P, Brittinger G *et al*. Minichromosome maintenance protein 6, a proliferation marker superior to Ki-67 and independent predictor of survival in patients with mantle cell lymphoma. *Br J Cancer* 2005; **93**: 939–945.
- 29 Setoguchi T, Kikuchi H, Yamamoto M, Baba M, Ohta M, Kamiya K *et al*. Microarray analysis identifies versican and CD9 as potent prognostic markers in gastric gastrointestinal stromal tumors. *Cancer Sci* 2011; **102**: 883–889.
- 30 Wong N, Yeo W, Wong WL, Wong NL, Chan KY, Mo FK *et al*. TOP2A overexpression in hepatocellular carcinoma correlates with early age onset, shorter patients survival and chemoresistance. *Int J Cancer* 2009; **124**: 644–652.
- 31 Yu J, Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G *et al*. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. *Cancer Res* 2007; **67**: 10657–10663.
- 32 Yuan RH, Jeng YM, Pan HW, Hu FC, Lai PL, Lee PH *et al*. Overexpression of KIAA0101 predicts high stage, early tumor recurrence, and poor prognosis of hepatocellular carcinoma. *Clin Cancer Res* 2007; **13** (Pt 1): 5368–5376.
- 33 Bogunovic D, O'Neill DW, Belitskaya-Levy I, Vacic V, Yu YL, Adams S *et al*. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci USA* 2009; **106**: 20429–20434.
- 34 Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. *J Clin Invest* 2004; **113**: 913–923.
- 35 Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS *et al*. Integrative genomic profiling of human prostate cancer. *Cancer Cell* 2010; **18**: 11–22.
- 36 Fujiwara T, Harigae H, Okitsu Y, Takahashi S, Yokoyama H, Yamada MF *et al*. Expression analyses and transcriptional regulation of mouse nucleolar spindle-associated protein gene in erythroid cells: essential role of NF-Y. *Br J Haematol* 2006; **135**: 583–590.
- 37 Hussain S, Benavente SB, Nascimento E, Dragoni I, Kurowski A, Gillich A *et al*. The nucleolar RNA methyltransferase Misu (NSun2) is required for mitotic spindle stability. *J Cell Biol* 2009; **186**: 27–40.
- 38 Davis JN, Wojno KJ, Daignault S, Hofer MD, Kuefer R, Rubin MA *et al*. Elevated E2F1 inhibits transcription of the androgen receptor in metastatic hormone-resistant prostate cancer. *Cancer Res* 2006; **66**: 11897–11906.
- 39 Malhotra S, Lapointe J, Salari K, Higgins JP, Ferrari M, Montgomery K *et al*. A tri-marker proliferation index predicts biochemical recurrence after surgery for prostate cancer. *PLoS One* 2011; **6**: e20293.
- 40 Adamo B, Anders CK. Stratifying triple-negative breast cancer: which definition(s) to use? *Breast Cancer Res* 2011; **13**: 105.
- 41 Rodriguez-Enriquez S, Pacheco-Velazquez SC, Gallardo-Perez JC, Marin-Hernandez A, Aguilar-Ponce JL, Ruiz-Garcia E *et al*. Multi-biomarker pattern for tumor identification and prognosis. *J Cell Biochem* 2011; **112**: 2703–2715.
- 42 von Euler H, Eriksson S. Comparative aspects of the proliferation marker thymidine kinase 1 in human and canine tumour diseases. *Vet Comp Oncol* 2011; **9**: 1–15.
- 43 Pflueger D, Terry S, Sboner A, Habegger L, Esgueva R, Lin PC *et al*. Discovery of non-ETS gene fusions in human prostate cancer using next-generation RNA sequencing. *Genome Res* 2011; **21**: 56–67.
- 44 Wadia PP, Coram M, Armstrong RJ, Mindrinos M, Butte AJ, Miklos DB. Antibodies specifically target AML antigen NuSAP1 after allogeneic bone marrow transplantation. *Blood* 2010; **115**: 2077–2087.
- 45 Sharma A, Yeow WS, Ertel A, Coleman I, Clegg N, Thangavel C *et al*. The retinoblastoma tumor suppressor controls androgen signaling and human prostate cancer progression. *J Clin Invest* 2010; **120**: 4478–4492.
- 46 Iyer J, Moghe S, Furukawa M, Tsai MY. What's Nu(SAP) in mitosis and cancer? *Cell Signal* 2011; **23**: 991–998.
- 47 Vanden Bosch A, Raemaekers T, Denayer S, Torreken S, Smets N, Moermans K *et al*. NuSAP is essential for chromatin-induced spindle formation during early embryogenesis. *J Cell Sci* 2010; **123** (Pt 19): 3244–3255.
- 48 Lapointe J, Li C, Higgins JP, van de RM, Bair E, Montgomery K *et al*. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci USA* 2004; **101**: 811–816.
- 49 Kim YH, Pollack JR. Comparative genomic hybridization on spotted oligonucleotide microarrays. *Methods Mol Biol* 2009; **556**: 21–32.
- 50 Sherlock G, Hernandez-Boussard T, Kasarskis A, Binkley G, Matese JC, Dwight SS *et al*. The Stanford Microarray Database. *Nucleic Acids Res* 2001; **29**: 152–155.
- 51 Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci USA* 2002; **99**: 6567–6572.

Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)