Increased Expression of SIM2-s Protein Is a Novel Marker of Aggressive Prostate Cancer

Ole Johan Halvorsen,1 Kari Rostad,4 Anne Margrete Øyan,2 Hanne Puntevoll,5 Trond Hellem Bø,6 Laila Stordrange,6 Sue Olsen,2 Svein Andreas Haukaas,3,7 Leroy Hood,9 Inge Jonassen,6,8 Karl-Henning Kalland,2,4 and Lars Andreas Akslen1,5

Abstract Purpose: The human SIM2 gene is located within the Down’s syndrome critical region of chromosome 21 and encodes transcription factors involved in brain development and neuronal differentiation. SIM2 has been assigned a possible role in the pathogenesis of solid tumors, and the SIM2-short isoform (SIM2-s) was recently proposed as a molecular target for cancer therapy. We previously reported SIM2 among the highly up-regulated genes in 29 prostate cancers, and the purpose of our present study was to examine the expression status of SIM2 at the transcriptional and protein level as related to outcome in prostate cancer. Experimental Design: By quantitative PCR, mRNA in situ hybridization, and immunohistochemistry, we evaluated the expression and significance of SIM2 isoforms in 39 patients with clinically localized prostate cancer and validated the expression of SIM2-s protein in an independent cohort of 103 radical prostatectomies from patients with long and complete follow-up. Results: The SIM2 isoforms (SIM2-s and SIM2-l) were significantly coexpressed and increased in prostate cancer. Tumor cell expression of SIM2-s protein was associated with adverse clinicopathologic factors like increased preoperative serum prostate-specific antigen, high histologic grade, invasive tumor growth with extra-prostatic extension, and increased tumor cell proliferation by Ki-67 expression. SIM2-s protein expression was significantly associated with reduced cancer-specific survival in multivariate analyses. Conclusions: These novel findings indicate for the first time that SIM2 expression might be important for clinical progress of human cancer and support the recent proposal of SIM2-s as a candidate for targeted therapy in prostate cancer.

The human SIM2 gene was identified within a region of chromosome 21 (21q22.2) referred to as the Down’s syndrome critical region (1) associated with trisomy 21 (2). Proteins encoded by the SIM2 gene belong to a family of transcriptional repressors (3, 4) and may control brain development and neuronal differentiation (5, 6). In animal studies, SIM2−/− knockout mice died following craniofacial abnormalities (7) and respiratory failure (8). Two differentially spliced isoforms of the SIM2 transcript, SIM2-long (SIM2-l) and SIM2-short (SIM2-s), have been reported (3), but details on their differential function in humans are not available. In mice, the ratio of SIM2-l to SIM2-s differs between normal tissues (9). Although SIM-s is missing one of two repressive domains present in SIM2-l, SIM2-s activates gene expression from a central midline element through ARNT, whereas a repressive effect on gene expression induced by hypoxia and dioxin response element was observed (9). In addition to a role in brain and neuronal development, SIM2 has recently been involved in the pathogenesis of solid tumors (10, 11), and overexpression of SIM2-s was associated with tumors of the colon, pancreas, and prostate (11, 12). Applying gene expression profiling, SIM2 ranked second among highly up-regulated genes in prostate cancer, as recently reported by our group (13). Contrary to a previous report (11), we now report a basal expression of SIM2 transcripts in benign prostate tissue, as well as coexpression and increase of both SIM2 isoforms in prostate cancer. Current evidence points to the SIM2-s isoform as a therapeutic target (11), and on this basis, we examined SIM2-s at the protein level by immunohistochemistry. A highly significant relationship between SIM2-s protein expression and adverse outcome was established in an independent series of
prostate cancer patients with long follow-up, and this is the first report describing SIM2 expression as a marker of disease progress in human cancer. Our findings are of potential practical importance because the SIM2-s isoform was recently proposed as a molecular target for antisense cancer therapy (11).

Materials and Methods

Patient series and tissues. During the period of 1997 to 2003, fresh prostate tissues were collected from consecutive patients treated by radical prostatectomy for clinically localized and biopsy verified prostate cancer at the Department of Surgery, Section of Urology, Haukeland University Hospital, Bergen, Norway. Fresh tissue samples were immediately frozen in liquid nitrogen and stored for later use at −80 °C. The percentage of tumor tissue was determined in the area from where fresh tissue was also collected. Samples were finally included in this series after histopathologic confirmation of benign or tumor tissue, evaluation of tumor content (>50%; mean, 76% in our cases), and availability of high-quality RNA. For cDNA microarray studies, 52 tissue samples from 33 patients (median age, 60 years; range, 47-69) were used, including 29 histologically verified primary tumors and 19 paired benign and malignant samples (13). The mean Gleason score for these 29 carcinomas was 6.2 (median, 6; range, 5-8); the median preoperative serum prostate-specific antigen (PSA) was 5.9 ng/ml; and 22 patients had clinical stage T1c, and 6 patients had T2 (missing information on serum prostate-specific antigen (PSA) was 5.9 ng/mL; and 22 patients used, including 29 histologically verified primary tumors and 19 paired samples from 33 patients (median age, 60 years; range, 47-69) were used. After histopathologic examination, 21 cases were classified as stage pT2, 8 cases were pT1, and 15 cases had positive surgical margins. For validation purposes (quantitative PCR), this series of 29 carcinomas was expanded to a total of 37 malignant tumors and 39 benign samples, including 27 tumor/benign pairs. Finally, validating SIM2-s protein expression, a consecutive series of 104 men (median age, 62.0 years) treated by radical prostatectomy for clinically localized prostate cancer during 1988 to 1994 with long and complete follow-up was included (14). One case that did not contain tumor tissue on the tissue microarrays when stained for SIM2-s was omitted. The following variables were recorded: patient age, clinical stage (tumor-node-metastasis category), largest tumor diameter, WHO histologic grade, capsular penetration, seminal vesicle invasion, involvement of surgical margins, pathologic stage (pathologic tumor-node-metastasis), presence of lymph node metastasis, and serum PSA before and after surgical treatment. Time from surgery until biochemical failure, defined as serum PSA elevation ≥0.5 ng/ml in two consecutive blood samples, was recorded, as was time to clinical locoregional recurrence, skeletal metastases, and cancer-specific survival. The last date of follow-up was December 31, 2001. Skeletal metastases were present in 15 patients, and nine patients died as a result of prostate cancer. No patients were lost to follow-up. None of the patients examined by cDNA microarray or quantitative PCR was part of this series (n = 103), which was a strictly independent cohort. The study was approved by The Data Inspectorate of Norway and The Regional Committee for Medical Research.

RNA purification, enzymatic modification, and fluorochrome labeling. Individual biopsies were ground to powder under liquid N2. Total RNA was extracted according to standard protocols (ref. 15; Invitrogen Trizol LS protocol and Qiagen RNeasy mini-kit protocol; Invitrogen, Carson City, CA). T7 RNA polymerase promoter-containing double-stranded cDNA and T7 RNA polymerase-amplified RNA (cRNA) were generated as previously described (16) and according to the Ambion T7 Megakit protocol. Aminomethyl-UT (aminomethyl-UTP from Ambion, Austin, TX) incorporation into cRNA followed by cross-coupling Cy5 and Cy3 by means of reactive Cy-NHS compounds (Amersham, Piscataway, NJ) was used for fluorochrome labeling of nucleic acids. Cy5 and Cy3 incorporations were measured by absorption readings at 649 and 550 nm, respectively, using a PowerWave Spectrophotometer. Simultaneous recording of absorptions at 260 nm/mM allowed calculation of specific labeling.

DNA microarray analysis. The Research Genetics human 40K cDNA microarray printed at the Institute for Systems Biology in Seattle has been described previously (13, 17). The Agilent human 1A oligonucleotide microarray (21K) was used for validation purposes according to the Agilent protocols except for a more stringent wash (0.1× SSC at 25 °C for 10 min). The oligonucleotide microarrays were scanned, and features were automatically extracted, recorded, and analyzed using the Agilent Microarray Scanner Bundle. Normalization, flooring, or filtration of data was done as described (13, 17).

Real-time quantitative PCR. Synthesis of hexamer-primed cDNA was done according to the MMLV reverse transcriptase kit instructions (Ambion). cDNA corresponding to 5 ng total RNA was used in each PCR reaction. Real-time quantitative PCR was done in a 96-well format in the ABI Prism 7900HT thermocycler according to User Bulletin #2 and SDS2.2 program manuals (Applied Biosystems, Foster City, CA). The SIM2 isoforms have the first nine exons and the first part of exon 10 in common, but the long isoform is spliced to an 11th exon on chromosome 21q22 (Supplementary Fig. S1). The primers used were SIM2-s, 5'-GCTGCTTTGGCCAGATTTGGA-3' (sense) and 5'-GCGAGAACAGGAGCCATTGGC-3' (antisense); SIM2-l, 5'-GAGCCTGCTTGGCCAACACTCA-3' (sense) and 5'-GTCGCTCTCGCCGAACCT-3' (antisense); and β-actin, 5'-CCGAGCACCACATGAGATCAGATC-3' (sense) and 5'-GCGAGCCAGACTGAGA-3' (antisense). Supplementary Table S1 shows the Genbank accession numbers and Taqman custom-made assays for the specific detection of the short and long isoforms of SIM2, and the β-actin Taqman assay was used as endogenous control. The Applied Biosystems assay Hs_00231927_m1 (Applied Biosystems) common to both SIM2 isoforms (across exons 3 and 4 boundary of chromosome 21q22) was also used.

Real-time PCR in the low-density array format. Taqman low-density arrays are customizable, 384-well microfluidic cards for real-time PCR (Applied Biosystems). Each low-density array card was configured for 96 different genes in duplicates, including Hs_00231925_m1. Hs_00231925_m1 targets the boundary of exons 3 and 4 of chromosome 21q22 (Supplementary Fig. S1). The primers used were SIM2-l, 5'-GGTGGGTGGCAGATGGA-3' (antisense); SIM2-s, 5'-GGTGGGTGGCAGATGGA-3' (antisense); and β-actin, 5'-ATC-GTGCACCGCCACTGAGC-3' (sense) and 5'-GCGAGCCAGACTGAGA-3' (antisense). Supplementary Table S1 shows the Genbank accession numbers and Taqman custom-made assays for the specific detection of the short and long isoforms of SIM2, and the β-actin Taqman assay was used as endogenous control. The Applied Biosystems assay Hs_00231927_m1 (Applied Biosystems) common to both SIM2 isoforms (across exons 3 and 4 boundary of chromosome 21q22) was also used.

RNA synthesis and in situ hybridization. SIM2 (accession no. NM005069) antisense (nucleotides 1395-1451) and sense (nucleotides 1395-1395) and β-actin (accession no. NM000101) antisense (nucleotides 1137-356) DIG-cRNA probes were made by a PCR-based approach. Initially, RNA was isolated from a prostate tumor sample with known elevated SIM2 mRNA expression, and single-stranded cDNA was transcribed using gene-specific primers (SIM2, 5’-TTCGAAT-GAAATGTGTCT-3’; β-actin, 5’-ATGTCACCGACACTGGGAC-3’) and Superscript III Reverse Transcriptase as described by the manufacturer (Invitrogen). Next, cDNA was PCR amplified using the following primers: SIM2, 5’-TTCGAATTGAAATGTGTCT-3’ and 5’-AGTGCCTCGTAA-TGTTAGG-3’; β-actin, 5’-ATGTCACCGACACTGGGAC-3’ and 5’-ATC- TCGGAGGACTGGGAC-3’. Ampli-Taq Gold PCR Master Mix (Applied Biosystems) was used with 0.15 μmol/l of each primer. Samples were subjected to an initial denaturation at 95 °C for 15 min and 50 cycles (95 °C for 30 s, 50 °C for 1 min, 72 °C for 2 min), with a final extension at 72 °C for 7 min. Additionally, one primer in the primer pair was designed with T7 promoter sequence (5’-TAATACGACTCACTATAGG-3’) in the 5’-end. The promoter sequence was hence incorporated into the PCR product. All PCR fragments were verified by sequencing. DIG-cRNA synthesis was done using 5 μg DNA and 1.9 mmol/l ATP, CTP, GTP, 1.3 mmol/l UTP, and 0.7 mmol/l DIG-UTP according to the MEGA script High Yield Transcription kit T7 manual instructions.
Tissue microarray and immunohistochemistry. After formalin fixation, radical prostatectomy specimens were totally embedded and studied by whole mount step sections. For immunohistochemistry, the area of highest tumor grade was selected for tissue microarray construction, using three parallel cores (0.6-mm diameter) from each case (14, 18). Tissue microarray slides were subjected to microwave epitope retrieval for 20 min in Tris-EDTA buffer at pH 9 and incubated for 60 min with an anti-SIM2-s antibody, SC-8715 (C15; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature, diluted 1:800 for 15 min, and 0.25% acetic anhydride in TEA-HCl for another 15 min. Fragmented DIG-cRNA probes were diluted to 100 ng/ml in hybridization solution (Sigma, St. Louis, MO) and incubated at 42°C overnight. Post-hybridization wash was done in 2× SSC at 52°C for 10 min, twice. Slides were RNase treated using 10 µg/ml RNase A at 37°C for 10 min. Re-fixation of the slides was done in 1:1 formamide/2× SSC at 42°C for 10 min followed by brief washing in 1× SSC and 0.5× SSC, respectively. The slides were blocked with 2% horse serum for 30 min at room temperature, before incubation with anti-DIG-AP (Roche, Basel, Switzerland) 1:250 for 1 h at room temperature. Staining was done by Liquid Permanent Red Chromogen (DakoCytomation, Copenhagen, Denmark) for 5 to 20 min. Hematoxylin was used as counter stain.

Statistical analysis. Associations between different variables were assessed by Pearson’s χ² test or the Mann-Whitney or Kruskal-Wallis tests, when appropriate (SPSS 11.5, SPSS, Inc., Chicago, IL). Correlations between continuous variables not categorized by quartiles or classifications were assessed by rank correlation (Spearman-rho = 0.79, 0.62 for SIM2-total, SIM2-s, and 0.86 and 0.72 for SIM2-l, and 0.89 and 0.69 for SIM2-total, respectively (Supplementary Table S4). There were no consistent associations between the expression of SIM2 isoforms and clinicopathologic features of prostate cancer in the subset of 37 cases, except a tendency for high expression of SIM2-s to be associated with increased preoperative serum PSA (P = 0.054, Mann-Whitney test).

Optimal cutoff values (based on highest sum of sensitivity and specificity) for classifying samples as malignant were determined by ROC analysis and found to be 2.0 for SIM2-s, 8.0 for SIM2-l, and 3.2 for SIM2-total. The sensitivity and specificity for classifying samples as malignant were 0.95 and 0.62 for SIM2-s, 0.86 and 0.72 for SIM2-l, and 0.89 and 0.69 for SIM2-total, respectively (Supplementary Fig. S2).

Real-time quantitative PCR of SIM2 transcripts. Both SIM2-s and SIM2-l isoforms were detected in benign (n = 39) and malignant (n = 37) prostate tissues at the mRNA level. SIM2-total, SIM2-s, and SIM2-l isoforms were all significantly elevated in malignant versus benign samples by 3.8-, 3.9-, and 6.7-fold, respectively (P < 0.001 for all, Mann-Whitney test; Fig. 1). The median (range) expression in benign versus tumor tissues was 2.2 (0.1-13.2) versus 8.2 (1.1-55.0), 1.3 (0.1-10.9) versus 5.2 (0.4-65.7), and 3.6 (0.1-80) versus 24.0 (1.36-1085.2) for SIM2-total, SIM2-s, and SIM2-l, respectively. The expression level of SIM2-s and SIM2-l isoforms correlated significantly in both benign and malignant prostate tissues, as did both isoforms with SIM2-total across all (i.e., tumor and benign) samples (P < 0.001 for all; Supplementary Table S3). SIM2 expression by cDNA microarray correlated to SIM2-s, SIM2-l, and SIM2-total mRNA by real-time quantitative PCR across all samples, reaching significance for the subset of benign tissues only (Supplementary Table S4).

In situ hybridization. Cases expressing SIM2-s at high levels by quantitative PCR were selected for SIM2 mRNA in situ hybridization. Positive expression was confirmed in malignant samples and validation by oligonucleotide arrays. SIM2-l isoforms were up-regulated in 29 malignant (n = 37) prostate tissues at the mRNA level. SIM2-total, SIM2-s, and SIM2-l isoforms and common primer SIM2-total. Columns, median; bars, 75 percentiles. A, SIM2-s; B, SIM2-l. C, SIM2-total. SIM2-s and SIM2-l isoforms and common primer SIM2-total were significantly elevated in malignant versus benign prostate tissues (P < 0.001, Mann-Whitney test).

Fig. 1. Relative mRNA quantity of SIM2 transcripts by real-time quantitative PCR versus β-actin used as endogenous control. Comparisons between 37 tumor and 39 benign prostate samples for SIM2-s and SIM2-l isoforms and common primer SIM2-total. Columns, median; bars, 75 percentiles. A, SIM2-s; B, SIM2-l. C, SIM2-total. SIM2-s and SIM2-l isoforms and common primer SIM2-total were significantly elevated in malignant versus benign prostate tissues (P < 0.001, Mann-Whitney test).
prostate glands and prostatic intraepithelial neoplasia compared with benign glands (Fig. 2). In benign glands, luminal cells were negative, whereas basal cells often showed some SIM2 expression (Fig. 2).

**SIM2-s protein expression by immunohistochemistry.** The SIM2-s nuclear staining index ranged from 0 to 9 (median staining index = 2.0) in prostate cancer, with variable cytoplasmic SIM2-s expression. A moderate/strong (defined as positive) nuclear staining of SIM2-s protein (staining index = 3-9) was noted in 44 of the 103 paraffin-embedded prostate carcinomas, as opposed to a weak or negative (defined as negative) staining (staining index = 0-2) in the remaining cases (Fig. 2). Positive nuclear SIM2-s expression was significantly associated with adverse prognostic variables like preoperative serum PSA >11.15 (median; \( P = 0.004 \)), high histologic grade (WHO; \( P = 0.043 \)), extra-prostatic extension (\( P = 0.044 \)), and increased tumor cell proliferation by Ki-67 expression (\( P = 0.013 \); Table 1). In addition, increased expression of SIM2-s was associated with reduced expression of p27 protein (\( P = 0.038 \), Mann-Whitney test).

**SIM2-s expression and survival.** In a univariate survival analysis of 103 prostate cancer patients, positive SIM2-s expression (cut off by median staining index) was significantly associated with reduced prostate cancer–specific survival (Fig. 3). The estimated 10-year (13-year) survival was 98.1% (98.1%) versus 79.5% (72.8%) in patients with negative versus positive SIM2-s expression, respectively (\( P = 0.008 \), log-rank test). Additionally, a trend for positive SIM2-s expression to be associated with time to skeletal metastasis (\( P = 0.084 \)) was found. Among preoperative serum PSA, histologic grade (WHO), pathologic stage, and SIM2-s expression, only histologic grade (\( P = 0.018 \)) and SIM2-s expression (\( P = 0.008 \)) were significantly associated with survival in univariate analysis. Although only one single event was recorded among low SIM2-s expressors, a multivariate survival model, including WHO histologic grade and SIM2-s expression, revealed that only SIM2-s remained in the model as a significant independent predictor of reduced cancer-specific survival (\( P = 0.028 \); hazard ratio, 6.9; 95% confidence interval, 0.8-57.7), whereas histologic grade was of borderline significance only (\( P = 0.059 \); hazard ratio, 3.7; 95% confidence interval, 0.9-15.1).

In our independent series of 37 cases of prospectively collected prostate cancers analyzed by real-time quantitative PCR, five biochemical failures were recorded (median follow-up, 2.3 years), and a trend for cases with high levels of SIM2-s mRNA to be associated with early recurrences was noted (\( P = 0.084 \), log-rank test), as was a similar tendency for high SIM2-l (\( P = 0.18 \), log-rank test).

**Discussion**

Although the SIM2 gene, which is located within the Down’s syndrome critical region, is known to be important for brain development and neuronal differentiation, studies have indicated that it might also be involved in the pathogenesis of solid tumors (10–12). We recently reported SIM2 as the second most consistently up-regulated gene in human prostate cancer (13). In our extended series, the short and long isoforms of SIM2 were significantly coexpressed and elevated in prostate tumors compared with benign tissue. The SIM2-l isoform has not been previously identified in prostate cancer. We here show for the first time that SIM2-s protein expression might be important for the clinical progress of human cancer, as shown for prostate tumors by multivariate survival analysis. Our findings may be of practical importance because SIM2-s was recently suggested as a candidate for targeted therapy. Thus, inhibition of tumor growth by antisense blocking of the SIM2-s isoform was shown in colon cancer xenografts in mice (11). Whether this effect is due to an influence on cell cycle regulation is not known, but high SIM2-s expression was significantly associated with increased tumor cell proliferation as indicated by our data. Alternatively, a stimulatory effect of SIM2-s...
antisense on tumor cell apoptotic regulation has been suggested (22). When looking at transcription factor binding sites by modules of the Genomatix program,10 an AHRR site (a potential binding site for PER, ARNT, SIM1, and SIM2) was predicted in the promoter regions of $p27$ and $p16$ and could represent a putative link between SIM2 and proliferation (data not shown).

Notably, an association between increased SIM2-s protein and reduced $p27$ expression was found in our study, indicating a possible repressive effect of SIM2. However, the significance of SIM2 in cell cycle regulation presently remains unknown.

The specific role of SIM2 in prostate cancer compared with other SIM2-overexpressing cancers like colon and pancreatic tumors has not been extensively explored. Differences in the expression of SIM2-s and SIM2-l isoforms have been reported for various normal tissues (9), and the two SIM2 isoforms may thus differ in a possible oncogenic or tumor-suppressive activity in a tissue-specific manner. In addition, SIM2-s and SIM2-l have different effects on central midline response elements and gene expression under control of hypoxia and dioxin (9), although the functions of the SIM2 proteins remain unknown in humans.

Given the significance of androgen responsiveness of prostate cancer cells, a preliminary transcription factor binding site analysis did not reveal androgen-responsive elements within the SIM2 promoter (data not shown). Conversely, no SIM2 binding site was found within the androgen receptor promoter.

In our study, increased expression of SIM2-s protein was found to be associated with adverse prognostic factors, such as increased preoperative serum PSA, high histologic grade, increased tumor cell proliferation, and invasive growth with extra-prostatic extension, indicating an association with a subset of aggressive tumors. Supporting this, high SIM2-s expression was associated with significantly reduced cancer-specific survival as indicated by multivariate analysis. All prostate cancer–related deaths, with one exception, belonged to the SIM2-s–positive group.

Both isoforms of SIM2 were expressed at low levels in benign prostate tissue. In a previous report, the SIM2-s and SIM2-l isoforms were not seen in normal colon, pancreas, or prostate, and SIM2-l was not detected in tumor tissues (11). Later, both isoforms have been detected in a pancreatic cancer cell line but not in normal tissues (12). We presently found that SIM2-s was paralleled by the expression of SIM2-l transcript in both benign and malignant prostate samples. Especially, the ratio between expression levels in malignant tumors compared with benign tissues was even higher for SIM2-l than for the SIM2-s isoform. The contrast between our findings and earlier reports may be explained by differences in methodologies such as the primer sequences applied, increased sensitivity of the assay, and differences in RNA quality. Our findings indicate that SIM2-l expression might also be significant for prostate cancer

Table 1. SIM2-s protein expression by immunohistochemistry and clinicopathologic variables in prostate cancer

<table>
<thead>
<tr>
<th>SIM-s</th>
<th>No.</th>
<th>Negative*</th>
<th>Positive*</th>
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<tr>
<td>Clinical stage</td>
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<td>79</td>
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*Cut point: median staining index.

$^{1}$Pearson's $\chi^2$.

$^{2}$Cut point: median tumor diameter; tumor diameter available in 102 cases.

$^{3}$Cut point: median preoperative serum PSA; preoperative serum PSA available in 98 cases.

$^{4}$Tumor cell proliferation by Ki-67 expression; cut point: lower quartile.

10http://www.genomatix.de
development and should be further validated for antisense strategies.

In conclusion, the two different isoforms SIM2-s and SIM2-l were both found in benign prostate tissue, and they were significantly coexpressed and elevated in prostate cancers. SIM2-s protein expression was found to be associated with adverse prognostic variables such as increased preoperative serum PSA and high histologic grade, extra-prostatic tumor extension, increased tumor cell proliferation by Ki-67 expression, and reduced patient survival as shown by multivariate analysis. These novel findings support the proposal of SIM2 as a candidate for targeted therapy of prostate cancer.

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