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ORIGINAL ARTICLE

HIF-1α and YBX1 Mediated Up-Regulation of NonO/p54nrb in Prostate Cancer Cells

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Abstract

This study examines the effect of hypoxia, a pivotal factor in prostate carcinogenesis, on the cellular roles of NonO/p54nrb and YBX1 proteins. NonO/p54nrb is a part of the paraspeckle protein, a nuclear domain that is encoded by the NONO gene in humans and has been demonstrated to interact with the Androgen receptor, SFPQ, and SPI1. In the nucleus, NonO/p54nrb is involved in a variety of processes, such as transcription initiation, RNA processing, transcription elongation, and termination while YBX1, binds to both DNA and RNA. Elevated levels of YBX1 and HIF-1α have been observed in prostate carcinoma. The expression profile of NonO/p54nrb was assessed using sqRT-PCR in HUVEC and a panel of nine diverse cancer cell lines from various tissues (prostate, breast, colon, liver, pancreas, and bone). The PC3 prostate cancer cell line exhibited the highest level of NonO/p54nrb expression. A hypoxic microenvironment was successfully induced with the mimetic agent $CoCl_2$, with its confirmation obtained by quantifying HIF-1α mRNA levels via Real-Time PCR. To assess the activity of the NonO/p54nrb promoter, a series of 5' deletion constructs −730/+529; -516/+529; -336/+529 and −159/+529 were cloned into the pMetLuc luciferase vector. The basal transcriptional activity of NonO/p54nrb promoter constructs increased under hypoxic conditions at 48 h. Hypoxic up-regulation was also observed at both mRNA and protein levels. The study also analyzed the effect of exogenously produced YBX1 transcription factor on NonO/p54nrb mRNA and protein levels. An upregulation was also observed in all YBX1-transfected promoter fragments. The findings from our study indicate that NonO/p54nrb is transcriptionally upregulated by HIF-1 in PC-3 cell line. Furthermore, the YBX1 transcription factor enhances NonO/p54nrb expression under both normoxic and hypoxic conditions, with a more pronounced increase observed specifically during hypoxia.

Extended author information available on the last page of the article

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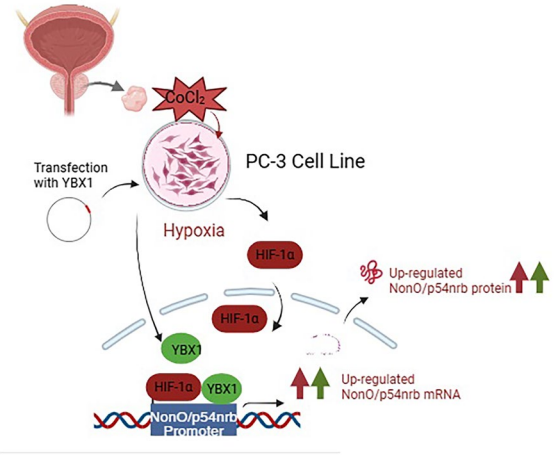
Graphical Abstract



Keywords Hypoxia · NonO/p54nrb · YBX1 · HIF-1α Prostate cancer · PC-3

Introduction

NonO/p54nrb, belonging to the Drosophila behavior/human splicing (DBHS) family, is a multifunctional nuclear protein. The DBHS protein family, the major protein component of paraspeckles, has three members in mammals: Polypyrimidine Tract-Binding Protein-Associated Splicing Factor (PSF), Non-POU domain-containing octamer-binding protein/nuclear RNA-binding protein (54 kDa) (NonO/p54nrb), and Paraspeckle Component 1 (PSPC1) (Fox and Lamond 2010). Paraspeckles, which are protein-rich nuclear organelles built around a specific Long non-coding RNAs (lncRNA) scaffold, regulate gene expression along with their molecular constituents in a wide range of cellular functions, including viral infection, differentiation, and cellular stress reactions (Dong et al. 1993, Fox et al. 2018).

Emerging evidence highlights the extensive involvement of the NonO/p54nrb protein across nearly every stage of gene regulation. Its functions encompass, but are not limited to, mRNA splicing, DNA denaturation-renaturation, transcriptional regulation, the nuclear arrest mechanism of defective RNA, and DNA repair. Beyond these molecular roles, NonO/p54nrb also participates in crucial biological processes such as cell proliferation and apoptosis. Significantly, dysregulation of NonO/p54nrb has been observed in numerous cancer types. Studies in the literature indicate that NonO/p54nrb has a wide range of transcriptional-level regulatory functions. In this way, it is quite interesting that it interacts with many different proteins and DNA regions and that the genes it regulates are especially cancer-related genes. These findings suggest its potential as a novel biomarker or a viable therapeutic target for various cancers (Bond and Fox 2009).

NonO/p54nrb plays a pivotal role in transcriptional regulation by binding to specific DNA elements, such as intracisternal A particles, and facilitating the recruitment of other transcription factors to their respective response elements. Furthermore, the PSF/NonO/p54nrb heterodimer is known to modulate the transcriptional activity of various nuclear receptors (NRs), including the thyroid hormone receptor and the androgen hormone receptor. In vivo studies have also demonstrated that NonO/p54nrb cAMP response element-binding proteins (CREB) are essential for the cyclic AMP (cAMP)-dependent activation of target genes (Lu and Sewer 2015). Post-transcriptional regulation of the nmt55/p54nrb protein has been observed in human breast tumors. This control leads to a decrease in its expression within ER-negative tumors, while ER-positive tumors exhibit expression of isoforms with altered amino-terminal ends. Researchers have posited that because nmt55/p54nrb's involvement in RNA binding and pre-mRNA processing is essential for normal cellular function, any loss or alteration of the protein may contribute to tumor growth or progression (Pavao et al. 2001). NonO/p54nrb stimulates Sterol regulatory element binding protein 1α (SREBP-1α) mediated transcription of lipogenic genes and lipid production in breast cancer cells. NonO/p54nrb binding to SREBP-1α is also critical for breast tumor development in vivo (Zhu et al. 2016). The post-transcriptional regulation of SKP2 and E2F8 RNA by NonO/p54nrb is also crucial for promoting the proliferation of breast cancer cells (Iino et al. 2020). In ESCC (esophageal squamous cell carcinoma), the suppression of cell growth and invasion by NonO/p54nrb downregulation is coupled with the induction of apoptosis. This dual effect is driven by NonO/p54nrb's activation of the Akt and Erk1/2 signaling cascades (Cheng et al. 2018). Highly expressed in lung cancer tissue and associated with a poor prognosis, NonO/p54nrb governs the energy metabolism of cancer cells by regulating the NAMPT gene (Kim et al. 2020). In patients with Hepatocellular Carcinoma (HCC), NonO/p54nrb expression has been reported to be frequently increased and associated with poor outcomes (Hu et al. 2020). pancEts-1 regulates Ets-1 expression by facilitating NonO/p54nrb-mediated ERG transactivation, which in turn leads to increased tumorigenicity and aggressiveness in gastric cancer cells (Li et al. 2018). Increased expression of lncRNA GAPLINC (Gastric Adenocarcinoma Associated, Positive CD44 Regulator, Long Intergenic Non-Coding RNA) correlates with increased tumor size in CRC (Colorectal Cancer). In CRC patients, GAPLINC promotes colorectal carcinoma invasion via binding to PSF/NonO/p54nrb and partially by inducing SNAI2 expression (Yang et al. 2016).

NonO/p54nrb promoter activity increased in malignant melanoma, where strongly upregulated NonO/p54nrb protein levels were determined. Analysis of the human melanoma B2 (HMB2) cell system with MIA (melanoma inhibitory activity) deficiency revealed strong downregulation of NonO/p54nrb. Knockdown of NonO/p54nrb protein in melanoma cell lines leads to low proliferation rates and a strong decrease in migration potential. These findings suggest an important role for NonO/p54nrb in the first stage of tumor formation or metastasis. NonO/p54nrb mRNA expression was determined in melanoma cell lines, primary tumor, metastasis, and melanoma tissue samples, and it was shown that NonO/p54nrb had increased expression and protein levels in all melanoma cell lines and tissue samples compared to normal cells (Schiffner et al. 2011, Nelson et al. 2012, Ren et al. 2014, Schmid et al.

2013, Schmid et al. 2013). Functional promoter analyses identified the transcription factor Y-box binding protein 1 (YBX1), a known regulator of MIA/CD-RAP-dependent chondrogenesis, which mediates the activation of NonO/p54nrb transcription by MIA (Schmid et al. 2013, Schmid et al. 2013). The YBX1-induced chemoresistance in colorectal cancer cells is significantly increased by NonO/p54nrb or RALY, as silencing these proteins restores sensitivity to chemotherapeutic agents despite YBX1 overexpression (Tsofack et al. 2011).

Prostate cancer is a leading cause of male cancer deaths, with hypoxia and its key effector, HIF-1α (Hypoxia-inducible factor 1-alpha), driving its aggressive nature by promoting processes like metastasis and therapeutic resistance. Metastasis is the primary cause of cancer fatalities, particularly in aggressive sarcomas. Studies show YBX1 is vital for Epithelial-Mesenchymal Transition (EMT) and metastasis, acting as a "metastatic circuit switch" in high-risk sarcomas. Crucially, YBX1 regulates HIF-1α expression in these cells, leading to increased invasion and metastatic potential (El-Naggar et al. 2015). In addition, HIF1A-AS3 directly integrated with YBX1 and inhibited its ability to bind to p21 and AJAP1 promoters to suppress their transcriptional activities, thereby promoting hypoxic Ovarian Tumor (OC) progression (Xie et al. 2023). The positive feedback loop between HIF1α and HIF1A-AS1 drives pancreatic cancer progression by promoting YBX1 phosphorylation through an enhanced AKT-YBX1 interaction, a mechanism associated with poor patient survival (Xu et al. 2021). A recent investigation into the role of HIF1α in the progression of Translocation Renal Cell Carcinoma (tRCC), found that the NonO/p54nrb-TFE3 fusion protein directly targets and upregulates HIF1α under hypoxic conditions. This upregulation in tRCC cells (UOK109) then promotes aerobic glycolysis and angiogenesis, highlighting a key mechanism driving the aggressive nature of this cancer (Chen et al. 2021).

NonO/p54nrb directly or indirectly affects mRNA modification processes in cancer. In this study, the expression of NonO/p54nrb in different cancer cells was firstly investigated. It has not been shown whether NonO/p54nrb is regulated by hypoxia and the YBX1 transcription factor in prostate cancer. It was shown whether the expression of this gene changed in hypoxic and normoxic conditions in selected cell lines, and also the response of the gene to hypoxia was checked at different stages. For this purpose, it was aimed to clone the NonO/p54nrb promoter and to create promoter deletion mutants of different lengths. The basal transcriptional activities of the promoter fragments were compared with their activities in a hypoxic environment. After the transcriptional activity, NonO/p54nrb mRNA and protein levels were compared in normoxic and hypoxic conditions, and to confirm the hypoxic response obtained, it was tested with the EMSA technique whether HIF1α directly binds to the HRE (Hypoxia response element) regions detected as a result of bioinformatic analyses in the promoter region. YBX1 expression plasmid together with NonO/p54nrb promoter fragments were transiently transfected into PC-3 cells under both normoxic and hypoxic conditions, and its contribution to NonO/p54nrb promoter activity was determined by measuring luciferase activity. In addition, the effect of exotopically produced YBX1 on NonO/p54nrb mRNA and protein levels under both normal and hypoxic conditions was also investigated.

Materials and Methods

Material

The Human Prostate Cancer Cell Lines (PC3 and Du145) were obtained from Dr. Kemal Sami Korkmaz at Ege University, Türkiye. The colon cancer cell line (HT-29) were provided by Prof. Dr. Tuğba Boyueğmez from Çanakkale 18 Mart University, Türkiye. The human umbilical vein endothelial cells (HUVEC) was kindly provided by Dr. Ayşe Begüm TEKİNAY from National Nanotechnology Research Center, Bilkent University, Türkiye. Human osteosarcoma cell line, Mg63 was kindly gifted by Prof. Kenneth Wann from Cardiff University, UK. Human osteosarcoma cell line, Saos-2 cells were kindly gifted from Prof. Deborah Mason from Cardiff University, UK. Hep3B (human hepatoma) and HeLa (human cervical cancer) cells were provided by Prof. Dipak Ramji (Cardiff University, Cardiff UK). PANC-1, (human pancreas ductal adenocarcinoma) cell line was purchased from ATCC (American Type Culture Collection). Invitrogen was the source of all tissue culture supplies. Nucleobond EXtra Midi Plus Plasmid DNA isolation kit (Macherey-Nagel) was used for DNA isolation. The transfection kit was sourced from Promega (ViaFect™ Transfection kit) for the plasmid DNA transfection process. GeneJET RNA Purification Kit was purchased from Thermo. The luciferase assay reporter kits were purchased from Clontech, located in Mountain View, California, USA. Furthermore, antibodies were acquired from Sigma-Aldrich (St. Louis, MO, USA), Santa Cruz Biotechnology (Dallas, TX, USA), and Abcam (Cambridge, UK).

Bioinformatic Analysis

The human NonO/p54nrb promoter was analyzed for potential transcription factor binding sites using MathInspector and TransFac 1.3. To evaluate the evolutionary conservation of these regulatory elements, mammalian NonO/p54nrb sequences were aligned using ClustalW within the BioEdit software suite.

Clinical expression profiles and the prognostic significance of NONO in the The Cancer Genome Atlas - Prostate Adenocarcinoma (TCGA-PRAD) dataset were assessed via the GEPIA2 (Gene Expression Profiling Interactive Analysis) database (http://gepia2.cancer-pku. cn/#analysis, accessed on 10 February 2026). Differential expression was analyzed using boxplots, and survival analysis was performed using Kaplan-Meier curves. Co-expression patterns between NONO/p54nrb, HIF1α, and YBX1 were evaluated using Spearman's correlation coefficients in GEPIA2. These associations were further validated through the TIMER 3.0 (Tumor Immune Estimation Resource) database (https://compbio.cn/timer3/), employing purity-adjusted partial Spearman's correlation to account for the potential confounding effects of tumor microenvironment infiltration ($n = 497$). Statistical significance for all analyses was defined as $p < 0.05$.

Cloning of Human NonO/P54nrb Promoter and YBX1 Gene

The nucleotide sequences for the human NonO/p54nrb gene promoter (GenBank NC_000023.11) and YBX1 gene region (GenBank NM_004559.4) were retrieved from the NCBI database for this study. Four promoter constructs of varying lengths were generated for cloning into the pMetLuc Reporter Vector (Clontech, PT4059-5), cloning over the 1259 bp region of the human NonO/p54nrb sequences. For cloning of the YBX1 gene into the pcDNA3.1 vector (Thermo Scientific, V79020), the cloning site encompassing the CDS region (332–1306 bp) was determined. To ensure cloning in the correct orientation, XhoI was added to all forward primers, and a HindIII restriction enzyme site was added to all reverse primers. Table 1 provides a comprehensive list of all primers used for cloning.

PCR Amplification of NonO/p54nrb Promoter and YBX1 Gene The human NonO/p54nrb promoter and YBX1 gene were amplified via PCR using genomic DNA (gDNA) isolated from the PC3 prostate cancer cell line as a template. The reaction was performed in a total volume of 50 µL containing 200 ng of genomic DNA template, 2 mM MgCl2, 2 µM primers, 1X buffer (Fermentas), and 2.5 U of Fermentas Taq DNA polymerase. The buffer consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9), and 1% (v/v) Triton-X-100. The reaction conditions were set denaturation (3 min at 94 °C), annealing (35 cycles, 1 min at 94 °C, 1 min at 54 °C, 1.5 min at 72 °C), and final extension (10 min at 72 °C), respectively. Following the purification of PCR products from the gel and the generation of sticky ends, the DNA was first cloned into the pGEM®-T Easy Vector (Promega, A1360). These constructs were then subcloned into either the pMetLuc-Reporter vector (for NonO/P54nrb promoter) or the pcDNA3.1 vector (for the YBX1 region), with the accuracy of all cloned regions confirmed by sequencing (REFGEN, Ankara).

Table 1 Forward and reverse primer sequences and product length used in the cloning of the NonO/P54nrb promoter and the YBX1 gene

Gene name	Cloning primers	Product lenght
NonO/p54^{nrb}	P1 FP1-CTCGAGAGGTTCTC AGTTGCATTAGT RP-AAGCTTTCTACCGACTG GTATCTCCT	1259 bp (−730/+529)
	P2 FP2-CTCGAGATCAATTGA TCCTTGTAGTCCTTGG RP-AAGCTTTCTACCGACTG GTATCTCCT	1045 bp (−516/+529)
	P3 FP3-CTCGAGATAGTGATG TTTGGACTGCTA RP-AAGCTTTCTACCGACTG GTATCTCCT	865 bp (−336/+529)
	P4 FP4-CTCGAGCAGGAGAA GCATCTAGAA AT RP-AAGCTTTCTACCGACTG GTATCTCCT	688 bp (−159/+529)
YBX1	FP-AAGCTTCGCAACCATGA GCAGCGG RP-CTCGAGCTCAGCCCCG CCCTGC	974 bp

Cell Culture and Hypoxia Treatment

PC3 prostate cancer cells were cultured in DMEM-F12 supplemented with 10% FCS at 37 °C and 5% CO_2. Cell viability was confirmed using Trypan Blue exclusion. For both mRNA and protein analyses, cells were seeded at a density of 2×10^6 cells per 25 cm^2 flask and allowed to attach overnight.

A chemically-induced hypoxic condition was established using $CoCl_2$ at a final concentration of 150 µM, consistent with established literature (Turkoglu and Kockar 2016). For quantitative cell viability analysis, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and treated with $CoCl_2$ (150 µM) for varying durations (24 to 72 h). The effect of the treatment was assessed using an MTT assay, with absorbance measured at 550 nm on a spectrophotometer to quantify cell viability (Turkoglu and Kockar 2016).

Transient Transfection and Co-transfection Assays

Transient transfections for both basal transcriptional activity and cotransfection assays were performed using the ViaFect™ Transfection kit. For basal activity, PC3 cells (250×10^3 cells/well) were transfected in 12-well plates with 0.5 µg of promoter constructs (P1 to P4). Untreated transfected and non-transfected cells served as controls. To induce a hypoxic response, cells were treated with 150 µM $CoCl_2$ for 24, 48, and 72 h. Secreted luciferase and SEAP (Secreted Embryonic Alkaline Phosphatase) activities were quantified using Luciferase Reporter Systems (Clontech) on a Luminometer (Thermo). Luciferase activity was normalized against corresponding SEAP values. A pMetLuc control vector was utilized to assess transfection efficiency, and both pMetLuc control and SEAP control vectors served as positive controls. All transfection experiments were independently performed in triplicate.

For subsequent mRNA and protein analyses, cells were seeded at 2×10^6 cells/25 cm^2 per flask and incubated overnight to ensure optimal cellular attachment. The following day, 5 µg of expression vectors encoding relevant transcription factors were introduced via the calcium phosphate precipitation method. After 6 hours, the transfection medium was replaced, and cells were subjected to $CoCl_2$-induced hypoxia (150 µM final concentration) for varying durations: 24,48, and 72 h. At each specified time point, RNA and protein isolation was performed, and samples were stored at −80 ˚C for future analysis.

RNA Preparation and Gene Expression Analysis

Total RNA extraction was performed using the GeneJET™ RNA Purification Kit (Thermo Scientific, K0731) according to the manufacturer's recommendations. After checking the integrity and purity of the RNAs, cDNA was synthesized from the appropriate RNAs using Revert Aid reverse transcriptase (Thermo Scientific, EP0441).

Following PCR optimization, sqRT-PCR was performed using the primers listed in Table 2 for gene expression profile analysis. PCR conditions were set as hold (94 °C, 5 min), denaturation (94 °C, 30 s), annealing (55 °C for hβ2; 61 °C for YBX1; 51 °C for NonO/p54nrb; 55 °C for HIF1α, 30 s), and elongation (72 °C, 10 min). Ethidium

Table 2 Primers and product lengths used in gene expression analyses

Gene name	Expression primers	Product lenght
NonO/p54^{nrb}	FP-ATATGCCACTCCGTGGAAAG RP-GAAGGAGCCTTCACTGCATC	260 bp
YBX1	FP-GGGACAAGAAGGTCATCGCA ACGAA RP-TGA ACT GGA ACA CCA CCA GGA CCT G	253 bp
hβ2	FP-TTTCTGGCCTGGAGGCTATC RP-CATGTCTCCATCCCACTTAACT	314 bp
HIF1α	FP-CCACCTATGACCTGCTTGGT RP-TGTCCTGTGGTGACTTGTCC	269 bp

bromide staining was used to visualize the PCR products after they had been separated on 0.8% agarose gels. For the quantitative assessment of mRNA levels, densitometric analysis of the agarose gel images was performed using ImageJ software. The signal intensity for NonO/p54nrb was measured for each cell line and normalized by dividing it by the intensity of the corresponding human β2 microglobulin (hβ2) band.

mRNA expression levels of YBX1, NonO/p54^{nrb}, HIF1α, and hβ2 were determined with Real time PCR using SYBR Green I Master (Roche, Basel, Switzerland). Product specificity was confirmed by melting curve analysis, and the results were analyzed relative to the human β2 microglobulin (hβ2) gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Western Blot Analysis

Total proteins were extracted from the cells for Western blot analysis, then separated on a 10% polyacrylamide gel and transferred to a PVDF membrane (Millipore) as previously described (Turkoglu and Kockar 2016). To ensure equal loading, the total protein concentration of each sample was pre-measured by Bradford assay, and an equal amount of protein was loaded into each well. Following blocking, the membranes were first incubated with a NonO/p54nrb (Thermo, PA5-27408). primary antibody and then with a Goat-anti-rabbit secondary antibody (Abcam). Protein bands were visualized using a chemiluminescence substrate (Pierce). Densitometric analysis was performed using ImageJ software. The signal intensity of the NonO/p54nrb bands was normalized against the corresponding β-Actin bands for each lane to correct for any minor variations in loading or transfer efficiency. The final results were expressed as fold-change relative to the control groups.

Immunofluorescence Staining

PC3 cells were cultured in DMEMF12 in 12-well plates (250×10^3 cells/well). Hypoxic conditions were applied to the relevant wells with $CoCl_2$, and 0.5 µg YBX1 transfections were performed with the ViaFect™ Transfection kit. Immunofluorescent staining was performed the next day (Poyrazlı et al. 2024). For immunofluorescence analysis, cells were cultured on coverslips. Both YBX1-transfected and non-transfected cells were exposed to $CoCl_2$ for 24 h. Following treatment, cells

were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at 25 ˚C.Subsequently, cells were incubated for 1 h at 25 ˚C with a 1:100 dilution of Anti-NonO/p54nrb polyclonal antibody. This was followed by a 1 h incubation at 25 ˚C with an Alexa Fluor 488 secondary antibody. Finally, cells were counterstained with DAPI and analyzed using a fluorescence microscope (Nikon DS-RI2) to assess NonO/p54nrb localization. To quantify protein expression, the mean fluorescence intensity (MFI) of NonO/p54nrb was measured using ImageJ software across multiple random fields.

EMSA Analysis

PC-3 cells treated with $CoCl_2$ at a final concentration of 150 µM produced nuclear extracts. Control groups comprised of untreated cells using the Biotin 3′ end DNA Labeling Kit (Thermo Scientific, USA), biotin was applied to oligonucleotides (Schmid et al. 2013). Competition reactions were performed by adding a 500-fold excess of unlabeled probes, with signal detection subsequently carried out using a Chemiluminescent Nucleic Acid Detection Module (Thermo, USA) as per the manufacturer's protocol. Bioinformatic analyses revealed that the potential binding site of the hypoxia-inducible transcription factor HIF-1α to the NonO/p54nrp promoter lies within the +471/+529 bp range and encompasses all four promoter fragments. The primer information designed for this region is as follows: forward primer 5'-TCGGG ACGGGAGAGGCCGTGTAGCGTCGCCGTTACTCCGAGGAGATACCAGTCG GTAGA-3' and reverse primer 5'-AGCCCTGCCCTCTCCGGCACATCGCAGCG GCAATGAGGCTCCTCTATGGTCAGCCATCT-3'.

Statistical Analysis

Statistical analyses were performed using Minitab 14 software. Data are presented as the mean ± standard deviation (SD) of at least three independent biological replicates. For comparisons between multiple experimental groups, one-way ANOVA (Analysis of Variance) was applied, followed by Tukey's post-hoc test for pairwise multiple comparisons. Statistical significance was defined as $p \leq 0.05$ and $p \leq 0.01$. For bioinformatic analyses (GEPIA2 and TIMER 2.0), correlation significance was determined using Spearman's rank correlation as described in the respective database protocols.

Results

Bioinformatic Analysis and Cloning of the NonO/p54nrb Promoter

To determine the putative promoter region of the NonO/p54nrb gene, nucleotide sequence databases for NonO/p54nrb cDNA sequences were examined (GenBank accession no. NC_000023). Firstly, the promoter regions of Homo sapiens, Mus musculus, and Rattus norvegicus were compared, and conserved regions of the promoter were examined. The NonO/p54nrb promoter region (−150/+529) was used in the

comparisons. The results of the bioediting comparisons of the promoter regions can be seen in Fig. 1A. As shown in Fig. 1A, the promoter regions are highly conserved; Homo sapiens and Mus musculus are 77% similar, while Homo sapiens and Rattus norvegicus are 80% similar. Mus musculus and Rattus norvegicus are 92% similar. It is noteworthy that the promoter region is highly conserved in the in silico analyses, and when the TF binding regions are compared, it is determined that the HIF and YBX1 transcription factor binding regions are also conserved in these three species (Fig. 1A).

The NonO/p54nrb promoter (–730/+529) was cloned into the pGEMT-easy vector using genomic DNA and a PCR-based technique. Four separate promoter fragments were cloned from the 1259 bp promoter region of NonO/p54nrb. Three different regions of specific lengths were identified from this sequence, with P1 (−730/+529) being the largest. The amplified promoter regions were cloned into the pGEMT-easy

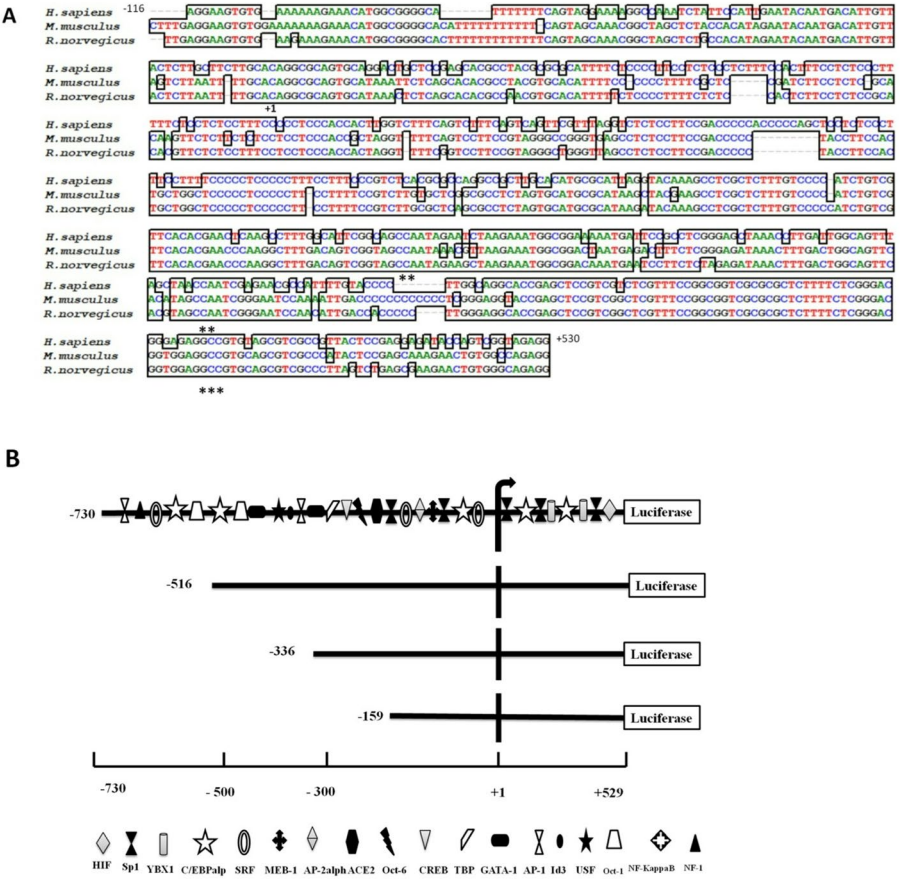


Fig. 1 **A** Comparison of the NonO/p54nrb promoter region in different organisms: +1:transcription start site; **: YBX1 binding sites, ***: HIF binding sites. **B** Schematic representation of the human NonO/p54nrb promoter region with different lengths, showing the binding sites of different transcription factors on this diagram

vector using a PCR strategy and confirmed by sequence analysis. These promoter fragments were then subcloned into the pMetLuc luciferase expression vector. Shematic represantation of four truncated promoter fragments were generated: promoter 1 (P1: −730/+529), promoter 2 (P2: −516/+529), promoter 3 (P3: −336/+529), and promoter 4 (P4: −159/+529). Possible transcription factor binding sites within the 1259 bp (−730/+529 bp) NonO/p54nrb promoter region were analyzed using the Ali Baba 3.1 program (Supplementary File). The identified binding sites are schematically represented in Fig. 1B.

NonO/p54nrb Expression Analysis in Different Human Cell Lines and Its Clinical Association in Prostate Adenocarcinoma (PRAD)

In our study, cDNA synthesis was performed using 1000 ng of RNA obtained from various cancer cell lines. These were derived from cervical cancer (HeLA), colon cancer (HT-29), breast cancer (MCF7), prostate cancer (PC3, Du145),, Hepatocellular carcinoma (Hep3B), pancreatic cancer (Panc), bone cancer (Saos, MG63), and Normal human umbilical vein endothelial cells (HUVEC) were also included in the study. PCR reactions were performed on different cell lines using specific NonO/p54nrb and H-β−2 Microglobulin expression primers. The products from the amplified regions were run on a 1% agarose gel and visualized with a UV image, and the bands were analyzed densitometrically. Expression levels of the NonO/p54nrb gene in different cancer cell lines were determined using densitometric analysis. As shown in Fig. 2A, the expression levels of the NonO/p54nrb gene vary in each cancer cell line. These differences in NonO/p54nrb expression suggest that NonO/p54nrb regulation is different in various cancer cells. Among the cell lines selected for NonO/p54nrb expression, PC3 cells exhibit the highest expression levels (Fig. 2B).

To assess the clinical relevance of these in vitro findings, the The Cancer Genome Atlas, Prostate Adenocarcinoma dataset (TCGA-PRAD) was analyzed via GEPIA2. Consistent with the experimental data, NONO mRNA levels were significantly upregulated in prostate adenocarcinoma (PRAD) tissues ($n=492$) compared to normal prostate tissues ($n=152$) ($p<0.01$; Fig. 2C). The prognostic value of NONO was further evaluated using Overall Survival (OS) analysis; however, no statistically significant association was observed between NONO expression levels and total survival duration ($p=0.69$; Fig. 2D).

Given the established role of the hypoxia-inducible factor HIF-1α and the transcription factor YBX1 in prostate cancer progression, their co-expression patterns with NONO were examined. Multi-gene comparison analysis revealed a synchronized expression profile among NONO, HIF-1α, and YBX1 (Fig. 2E). Spearman correlation analysis using the GEPIA2 dataset indicated a positive association between NONO and HIF-1α ($R=0.44$, $p=4.2\times10^{-27}$).

To validate this interaction while accounting for the tumor microenvironment, a purity-adjusted analysis was performed via the TIMER 2.0 database (Fig. 2F). This analysis yielded a robust partial Spearman's rho of 0.549 ($n=497$, $p<0.001$), suggesting that the correlation between NONO and HIF-1α is independent of non-tumor cell infiltration. These data collectively indicate a significant molecular asso-

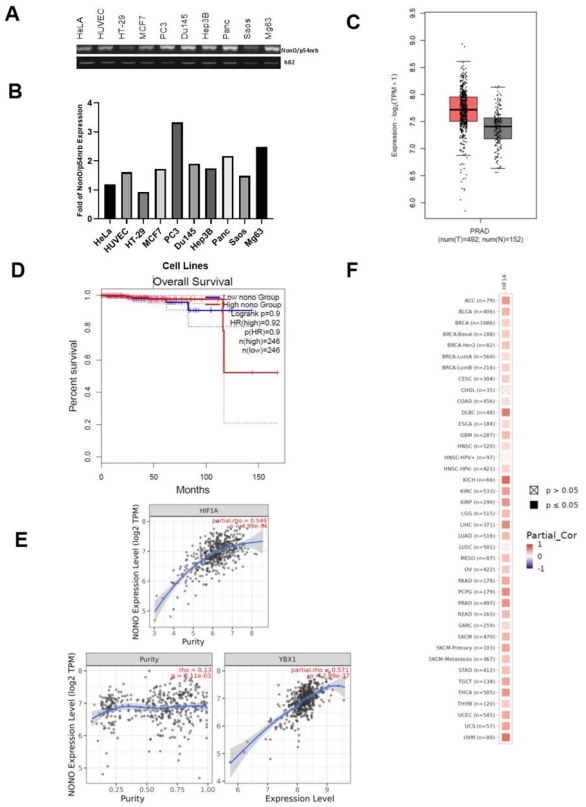


Fig. 2 Characterization of NonO/p54nrb expression different cell lines and its clinical association in Prostate adenocarcinoma (PRAD), **A** Representative agarose gel image of NONO mRNA expression in cancer cell lines and corresponding densitometric quantification. Agarose gel image of the NonO/p54nrb gene and the Hβ2 control gene in 10 different (Hela: Cervical cancer, HUVEC: Normal human umbilical vein endothelial cells, HT-29: Colon cancer cell line, MCF7: Breast cancer cell line, PC3: Prostate cancer cell line, Du145: Prostate cancer cell line, Hep3B: Hepatocellular carcinoma cell line, Panc: Pancreatic cancer, Saos: Bone cancer, Mg63: Bone cancer cell lines, **B** Densitometric analysis of the NonO/p54nrb gene in different cancer cell lines (NonO/p54nrb/Hβ2). **C** Boxplot analysis showing significant upregulation of NonO/p54nrb in PRAD tissues ($p < 0.01$). **D** Kaplan-Meier curves for Overall Survival (OS) based on NonO/p54nrb expression levels; while NonO/p54nrb shows high expression in tumors, its independent impact on OS in PRAD is presented. **E** Multi-gene Comparison Analysis: Scatter plot matrices showing the interrelated expression patterns of NonO/p54nrb, HIF-1α, and YBX1 within the GEPIA2 dataset. The synchronized expression of these three factors suggests a functional co-regulation in the prostate tumor microenvironment. **F** Purity-adjusted Spearman correlation analysis from the TIMER 2.0 database confirming the strong association (Rho = 0.549, $p < 0.001$)

ciation between the NONO, HIF-1α, and YBX1 axes in the context of prostate adenocarcinoma.

Verification of Hypoxia and Its Effects on PC3 and HUVEC Cell Proliferation

As a key regulator of genes involved in angiogenesis, hormone synthesis, glycolysis, and cell survival, HIF-1α was used as an indicator to confirm the occurrence of

hypoxic conditions in our chemical model. The expression of HIF-1α mRNA was monitored by real-time PCR, using specific primers and cDNA derived from the isolated RNA of chemically hypoxic cells. The obtained results were evaluated by the Livak method for Ct values. The occurrence of hypoxic conditions for PC3 (Fig. 3A) and HUVEC (Fig. 3B) cells was confirmed by examining the HIF-1α expression profile. The effects of hypoxic and normoxic environments on cell proliferation in PC3 and HUVEC cells, which are the cells we used as models, were determined by the MTT test. No statistically significant effect was observed in the PC3 cell ine. However, HUVEC cells were observed to be sensitive to hypoxic conditions.

Response of NonO/p54nrb to Hypoxia in PC-3 and HUVEC Cell Lines at mRNA and Protein Levels

After confirming that hypoxic conditions occurred in PC3 cells at intervals of 24, 48, and 72 h by looking at HIF-1α expression, cell pellets were taken at certain time intervals to perform RNA isolation with PC3 and HUVEC cells. Cells were grown in appropriate media by routine passage once a week. Then, they were placed in experimental vessels, and hypoxic flasks were treated with $CoCl_2$ to a final concentration of 150 µM to create hypoxic conditions. At specific time intervals (24, 48, and 72 h), cell pellets were collected for RNA isolation. RNA amounts were determined, and samples were run on RNA gel electrophoresis. The results were visualized on a UV imager. cDNA synthesis was performed from RNA samples obtained under hypoxic and normoxic conditions at the specified time intervals. Real-time PCR reactions were performed using the resulting cDNAs using primers specific for human NonO/p54nrb and Hβ2 Microglobulin. Hβ2 Microglobulin was used for normalization. The cT values obtained were evaluated according to the Livak method.

NonO/p54nrb gene expression at the mRNA level in PC3 (Fig. 4A) and HUVEC (Fig. 4B) cells under hypoxic conditions at time intervals of 24, 48, and 72 h is shown. A statistically significant increase was detected under hypoxic conditions in the PC3 cell line. No significant increase in NonO/p54nrb mRNA levels was observed in the HUVEC cell line compared to the control group under hypoxic conditions at time intervals of 24, 48, and 72 h.

NonO/p54nrb protein expression in both cell lines was analyzed by Western blot. Western blot membranes, photographed with a UVP imaging system, were analyzed densitometrically. Protein levels were determined in PC3 (Fig. 4C) and HUVEC (Fig. 4D) cells at 24, 48, and 72 h under hypoxic and normoxic conditions. An increase in NonO/p54nrb protein levels was observed in PC3 cells at 24 h (Fig. 4C), while an increase was observed in HUVEC (Fig. 4D) cells at 48 h.

Transfection of Different Promoter Fragments and Determination of Their Basal Activities

Transfection of the four truncated promoter fragments (P1 to P4), as shown in Fig. 5, was carried out using 0.5 µg of each fragment and Lipofectamine 2000 Reagent. After 48 and 72 h, medium samples were taken from the wells, and the activities of the promoter fragments were determined by measuring luciferase activity. Since the

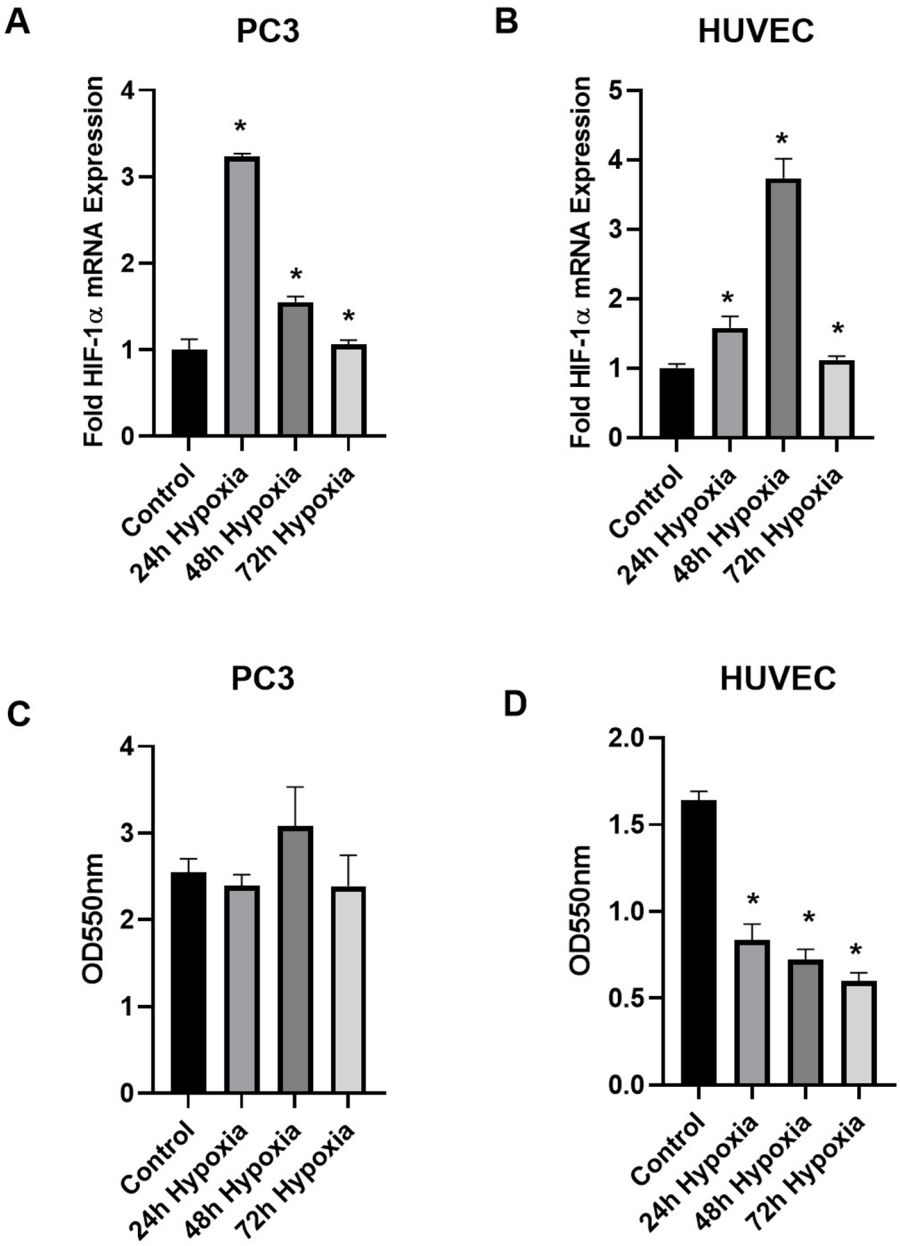


Fig. 3 Validation of the chemical hypoxia model and determination of cytotoxic effects on cells. **A** Validation of hypoxic conditions by HIF-1α expression in the PC-3 cell line. **B** Validation of hypoxic conditions by HIF-1α expression in the HUVEC cell line. **C** Determination of the effects of hypoxic and normoxic conditions on cell proliferation in PC3 cells by MTT analysis. **D** Determination of the effects of hypoxic and normoxic conditions on cell proliferation in Huvec cells by MTT analysis

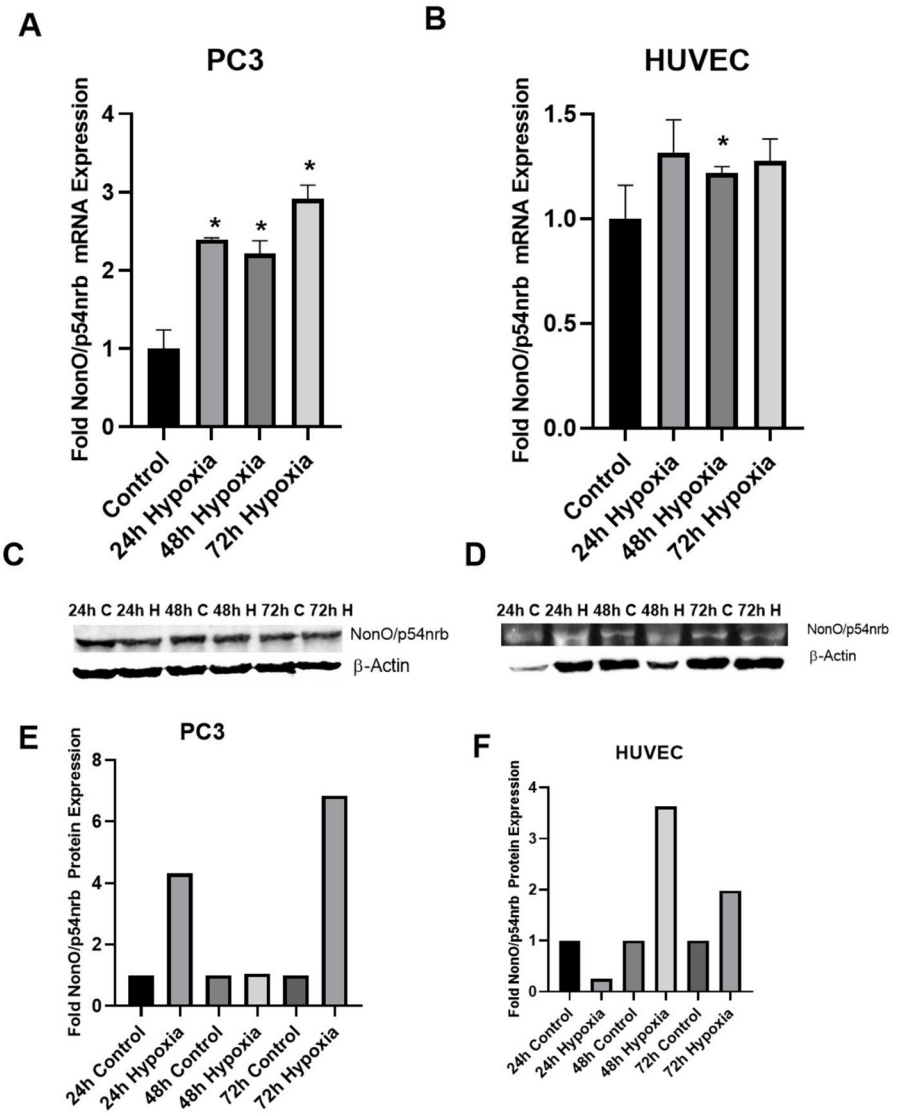


Fig. 4 Analysis of NonO/p54nrb expression at both mRNA and protein levels under chemical hypoxia induced by $CoCl_2$. **A** Quantitative RT-PCR analysis of NonO/p54nrb mRNA levels in PC-3 cells and **B** HUVEC cells. **C**, **D** Representative Western blot images showing NonO/p54nrb protein levels in PC-3 and HUVEC cells, respectively, with beta-actin as the loading control. **E**, **F** Densitometric quantification of Western blot signals for (**E**) PC-3 and (**F**) HUVEC cells, normalized to the internal control. Data represent the mean ± SD of three independent biological replicates. Statistical significance: $*p < 0.05$ compared to the normoxic control group

SEAP vector was also transfected into the cells along with the vectors containing the promoter fragments, the SEAP activity was used for normalization. The results were normalized by dividing the luciferase activity by the SEAP activity, and the relative luciferase activity was determined. As a result of the activity analyses, the basal

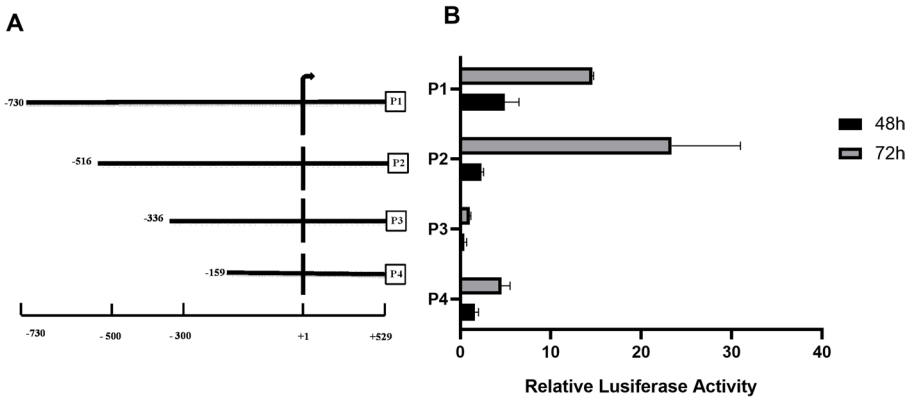


Fig. 5 Basal activity of NonO/p54nrb promoter fragments in PC3 cells (P1: −730/+529, P2: −516/+529, P3: −336/+529, and P4: −159/+529). **A** Representative diagram of promoter fragments, **B** Relative luciferase activity of promoter fragments (Luc/SEAP)

activities of all promoter fragments were determined in the PC3 cell line. P1 and P2 were identified as the most active promoter fragments.

Hypoxic Activities of NonO/p54nrb Promoter Contructs and Analysis of HIF-1 Binding Site in NonO/p54nrb Promoter

The transcriptional activity of truncated NonO/p54nrb promoter fragments was evaluated in PC3 cells under both normoxic and hypoxic conditions. The fragments analyzed included P1 (−730/+529), P2 (−516/+529), P3 (−336/+529), and P4 (−159/+529 bp) (Fig. 6A). While the P1 and P2 fragment exhibited the highest absolute luciferase activity, the P4 fragment was found to elicit the most robust hypoxic response when calculated as the fold-induction ratio (hypoxia/normoxia). Although the P1 fragment exhibited the highest absolute luciferase activity, the P4 fragment demonstrated the most robust hypoxic response (Fig. 6B) when assessed by the fold-induction ratio (hypoxia/normoxia). In silico analysis identified a conserved HIF-1α binding site within the +471/+529 bp region, which is common to all four promoter fragments. The presence of this site within the minimal P4 region likely accounts for its dynamic responsiveness to hypoxia, despite its lower basal activity compared to longer constructs. To validate this DNA-protein interaction, an Electrophoretic Mobility Shift Assay (EMSA) was performed. Initially, primers for the EMSA experiment were designed based on the +471/+529 bp region of the NonO/p54nrp promoter. The DNA-protein complex is shown in Fig. 5. As observed in Fig. 5, two complexes (C1 and C2) were formed in the hypoxic and normoxic extracts. Contrary to expectations, the complexes were stronger in the normoxic nuclear extract. This suggests that different transcription factors bind to the potential HIF-1α region selected under normal conditions. Indeed, when this region was examined, numerous potential binding sites for the SP1 transcription factor were observed. When the specificity of the complexes was tested using a 1000-fold biotin-free probe (wells 2 and 4), the complexes disappeared. Competition experiments were also conducted with

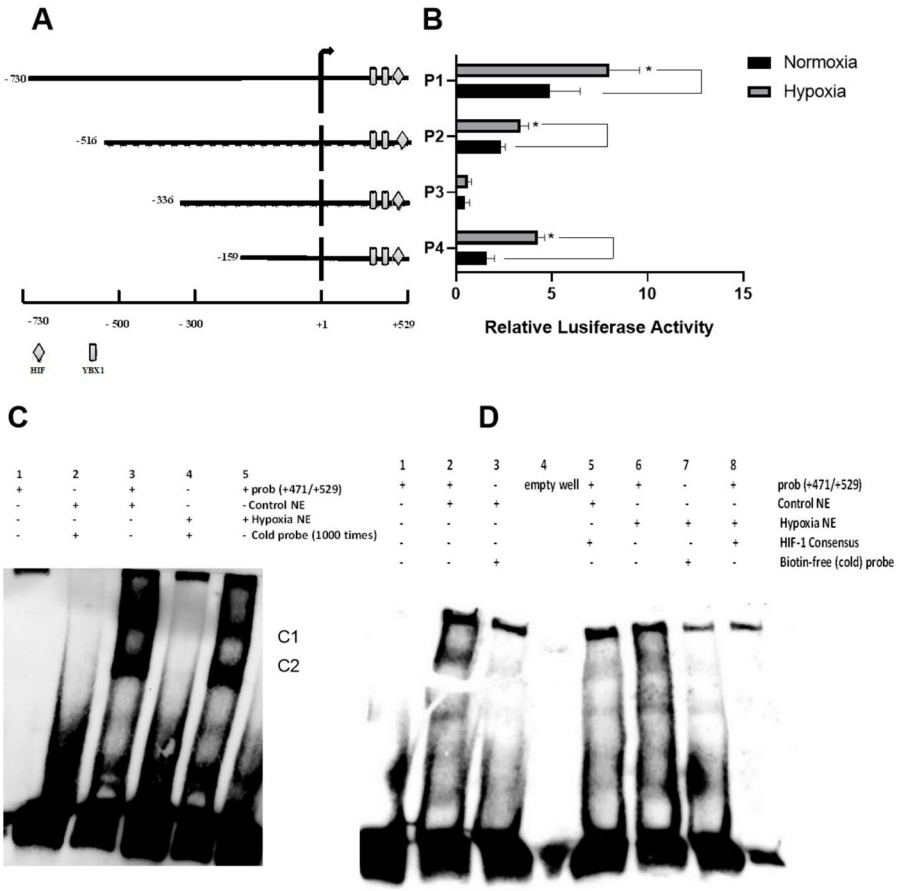


Fig. 6 Demonstration of hypoxic activities of promoter fragments and HIF binding by the EMSA technique. **A** HIF and YBX1 transcription factor binding sites are shown on the representative diagram of promoter fragments. **B** Activities of promoter fragments in the PC3 cell line in the chemical hypoxia model. **C** Image of NonO/p54nrb DNA protein complex. 1: Free probe, 2: Probe + N.E (Normoxia), 3: Cold probe + N.E (Normoxia) + Probe, 4: Probe + N.E (Hypoxia), 5: Cold probe + N.E (Hypoxia) + Probe. **D** EMSA competition experiment using HIF-1α consensus. 1: Free probe, 2: probe + N.E (Normoxia), 3: probe + N.E (Normoxia)+ cold probe, 4: Empty well, 5: probe + N.E (Normoxia) + HIF-1α consensus, 6: probe + N.E (Hypoxia), 7: probe + N.E (Hypoxia)+ cold probe, 8: probe + N.E (Hypoxia) + HIF-1α consensus

HIF-1α consensus probes to confirm true binding. Figure 5 shows similar complexes forming. The complexes disappeared again when a 1000-fold biotin-free probe was used. In the competition experiment using the HIF-1α consensus probe, the complexes disappeared completely, especially in the hypoxic nuclear extract (well 8). This demonstrated that HIF-1α indeed binds to this region.

Regulation of the YBX1 Transcription Factor on the NonO/p54nrb Gene

Confirmation of hypoxic conditions induced by $CoCl_2$ was achieved by using HIF-1α-specific primers in real-time PCR. HIF-1 levels were also analyzed in YBX-1-transfected groups under both normal and hypoxic conditions. Compared with normal conditions, levels of HIF-1α, the main regulator of hypoxia, were statistically significantly increased under hypoxic conditions (Fig. 7A). To verify the efficacy of the transfection, we first assessed the levels of YBX1 following the introduction of the ectopic expression plasmid. Quantitative RT-PCR analysis confirmed a significant increase in YBX1 mRNA levels, successfully validating the ectopic expression model (Fig. 7B). After confirming ectopic expression by YBX1 up-regulation and hypoxic conditions by HIF up-regulation, NonO/p54nrb mRNA expression was analyzed in YBX1-transfected cells. NonO/p54nrb expression was increased compared to normal conditions at both 48 and 72 h, but the upregulation was stronger at 72 h (Fig. 7C). According to the western blot analysis performed to see the change in protein level, an increase in NonO/p54nrb protein level was detected in YBX1 transfected hypoxic conditions at 72 h, in correlation with the mRNA level (Fig. 7D). According to the results of the immunofluorescence technique applied to analyze the localization of NonO/p54nrb protein in the cell and its change in hypoxic conditions, NonO/p54nrb protein levels were also found to increase in YBX1 transfected hypoxic groups compared to YBX1 transfected normoxic groups (Fig. 7E-F). To determine the effect of the YBX1 transcription factor on NonO/$p54^{nrb}$ promoter constructs, YBX1 transfected to the cells and lusiferase activities were measured by luminometre. The longest promoter, which contains P1 (−730/+529) promoter activity, was increased under hypoxic conditions at 48 and 72 h. NonO/$p54^{nrb}$ promoter fragments were transfected, and the luciferase and seap activities of the promoter fragments were assessed in experiments conducted under both normal and hypoxic conditions. When promoter activities were compared under normal and hypoxic conditions, upregulation was observed in all YBX1-transfected promoter fragments. The highest promoter activity was observed in the smallest fragment, P4 (Fig. 7G). All promoter fragments contain both HIF and YBX1 transcription factor binding sites (Fig. 6A). The highest activity increase observed in P4 may have been due to the removal of an existing silencer.

Discussion

Cells, tissues, or organs must adapt to changes in oxygen levels. Hypoxia, defined as decreased oxygen levels, leads to increased anaerobic respiration, cell division, red blood cell production, and cellular survival. It also supports angiogenesis to regulate oxygen levels. HIF-1 proteins are key regulators of hypoxia, which, in turn, regulate the transcriptional activation of various genes expressed in such molecularly mediated conditions (Demirel and Çetinkaya 2014). The nuclear HIF-1α subunit of the HIF-1 gene family becomes stable in the cytosol under hypoxic conditions, is phosphorylated, and enters the nucleus to form a heterodimer complex. In this way, it binds to the HRE (hypoxia response element) regions in the promoters of genes

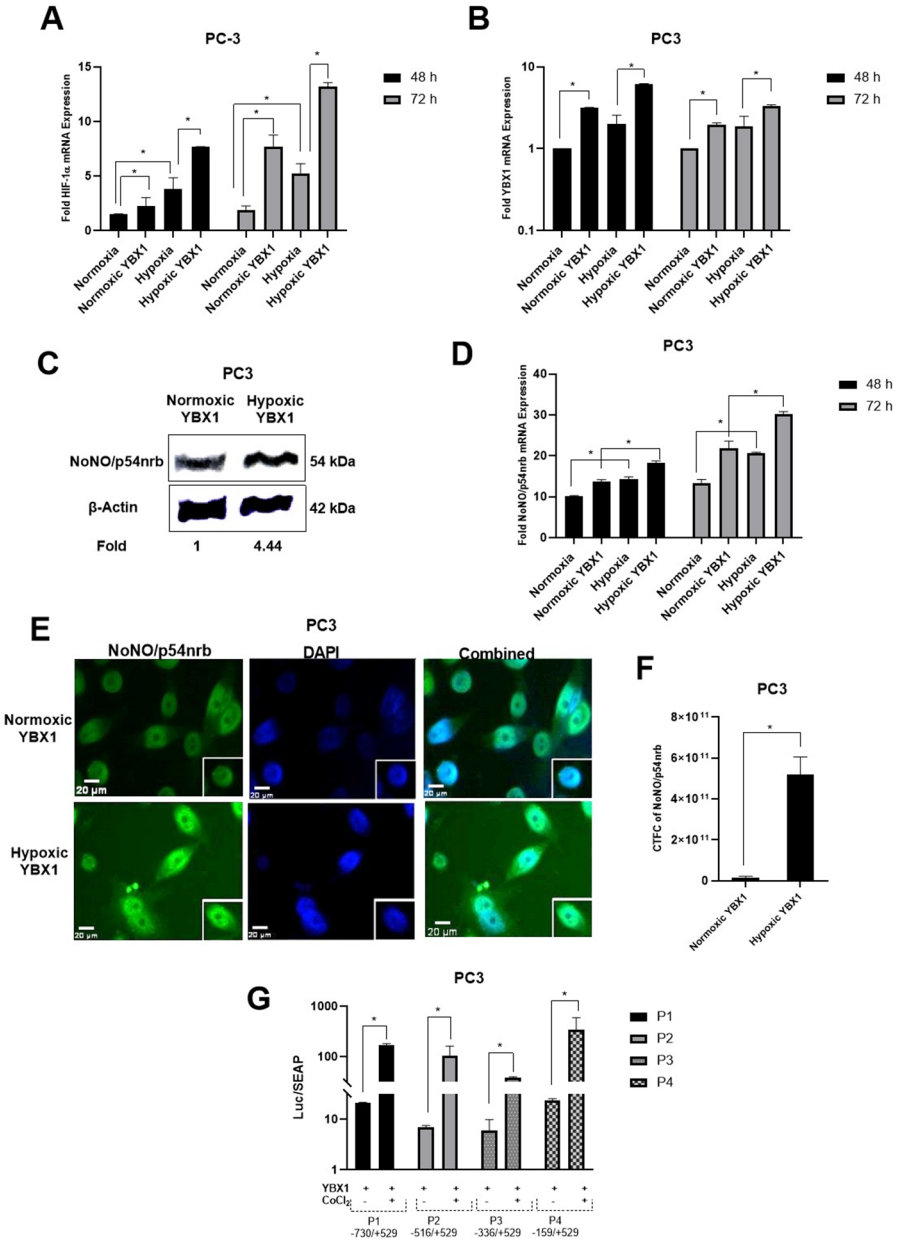


Fig. 7 Regulation of NonO/p54nrb by YBX1 under chemical hypoxia in PC-3 cells. **A** Quantitative RT-PCR validation of the chemical hypoxia model via HIF-1α expression. **B** Quantitative RT-PCR validation of ectopic YBX1 expression. **C** Quantitative RT-PCR analysis of NonO/p54nrb mRNA levels in PC-3 cells transfected with YBX1-expression plasmid under normoxic and hypoxic conditions. **D** Western blot analysis and **E** representative immunofluorescence (IF) images of NonO/p54nrb protein levels following YBX1 transfection; scale bar = 20 μm. **F** Quantification of IF signal intensity. **G** Dual-luciferase reporter assay demonstrating the activities of NonO/p54nrb promoter fragments in PC-3 cells transfected with YBX1 and subjected to chemical hypoxia. Data represent mean ± SD from three independent experiments. *$p < 0.05$

expressed under hypoxic conditions for transcription. Under normoxic conditions, it is unstable and is targeted by the VHL protein, resulting in proteasomal degradation. It cannot bind to the HRE region. Immunohistochemical studies on various human cancers (breast, colon, lung, prostate, skin, etc.) have reported that HIF-1α protein levels are present at a certain level in benign tumors, while the amount increases in malignant tumors and is much higher in metastases (Demirel and Çetinkaya 2014).

The regulation of HIF is affected by the accumulation of acidic metabolites or reactive oxygen species (ROS) in the early stages of prostate cancer, and its amount increases and accumulates inside the cells to manage the tumor microenvironment (Pouysségur et al. 2006, Tafani et al. 2016, Mohamed et al. 2023). In their study in 2017, Bizzarro et al. showed that HIFα is responsible for EMT in prostate cancer (Bizzarro et al. 2017). Hypoxia is considered a key precursor to prostate carcinogenesis associated with an aggressive phenotype (Deep and Panigrahi 2015). Studies on HIF-1α regulation, which may be associated with poor outcomes and poor prognosis in prostate cancer, are also quite limited. Previous studies have identified important roles for YBX1 in EMT and metastasis of epithelial malignancies (El-Naggar et al. 2015).

NonO/p54nrb has been revealed to mediate the regulation of the MIA/CD-RAP complex to promote chondrogenesis and malignant melanoma (Schmid et al. 2013). Overexpression of YBX1 has been shown to induce chemotherapeutic resistance in colorectal SW480 and HT29 cancer cells. Importantly, NonO/p54nrb or RALY-silenced SW480 and HT29 colorectal cancer cell lines are sensitive to chemotherapeutic agents despite overexpression of YBX1 (Tsofack et al. 2011).

Previous studies have identified important roles for YBX1 in EMT and metastasis of epithelial malignancies. The data reveal YBX1 as a critical regulator of HIF-1α expression in sarcoma cells. This mechanism leads to HIF-1α-mediated sarcoma cell invasion and enhanced metastatic capacity (El-Naggar et al. 2015). In addition, HIF1A-AS3 directly integrated with YBX1 and inhibited its ability to bind to p21 and AJAP1 promoters to suppress their transcriptional activities, thereby promoting hypoxic Ovarian Tumor (OC) progression (Xie et al. 2023). HIF1A-AS1 promoted the phosphorylation of YBX1 (pYBX1) by facilitating the interaction between the serine/threonine kinase AKT and YBX1 (Xu et al. 2021). The NonO/p54nrb-TFE3 fusion protein is reported to promote aerobic glycolysis and angiogenesis in Translocation Renal Cell Carcinoma (tRCC) by increasing HIF1α expression (Chen et al. 2021).

Studies in the literature indicate that NonO/p54nrb has a wide range of transcriptional-level regulatory functions. In this way, it is quite interesting that it interacts with many different proteins and DNA regions and that the genes it regulates are especially cancer-related genes, but it is not fully known how NonO/p54nrb, a multifunctional protein, is expressed in various cancer cells and how it functions in cellular functions. NonO/p54nrb has also been reported to be differentially expressed between normal and tumor tissues in bladder cancer and prostate cancer, and is upregulated in these tissues.

In our study, we first examined the expression level of NonO/p54nrb mRNA in HELA, HUVEC, HT-29, MCF7, PC3, Du145, Hep3B, Panc, Saos, and MG63 cell lines. NonO/p54nrb mRNA expression was detected in all cell lines studied. NonO/

p54nrb expression was highest in PC3 cells and lowest in HT-29 cells. These differences in NonO/p54nrb expression suggest that NonO/p54nrb regulation is different in various cancer cells. Consistent with these observations, bioinformatic analysis of the TCGA-PRAD dataset via GEPIA2 database confirmed that NonO/p54nrb is significantly upregulated in prostate adenocarcinoma tissues ($p < 0.01$). Moreover, analysis of the TIMER 2.0 database revealed a positive correlation between NonO/p54nrb and HIF1α as well as a significant association with YBX1 (Rho = 0.549, $p < 0.001$). This co-expression pattern, which remains significant after adjusting for tumor purity, suggests that the NONO- HIF1α -YBX1 axis is an intrinsic feature of the PRAD molecular landscape. The synchronized expression of these three factors implies they may function within a coordinated regulatory network, potentially driving the molecular mechanisms of prostate cancer progression under hypoxic microenvironmental conditions.

Our studies were conducted at the mRNA, protein, and promoter levels to demonstrate the contribution of hypoxia, a key process in the development of prostate cancer, to the regulation of NonO/p54nrb, which is highly expressed in the PC-3 cell line. The HIF1-α binding site on the NonO/p54nrb promoter region was analyzed bioinformatically, and it was determined that the HIF-1α transcription factor is located within the +471/+529 bp promoter region. In silico analyses, it is noteworthy that the NonO/p54nrb promoter region is highly conserved in *H. sapiens*, *M. musculus*, and *R. norvegicus*, and when TF binding sites are compared, it is determined that the HIF and YBX1 transcription factor binding sites are also conserved in these three species.

In a study of PC-3 and HUVEC cell lines, a chemical hypoxia model was created to analyze NonO/p54nrb. This model's validity was confirmed via Real-Time PCR (Fig. 3A and B), and the cytotoxic effects of the hypoxia were measured with the MTT test. The chemical hypoxia method has been successfully used in previous studies across various cell lines (Turkoglu and Kockar 2016, Poyrazlı et al. 2024, Okuyan et al. 2020, Altuntaş et al. 2023). As determined by the MTT assay, $CoCl_2$ exhibited no cytotoxic effects on either PC-3 or HUVEC cells after 24, 48, or 72 h of treatment (Fig. 3C and D). A statistically significant increase in NonO/p54nrb mRNA levels was detected in PC3 cells at 24, 48, and 72 h under hypoxic conditions (Fig. 4A). An increase in NonO/p54nrb protein levels was observed in PC3 cells at 24 h (Fig. 4C). The observed increase in both mRNA and protein levels indicated that NonO/p54nrb is regulated under hypoxic conditions in PC-3 cells. Studies on the HUVEC cell line have shown that this cell line is sensitive to chemical hypoxia (Fig. 3D). The best response to 24, 48, and 72 h of chemical hypoxia was observed at 48 h (Fig. 3B), and NonO/p54nrb levels increased at both the mRNA (Fig. 4B) and protein levels during this period (Fig. 4D). In healthy HUVEC cells, NonO/p54nrb is also regulated under hypoxic conditions.

The core promoter, a fundamental component in the initiation of transcription, was historically considered a uniform structure operating via a singular, universal mechanism. However, contemporary research has robustly demonstrated that core promoters exhibit widespread diversity in both their structure and function (Thomas and Chiang 2006, Smale and Kadonaga 2003). This evolving understanding challenges previous assumptions, highlighting the intricate regulatory potential embed-

ded. To study the function and transcriptional regulation of p54nrb in a non-small cell lung cancer cell line (NSCLC), the 2952 bp p54nrb promoter region, from −1671 to +1261, was cloned. Deletion studies in the promoter region showed that an important regulatory region exists in the +311/+414 fragment of the p54nrb promoter, and deletion of the +109/+203 region resulted in a partial decrease in luciferase activity (Lin et al. 2013). To analyze the transcriptional regulation of the NonO/p54nrb core promoter, we cloned four different 5' deletion promoter constructs and compared their transcriptional activities (Fig. 5). As a result of the activity analyses, the basal activities of all promoter fragments were determined in the PC3 cell line, and P1 and P2 were identified as the most active promoter fragments (Fig. 5B). In order to demonstrate the up-regulation at the mRNA and protein levels obtained under hypoxic conditions at the promoter level, the hypoxic response of all promoters was compared in transfection studies. The hypoxic response was calculated as the ratio of activity under hypoxia to that under normoxia to normalize for differences in basal promoter activity. The P4 promoter fragment was found to elicit the highest hypoxic response. Transcription factor binding sites were analyzed on the largest promoter fragment, and it was determined that the HIF-1α transcription factor, located within the +471/+529 bp region, encompassed a common region within four separate deletion promoter fragments. Initially, primers for the EMSA experiment were designed based on the +471/+529 bp region of the NonO/p54nrp promoter, and direct binding of HIF-1α was seen.

NonO/p54nrb has been described to regulate the MIA/CD-RAP complex to promote chondrogenesis. YBX1 is a regulator of MIA/CD-RAP-dependent chondrogenesis. Functional promoter analyses identified YBX1 as the transcription factor that mediates MIA protein for the activation of NonO/p54nrb transcription (Schmid et al. 2013, Schmid et al. 2013). In addition, overexpression of YBX1 has been shown to induce chemotherapeutic resistance in colorectal SW480 and HT29 cancer cells. NonO/p54nrb and RALY have been shown to significantly increase chemotherapeutic resistance in colorectal cancers overexpressing YBX1 protein (Tsofack et al. 2011).

In PC-3 cells overexpressing YBX1, NonO/p54nrb mRNA and protein levels were increased under normal conditions. In the PC-3 hypoxia model, where chemical hypoxia was induced and validated, NonO/p54nrb mRNA and protein levels were increased (Fig. 7). In co-transfection studies using promoter fragments, upregulation was detected when values obtained from co-transfections under normal conditions were compared with hypoxic values Fig. 7F). When YBX1 was ectopically expressed, nonp54 levels were induced, and this was seen more strongly under hypoxia. This suggests that nonp54 is regulated both directly via HIF and via the YBX1 HIF axis.

Indeed, HIF1A-AS3 has been shown to directly integrate with YBX1 and inhibit its ability to repress transcriptional activities by binding to the p21 and AJAP1 promoters, promoting hypoxic ovarian tumor (OC) progression (Xie et al. 2023). HIF1A-AS1 facilitates YBX1 phosphorylation through AKT interaction and forms a positive feedback loop with HIF1α, a mechanism associated with increased transcription, upregulation, and poor prognosis in pancreatic cancer (Xu et al. 2021).

In PC-3 cells, our findings demonstrate a novel HIF-1-mediated mechanism that upregulates NonO/p54nrb at the mRNA, protein, and transcriptional levels. Further-

more, the YBX1 transcription factor increases NonO/p54nrb expression under both normal and hypoxic conditions, but this increase is stronger in hypoxia.

Conclusion

Hypoxic gene regulation is critical for understanding pathophysiological processes such as cancer. During the transition from normal physiological conditions to hypoxia, cells alter the regulation of numerous genes. Genes regulated by the master regulatory transcription factor HIF1-α are abundant in the genome. This is the first time that NonO/p54nrb, a key gene in prostate cancer, is regulated by both HIF1α and YBX1 transcription factors. We believe that these data will significantly contribute to the elucidation of the molecular mechanisms of prostate cancer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10528-026-11349-7.

Author contributions SAT designed the research; SB, ME, FP and YVY performed the experiments; SAT and FK checked the results; SAT wrote the paper.

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Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not applicable.

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