NKX3.1 Homeodomain Protein Binds to Topoisomerase I and Enhances Its Activity

Cai Bowen,1 August Stuart,1 Jeong-Ho Ju,1 Jenny Tuan,1 Josip Blonder,2 Thomas P. Conrads,2 Timothy D. Veenstra,2 and Edward P. Gelmann1

1Departments of Oncology and Medicine, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20007. 2Laboratory of Proteomics and Analytical Technologies, Science Applications International Corporation-Frederick, Inc., National Cancer Institute at Frederick, Frederick, Maryland

Abstract
The prostate-specific homeodomain protein NKX3.1 is a tumor suppressor that is commonly down-regulated in human prostate cancer. Using an NKX3.1 affinity column, we isolated topoisomerase 1 (Topo I) from a PC-3 prostate cancer cell extract. Topo I is a class 1B DNA-resolving enzyme that is ubiquitously expressed in higher organisms and many prokaryotes. NKX3.1 interacts with Topo I to enhance formation of the Topo I-DNA complex and to increase Topo I cleavage of DNA. The two proteins interacted in affinity pull-down experiments in the presence of either DNase or RNase. The NKX3.1 homeodomain was essential, but not sufficient, for the interaction with Topo I. NKX3.1 binding to Topo I occurred independently of the Topo I NH2-terminal domain. The binding of equimolar amounts of Topo I to NKX3.1 caused displacement of NKX3.1 from its cognate DNA recognition sequence. Topo I activity in prostate-specific NKX3.1+ and NKX3.1− mice was reduced compared with wild-type mice, whereas Topo I activity in livers, where no NKX3.1 is expressed, was independent of NKX3.1 genotype. Endogenous Topo I and NKX3.1 could be communoprecipitated from LNCaP cells, where NKX3.1 and Topo I were found to colocalize in the nucleus and comigrate within the nucleus in response to either γ-irradiation or mitomycin C exposure, two DNA-damaging agents. This is the first report that a homeodomain protein in the context of known mechanisms of molecular carcinogenesis. Homeodomain proteins bind both to DNA and to other proteins and determine gene expression in a time- and location-dependent manner. For example, NKX3.1 binds to its cognate DNA-binding sequence, whereby it down-regulates transcription, or DNA repair. [Cancer Res 2007;67(2):455–64]

Introduction
NKX3.1 is a prostate-specific protein that is a member of the NK family of homeodomain proteins that include several organ-specific differentiation factors (1). NKX3.1 is expressed almost exclusively in developing and mature prostate luminal epithelium (2–4). The NKX3.1 gene is located on chromosome 8p21.2 and is a target for mutations that reduce expression of mRNA (14). Unlike most human tumor suppressor genes that undergo biallelic disruption to produce complete loss of suppressor function, NKX3.1 is activated by partial down-regulation (10).

To understand the mechanism by which reduced NKX3.1 expression can contribute to prostate carcinogenesis and tumor progression, we have sought to understand the function of the protein in the context of known mechanisms of molecular carcinogenesis. Homeodomain proteins bind both to DNA and to other proteins and determine gene expression in a time- and location-dependent manner. For example, NKX3.1 binds to its cognate DNA-binding sequence, whereby it down-regulates transcription, and also binds to serum response factor, whereby it activates transcription of genes downstream of serum response elements (16). To characterize the functional spectrum of NKX3.1 in cells of prostate origin, we isolated proteins from a cellular extract that bound to NKX3.1. One of the proteins found to bind NKX3.1 was topoisoasemerase (Topo) I.

Topo I is a DNA-resolving enzyme that participates in a wide range of functions, including transcription (17, 18), DNA replication (19, 20), and DNA repair (21). Human Topo I is a type IB enzyme, indicating that after binding, it cleaves a single DNA strand where, in the course of catalysis, a covalent bond between a tyrosine and the 3′ phosphate in the cleaved DNA strand is formed (19). After single-strand cleavage, Topo I unwinds the DNA, relaxates the broken strand, and releases, all in an ATP-independent reaction. Topo I has been shown to play a role in repair of UV-induced DNA damage and enhanced cell survival after UV irradiation (22). Topo I has also been shown to bind other tumor suppressor proteins, such as p53 (23) and p14ARF (24). Here, we describe the first known interaction of a homeodomain protein with Topo I. This report focuses on the interaction of the proteins in vitro and in cells. We found that NKX3.1 markedly enhanced the formation of the
Topo I-DNA complex and resultant DNA cleavage. We speculate that the effect of NKX3.1 on Topo I may have an effect on the ability of the cell to repair DNA or to respond to stress.

Materials and Methods

Cell culture. The human prostate cell lines PC-3 and LNCaP and human kidney cell line 293T were cultured in modified IMEM (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) at 5% CO2 and 37°C. Affinity chromatography. Maltose-binding protein (MBP) and MBP-NKX3.1 fusion proteins (13) expressed in *Escherichia coli* BL21 were bound to amylase resin beads (NEB, Beverly, MA). Protein-loaded beads were washed with column buffer containing 20 mmol/L Tris-HCl, 200 mmol/L NaCl and 1 mmol/L EDTA. Crude lysates from PC-3 cells were obtained by treating cells with 20 mmol/L Tris-HCl, 1% Triton X-100, 10% glycerol, 137 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na2VO4, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Lysates were added to pre-equilibrated MBP or MBP-NKX3.1-loaded amylase beads. After incubation and exhaustive washing, retained proteins were then released by boiling and analyzed by SDS-PAGE. Selected Coomassie-stained bands were subjected to in-gel digestion using trypsin, and the resultant peptides were analyzed using a capillary liquid chromatography (LC)-microelectrodes sample mass spectrometry (MS) system using a LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) with Agilent 1100 micropipetillary LC system (Palo Alto, CA). The peptides are analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights (MS) and tandem MS (MS/MS) to determine amino acid sequence in successive instrument scans. The resultant peptides were also analyzed by MALDI-TOF/TOF using a Voyager 4700 mass spectrometer (Applied Biosystems, Inc., Framingham, MA). The MS/MS spectra were analyzed using the SwissProt, and National Center for Biotechnology Information protein sequence databases were used to search the LC/MS and MALDI-MS data using SEQUEST and MASCOT, respectively.

DNA relaxation assay. The reaction was done according to the protocol from Topogen (Columbus, OH) in final volumes of 20 μL with 10 ng (~5.5 nmol/L) Topo I. Topo I is supplied as units per volume; therefore, all assignments of molarity were calculated based on the midpoint of the range provided by the supplier for each lot of enzyme received. NKX3.1 recombinant proteins were preincubated with Topo I at room temperature for 10 or 60 min as indicated before the addition of 250 ng (7.1 nmol/L) supercoiled pHOT1 plasmid DNA at 37°C. DNA was electrophoresed in 1% agarose gels at 15V (2 V/cm) for 18 h. Gels were stained with ethidium bromide and imaged under UV light. pHOT1 contains a single TAAGTC suicide substrate and 14 to 110 nmol/L (3- to 20-fold) Topo I in buffer containing 50 mmol/L Hepes (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L EDTA, 0.1% Triton X-100, 5% sucrose, 0.1% bovine serum albumin (BSA), 5 mmol/L DTT, 5 ng/μL deoxyinosine-deoxythymidine. Samples were incubated 15 min after addition of 1.5 mmol/L of labeled probe. Samples were resolved in 8% native gel and visualized by autoradiography. A timed competition assay was done with end-labeled oligonucleotide with the NKX3.1 binding motif incubated for 15 min with NKX3.1 at room temperature. Topo I or ovalbumin was then added and incubated for different amounts of time. Samples were resolved in 8% native gel and visualized by autoradiography.

Coimmunoprecipitation of Topo I and NKX3.1. Nuclear extract was isolated from 105 LNCaP cells cultured in modified IMEM with 10% FBS. Cells were rinsed with PBS and then resuspended in 2.5 mL of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L EDTA, 0.1% Triton X-100, and proteinase inhibitor at room temperature for 10 min. The nuclei were isolated by centrifugation at 800 × g at 4°C and resuspended in two volumes of 20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.2 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and proteinase inhibitor and stirred at 4°C for 30 min. The suspension was clarified by centrifugation at 4°C at 12,000 × g for 30 min. Coimmunoprecipitation was done with the Seize Primary Immunoprecipitation kit (Pierce, Rockford, IL) according to instructions provided, except that eluted proteins were incubated at 4°C for 30 min in the presence of 1% volume of 2% sodium deoxycholate. Washed and air-dried coimmunoprecipitated pellets were resuspended in loading buffer and resolved by SDS-PAGE. Immunoprecipitated Topo I and NKX3.1 were analyzed with mouse anti-human Topo I (BD Biosciences Pharmingen, San Jose CA) and mouse anti-human NKX3.1 antibody (Zymed, San Francisco, CA).

**Immunoprecipitation-relaxation assay.** Immunoprecipitation was done by first combining 10 μg goat anti-human NKX3.1 antibody with an equal amount of goat IgG with 50 μL protein A/G agarose slurry in radioimmunoprecipitation assay buffer overnight at 4°C. One milligram of LNCaP cell nuclear extract was incubated with the antibody-conjugated agarose for 2 h at 4°C with rotation. The relaxation assay was carried out with the protein complex-bound agarose as described above.

**Protein colocalization.** LNCaP cells growing in IMEM supplemented with 5% FBS were treated with 20 Gy γ-irradiation and fixed with either formalin or cold methanol. The enhanced green fluorescent protein (EGFP)-Topo I fusion protein was introduced using the pTi1 plasmid kindly provided by William T. Beck (University of Illinois, Chicago, IL). Cells to be fixed in formalin, but not those fixed in methanol, were permeabilized with 0.05% Triton X-100 for 10 min and incubated with both rabbit anti-human Topo I antibody (F14, LAE Biotech International, Rockville, MD) for endogenous Topo I and/or mouse anti-human NKX3.1 antibody simultaneously at 1:1,000 dilution at room temperature for 1 h followed by continuously incubation at 4°C overnight. The rabbit antiserum was visualized by fluorescein-conjugated antirabbit secondary antibody (Vector Laboratories, Burlingame, CA). The mouse antibody was detected by biotinylated secondary antmouse antibody (Vector Laboratories) and then amplified by Texas red avidin (Vector Laboratories). Fluorescent images were examined under an Olympus IX-70 laser scanning confocal microscope (Olympus America, Center Valley, PA).
DNA relaxation assay with murine tissue extracts. DNA relaxation was done with nuclear extracts of prostate and liver from 8-week-old Nkx3.1 gene–targeted mice. The nuclear extracts were isolated as recommended by Topogen. Briefly, dissected murine prostate tissues were rinsed with cold PBS containing protease inhibitors and then submerged in TEMP buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 4 mmol/L MgCl2, 0.5 mmol/L PMSF]. Following 15 min of incubation in TEMP on ice and homogenization, the nuclei were resuspended and incubated for 30 min on ice in two volumes of TEP buffer (TEMP lacking MgCl2) and an equal volume of 1 mol/L NaCl. Supernatant was collected by centrifugation for 30 min at 4°C. DNA relaxation was carried out as described before with 200 ng nuclear extract protein and 250 ng pHOT1 plasmid DNA for 30 min at 37°C. DNA was resolved on 1% agarose gels containing ethidium bromide.

**Glutathione S-transferase pull-down assay.** The glutathione S-transferase (GST) fusion proteins, GST-Topo 70, GST-Topo D26, and GST-Topo A2, were kindly provided by Daniel T. Simmons, Ph.D. (University of Delaware, Newark, DE). For pull-down assays, equimolar amounts of either GST or GST fusion proteins on glutathione sepharose beads (Amersham Biosciences, Uppsala, Sweden) were incubated with 20 μL [35S]methionine-labeled wild-type (WT) Nkx3.1 translated with TNT Quick Coupled Transcription/Translation System (Promega, Madison WI) in PBS containing 1% BSA and 5 units/ml DNase for 1 h at room temperature. Topo I was affinity assayed using polyhistidine-tagged Nkx3.1 recombinant peptides constructed and produced in our laboratory. Equimolar amounts of either His or His-Nkx3.1 fusion peptides were immobilized on MagZ binding technology, and mouse anti-human specific antimurine cytokeratins 8 and 18 antibody (Santa Cruz Biotechnology). Antibodies: mouse anti-human Topo I (BD PharMingen, San Diego, CA), rabbit anti-human Nkx3.1 antiserum (3), mouse anti-human cytokeratin (C11) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), epithelial-specific antimurine cytokeratins 8 and 18 antibody (Santa Cruz Biotechnology), and mouse anti-human β-actin antibody.

**Results**

**Isolation of Topo I by Nkx3.1 affinity.** To isolate cellular proteins that bind to Nkx3.1, we passed a lysate of PC-3 prostate cancer cells through an affinity column made with a fusion protein of Nkx3.1 and bacterial MBP bound to amylase beads. We selected PC-3 prostate cancer cells that do not express endogenous Nkx3.1 instead of LNCaP prostate cancer cell that do because we did not want interference with our affinity reagent by endogenous protein. We have shown previously that the Nkx3.1-MBP fusion protein is functional and can bind the high-affinity TAAGTA Nkx3.1 DNA-binding site (15, 25). Nkx3.1-MBP adheres to an amylase column and can be eluted along with adherent proteins. As a control, PC-3 cell extracts were passed through a parallel amylase column that was preloaded with electrophoretically pure MBP. PAGE of the proteins and the affinity reagents that remain on the two columns after exhaustive washing were eluted and are shown in Fig. 1. The Nkx3.1-MBP fusion protein retained more proteins than the MBP column. Several bands were subjected to in-gel digestion and the digests were analyzed by nanoflow reversed-phase LC coupled online with electrospray ionization MS/MS. A total of 19 unique peptides corresponding to 35% sequence coverage of Topo I was obtained in the MS analysis. Mascot analysis of the peptide mapping results indicated the identification of Topo I with a confidence interval of 90% (Supplementary Table S1). No other protein was identified with a confidence interval above 0%. The peptide mapping results, confirmed by the LC/MS/MS results, unequivocally show that the major protein within the selected band in Fig. 1 is Topo I. A Western blot of PC-3 cell extract showed a 70-kDa truncated Topo I peptide fragment (Supplementary Fig. S1). The ~70-kDa migration of the Topo I band is consistent with a known spontaneous in vitro proteolytic cleavage fragment of the 91-kDa full-length protein (26). A band that migrated nearly in parallel from the MBP column did not contain peptide sequences of Topo I. All the peptides isolated from the band migrating at 70 kDa were derived from amino acids COOH terminus to residue 174, the site of cleavage for the truncated Topo I.

**Physical association between Nkx3.1 and Topo I.** Human Topo I is a 91-kDa, 765-amino acid protein with four major domains, two of which, the core and COOH-terminal domains, cooperate to form a “C” clamp around DNA that is a precursor for DNA cleavage, unwinding, and religation. Because both Nkx3.1 and Topo I are DNA-binding proteins, we sought to determine whether the interaction of the two was dependent on nucleic acid. The physical association of Nkx3.1 and GST-Topo 70 was studied in the absence and presence of camptothecin, a natural product chemotherapeutic that binds the Topo I-DNA complex, but not to either component alone. Camptothecin had no effect on the interaction between Nkx3.1 and GST-Topo 70 (Fig. 2A). We also treated the complex with either DNase or RNase and observed no effect on the interaction (Fig. 2A). Equivalent amounts of GST and GST-Topo 70 were loaded onto columns as shown in Fig. 2A (inset). Lastly, we did the converse experiment in the presence of DNase, binding in vitro transcribed and translated [35S]Topo I to a His-Nkx3.1 fusion protein bound to a MagZ binding column and showed preferential binding to the fusion protein compared with a polyhistidine peptide alone (Fig. 2B).

Nkx3.1 binds to Topo I and can combine with the Topo I-DNA complex. This interaction was suggested by an EMSA using the 50-mer Tetrathymina sequence as a probe and purified proteins. The amount of Nkx3.1 used in this assay alone did not bind to the Tetrathymina 50-mer probe (data not shown). However, Nkx3.1 enhanced Topo I binding as shown by the increasing intensity of

Figure 1. PC-3 proteins binding to Nkx3.1 affinity beads. A PC-3 cell extract was subjected to affinity chromatography using MBP or Nkx3.1-MBP fusion proteins bound to amylase resin beads. After washing, the selectively retained proteins were released by boiling and separated on a polyacrylamide gel followed by Coomassie blue staining. Bands associated with the Nkx3.1-MBP beads but not on the MBP alone lane presumably contained proteins that bound to Nkx3.1 and were identified using in-gel digestion and nanoflow reversed-phase LC coupled online with MS/MS. Arrow, band that is 70-kDa proteolytic cleavage product of human Topo I (26).
the two species formed by Topo I and the DNA probe and the depletion of the probe-alone band. Moreover, a supershifted complex can be seen, suggesting the formation of a complex of probe, Topo I, and NKX3.1 (Fig. 2C). Binding of Topo to NKX3.1 is of sufficiently high affinity to compete NKX3.1 from its cognate DNA sequence at a Topo I/NKX3.1 molar ratio as low as 2:1 (Fig. 2D). This was further confirmed by allowing the complex between NKX3.1 and DNA to form and then adding either Topo I or

**Figure 2.** Physical interaction of NKX3.1 and Topo I. A, equimolar of GST and GST-Topo I (70; 300 nmol/L) fusion proteins immobilized on glutathione sepharose beads were used in pull-down assays as described in Materials and Methods. [35S]methionine-labeled NKX3.1 in PBS containing 1% BSA in the presence or absence of 1 μmol/L camptothecin (CPT), 5 units/mL DNase, or 1 μg/mL RNase for 1 h at room temperature. Inset, equivalent amounts of input glutathione-bounded fusion proteins GST and GST-Topo I (70) revealed by silver stain. B, either 4 μg His-NKX3.1 fusion protein or equimolar polyhistidine peptide tag was immobilized on Mag2 binding particles and incubated with [35S]Topo I in PBS containing 1% BSA and 5 units/mL DNase. C, 110 nmol/L Topo I was incubated with a 50-mer oligonucleotide containing the Tetrahymena Topo I recognition site in the presence of increasing amounts of NKX3.1. D, 120 nmol/L NKX3.1 was incubated with oligonucleotides containing the cognate recognition sequence in the presence of increasing amounts of Topo I. E, NKX3.1-DNA complexes were formed for 15 min before the addition of either Topo I or ovalbumin for the indicated times. Complexes were subjected to 8% native gel electrophoresis as described in Materials and Methods.

**Figure 3.** Deletion analysis of Topo I for NKX3.1 binding. A, a map of full-length Topo I and its truncated derivatives used as GST fusion proteins in pull-down and DNA relaxation assays. Right, silver stain of each fusion protein preparation. B, GST-Topo I (70; 330 nmol/L) and equimolar amounts of GST-Topo I variants and of GST were immobilized on glutathione sepharose beads. In vitro translated NKX3.1 labeled with [35S]methionine was incubated with beads in the presence of 5 units/mL DNase in PBS containing 1% BSA for 1 h at room temperature. C, DNA relaxation assays were done as described in Materials and Methods with 7 nmol/L GST-Topo I and equimolar amounts of truncated fusion proteins bound to glutathione sepharose in the presence or absence of 30 nmol/L NKX3.1 or 30 nmol/L ovalbumin.
ovalbumin for different time intervals to show the specificity of Topo I interference with the binding of NKX3.1 to DNA (Fig. 2E).

Topo I has been shown to bind to other proteins, including suppressors, such as p53 (27) and p14ARF (24), which both complex with the NH2-terminal domain of Topo I. We used deleted fragments of Topo I fused to GST to determine the domains that interact with NKX3.1 (Fig. 3A). Topo A2 contains NH2 terminus (1–175), which has no catalytic activity. Topo 70 (175–765) consists of core domain, linker region, and COOH-terminal domain and is enzymatically active. Topo D26 (320–765) contains only a partial core domain that terminates at the core subdomain II. The core subdomain II (residues 233–319) and core subdomain I (residues 215–232 and 320–433) form the Topo I lobe or "cap" of the enzyme, whereas core domain III and the COOH-terminal domain form the bottom lobe of the enzyme (28, 29). The core and catalytic domains alone can complex to DNA and together cause cleavage and unwinding. The NH2-terminal region is unstructured and not required for catalytic activity but is required for nuclear localization and the physiologic response of Topo I to DNA damage (27). The region 191 to 206 regulates strand rotation and is critical for blunt-end ligation by Topo I (30). The linker region is not essential for enzymatic activity but forms a coiled coil motif that juts out from the active part of the enzyme and is believed to regulate the rate of DNA unwinding (31).

Using GST fusion constructs of Topo I fragments D26, A2, and 70 obtained from Daniel T. Simmons (Fig. 3A; ref. 32), we asked which fragments complexed with in vitro translated [35S]NKX3.1. NKX3.1 bound to GST-Topo D26 and GST-Topo 70 but not to GST-Topo A2 (Fig. 3B). The Topo I fusion constructs were also tested in plasmid relaxation assays with and without NKX3.1. Topo D26, which
showed the strongest physical association with NKX3.1 in the GST pull-down assay, did not relax plasmid DNA. Topo 70 that also bound NKX3.1 had supercoiled DNA relaxing activity that was enhanced by NKX3.1 but not by ovalbumin (Fig. 3C).

**NKX3.1 enhances Topo I cleavage of DNA.** To establish whether NKX3.1 affected Topo I activity, we did a classic cleavage reaction where a partial duplex “suicide” substrate is cleaved by Topo I that becomes covalently bound to the DNA. After purification of the Topo I-DNA complex, proteinase digestion produces a radiolabeled single DNA strand that is bound to a Topo I fragment and thus retarded on gel migration (31, 33, 34). Stoichiometric amounts of NKX3.1 activated substrate cleavage by Topo I, whereas similar concentrations of BSA had no detectable effect (Fig. 4A). We then examined the Topo I cleavage reaction in the presence of KCl, which at higher concentrations causes dissociation of the Topo I-DNA complex, although complexes already formed can proceed to complete religation (28). In the presence of 200 and 250 mmol/L KCl, NKX3.1 enhanced formation of the cleavage complex. Equimolar concentrations of ovalbumin, a nonspecific protein, had a minor effect at 200 mmol/L KCl and no effect at 250 mmol/L KCl (Fig. 4B). In the presence of NKX3.1, formation of the cleavage complex was accelerated and proceeded more efficiently over a 30-min reaction (Fig. 4C). When a religation substrate oligonucleotide was added to the reaction, NKX3.1 facilitated formation of the cleavage complex and completion of the religation reaction within 5 min (Fig. 4D). Topo I is also assayed by relaxing supercoiled DNA often using a pUC12 plasmid that includes a 50-nucleotide insert derived from the Tetrahymena rRNA gene repeat as a substrate (pHOT1; ref. 35). In a dose-dependent manner, NKX3.1 enhanced plasmid relaxation by Topo I. There was a minimal effect of adding equimolar amounts of a nonspecific protein, ovalbumin. As seen in Fig. 4E, 15 nmol/L ovalbumin reduced the supercoiled DNA by 10%; 3.7 nmol/L NKX3.1 reduced the supercoiled DNA by 87%.

We next examined the interaction of NKX3.1 with Topo I cleavage activity by varying the amounts of each protein. The result is seen in Fig. 5A, where NKX3.1 alone neither cleaved nor bound the radiolabeled suicide substrate but had a marked affect on Topo I activity. The amount of Topo I used generated little to no cleavage during the 30-min reaction (Fig. 5A, top inset). Fig. 5A (top inset, Y axis) indicates relative amount of uncleaved DNA determined by densitometry of the gel in Fig. 5A (bottom inset). The effect of NKX3.1 was seen at stoichiometric concentrations. We also titrated Topo I in the presence of 120 nmol/L NKX3.1 and showed that NKX3.1 changed the efficiency of the cleavage reaction catalyzed by Topo I (Fig. 5B).

NKX3.1 is a DNA-binding protein with a preferred hexanucleotide recognition sequence TAAGTC (15). pHOT1 contains a single TAAGTC NKX3.1 hexanucleotide binding sequence. We mutated this sequence to a CAAATC that does not bind NKX3.1 (15) to control for possible NKX3.1 binding to the plasmid. In addition, we used a plasmid pCMV that was constructed with pcDNA3 and

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effect of NKX3.1 on DNA cleavage and relaxing activity of Topo I. Recombinant NKX3.1 was preincubated with Topo I for 30 min before addition of 0.1 pmol 5′-[32P]-end-labeled suicide substrate followed by an additional 30 min at 37°C. The reactions were terminated by addition of 1% SDS. The DNA substrate was digested with proteinase K, heat denatured, and electrophoresed on 16% polyacrylamide gels with 7 mol/L urea. A, titration of NKX3.1 was carried out in the presence or absence of constant amounts of Topo I and constant amounts of the radiolabeled CL14/CP25 partial duplex oligonucleotide cleavage reagent. Topo band, complexed labeled partial duplex bound to Topo I. B, titration of Topo I in the presence or absence of a constant amount of NKX3.1. The fractions of cleaved DNA were determined by dividing the amount of cleaved DNA by the input substrate in the control lane (A and B, far left). C, DNA relaxation assays were done with pHOT1 and a CMV promoter–containing plasmid. In each case, the TAAGTC site in each plasmid was mutated to CAAATC by site-directed mutagenesis. pHOT1 native (WT) and mutant (M) constructs each at 7.1 nmol/L and pCMV constructs at 5.4 nmol/L were incubated with reaction buffer (lanes 1–4), with 5.5 nmol/L Topo I (lanes 5–8), or with both 5.5 nmol/L Topo I and 23.5 nmol/L NKX3.1 (lanes 9–12). D, plasmid pHOT1 relaxation assay with either Topo-68 or full-length enzyme at the indicated concentrations in the presence or absence of 190 nmol/L NKX3.1. E, plasmid relaxation assay was done with 5 nmol/L Topo I and mutant Topo I(Y723F) (Topogen) with 30 nmol/L NKX3.1. The procedure was the same as described above. There was no effect of Topo I(Y723F) in the presence or absence of NKX3.1.
Renilla luciferase. This plasmid also has a single TAAGTC site that was mutated as well. Both plasmids were unwound by Topo I in a reaction that was enhanced by the presence of NKX3.1. Loss of the NKX3.1 binding sites on either of the plasmids had no effect on NKX3.1 interaction with the reaction (Fig. 5C). Topo I contains an NH2-terminal domain important for nuclear localization and protein-protein interactions (19). The NH2-terminal domain is also a substrate for proteases and confers instability to Topo in vitro. We compared quantitatively the functional interaction of Topo-68, an NH2-terminal (Δ1–190) truncated Topo and full-length Topo I with NKX3.1 (26, 36). In a DNA relaxation assay we found that Topo-68 was activated by NKX3.1 perhaps even to a greater degree than full-length Topo I (Fig. 5D). Lastly, to show further that the DNA relaxation effects seen were dependent on Topo I activity, we compared the effect of NKX3.1 on Topo I and on the mutant Y723F protein that has lost the active site tyrosine. As shown in Fig. 5E, the Y723F mutation abrogated Topo I activity in the presence or absence of NKX3.1, further showing the specificity of the interaction between NKX3.1 and Topo I.

The competition of Topo I with the NKX3.1 cognate DNA sequence for NKX3.1 binding suggested that Topo I bound NKX3.1 at or near the homeodomain (Fig. 2D and E). We used purified recombinant NKX3.1 peptide fragments in a Topo I relaxation assay with pHOT1 as a substrate (Fig. 6, left and middle). We used two concentrations of NKX3.1 and its peptide fragments with 5 pmol Topo I. Under these conditions, NKX3.1 potentiated DNA relaxation in a 30-min reaction. Deletion of the inhibitory COOH-terminal domain distal to the homeodomain [NKX3.1(1–184)] had very little effect on the interaction with Topo I. Deletion of the COOH terminus and the homeodomain NKX3.1(11–123) abrogated the interaction with Topo I, even at a molar ratio of NKX3.1/Topo I of 7:1, suggesting that the homeodomain is critical for the interaction with Topo I. The NKX(1-123) construct was used as fusion product with a thioredoxin tag. The thioredoxin fragment alone had no effect on Topo I activity and had no inhibitory effect when added to DNA relaxation assays with Topo ± NKX3.1 (data not shown). A DNA relaxation assay with either 3.8 or 19 pmol electrophoretically pure NKX3.1(1–123) without the thioredoxin tag was compared with 3.8 pmol NKX3.1(1–184) and showed no detectable effect of NKX3.1(1–123) on Topo I DNA relaxing activity (data not shown). 

We also repeated the experiment with NKX3.1(1–123) cleaved from pure NKX3.1(1–123) and confirmed the near absence of interaction with the thioredoxin tag and confirmed the near absence of interaction with Topo I relaxation activity.

NKX3.1(114–184) containing the homeodomain and upstream 10 amino acids showed comparable enhancement of Topo I activity to the full-length NKX3.1 protein. Interestingly, the 10 amino acids NH2 terminus to the homeodomain were important to optimize the interaction because the homeodomain alone [NKX3.1(124–184)] was inactive at an approximately 3.5 molar ratio but active at an approximately 7:5 molar ratio with Topo I. Lastly, we showed that binding between the NKX3.1 peptide fragments and Topo I paralleled the capacity of NKX3.1 to enhanced DNA relaxation activity. His-NKX3.1 fusion peptides that corresponded to each of the expression constructs were used to pull-down [35S]Topo I. Substantial binding was seen for the His fusions with NKX3.1, NKX3.1(1–184), and NKX3.1(114–184) (Fig. 6, right).

Topo I activity in tissues. The effect of the homeoprotein on the activity of endogenous Topo I was examined in tissues from Nkx3.1 gene–targeted mice. Equal amounts of protein extracts from prostate tissues were used in DNA relaxation assays. Samples from different individual animals showed that intact mice had high levels of DNA relaxing activity. Prostate extracts from Nkx3.1+/− mice had reduced levels of activity, whereas extracts from Nkx3.1−/− mice had lower activities than the heterozygous mice (Fig. 7A). In contrast, DNA relaxing activity in extracts from liver, where Nkx3.1 is not expressed, did not vary depending on the genotype (Fig. 7B). The degree of DNA relaxation by different extracts is shown in Fig. 7C. The effect of Nkx3.1 deletion was seen in prostate tissue extracts but not in liver. The reduced levels of Nkx3.1 protein in the prostate tissues was confirmed by Western blot of the extracts used for the relaxation assays (Fig. 7D). Moreover, the relative amounts of epithelial cells that contributed to the extracts were similar as shown by the Western blots for cytokeratins 8 and 18.
NKX3.1 and Topo I interaction in cells. Endogenous NKX3.1 and Topo I from LNCaP cells could be coprecipitated with NKX3.1 antibody. Immunoprecipitates were blotted simultaneously with antibodies to NKX3.1 and Topo I. Activity of the immunoprecipitate was verified with pHOT as a substrate in a DNA relaxation assay (Fig. 8). Figure 8 (top) shows communoprecipitation of Topo I and NKX3.1 analyzed by Western blot. Figure 8 (bottom) displays the DNA relaxation result done with the indicated immunoprecipitates.

In LNCaP cells that express endogenous NKX3.1, we determined the subcellular localization of NKX3.1 and Topo I. The subcellular location of Topo I has been generally found in the nucleolus with additional punctate distribution in the nucleoplasm (37, 38). The nucleolus is a nuclear nonmembrane protein structure, and its formation is cell cycle and cell growth dependent (39). NKX3.1 and Topo I were localized by antibody staining to aggregates in the nucleus as shown on Fig. 9 (left). Exposure to 20 Gy γ-irradiation resulted in delocalization from the nuclear aggregates but continued colocalization of NKX3.1 and Topo I up to 45 min after irradiation (Fig. 9). We also transfected LNCaP cells with a fusion construct of Topo I and EGFP. The EGFP-Topo I fusion protein localized similarly to Topo I. NKX3.1 was detected by antibody staining. The two proteins were seen to colocalize in the nuclei (Fig. 9B). After exposure to mitomycin C, which generates double-stranded DNA breaks, both signals delocalized but remained nuclear and continued to colocalize to a substantial degree as suggested by the high-magnification images on Fig. 9B (bottom).

Discussion

NKX3.1 is a DNA-binding protein that is a transcriptional repressor (15). NKX3.1 binds to other transcription factors, such as serum response factor, and thereby can synergize transcriptional activation (16). However, the role of serum response factor in the prostate has not been defined. NKX3.1 has also been shown to interact with SP transcription factors to regulate expression of prostate-specific antigen (40). Because NKX3.1 is expressed early in murine embryonic development as a marker of somitogenesis, the serum response factor interaction may be related to early developmental events and not differentiation of the prostate gland (41). NKX3.1 also interacts with the prostate-derived ETS-related factor and may thereby affect gene expression in the prostate (42).

In an attempt to identify other proteins that interact with NKX3.1, we did affinity chromatography and isolated Topo I among other proteins. We have shown that the NKX3.1 homeodomain protein binds to Topo I and enhances formation of the Topo I-DNA complex. NKX3.1 and Topo I colocalize in cells and seem to be linked in the cellular response to DNA-damaging agents. Based on our binding studies, it seems that NKX3.1 accelerates the formation of the Topo I-DNA complex and is also a noncovalent component of that complex. The protein-protein interaction involving the
NKX3.1 homeodomain and Topo I effectively competes with the
cognate NKX3.1 DNA sequence for binding to the homeodomain.
We can speculate that through high-affinity binding, NKX3.1 may
enhance transcription mediated by Topo I in developing prostate
epithelial cells, but at times of transcriptional quiescence, NKX3.1
may become less engaged in transcription and therefore bind
directly to DNA recognition sites where it acts as a repressor (15).

As a protein that interacts with DNA and with at least two
transcription factors, it is clear that NKX3.1 has complex functions
that will have to be described to understand how the protein
affects oncogenic pathways. To link the Topo I interaction to an
oncogenic pathway, we sought to determine whether NKX3.1
affected transcriptional quiescence, NKX3.1
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As a protein that interacts with DNA and with at least two
transcription factors, it is clear that NKX3.1 has complex functions
that will have to be described to understand how the protein
affects oncogenic pathways. To link the Topo I interaction to an
oncogenic pathway, we sought to determine whether NKX3.1
cellular localization was affected by DNA-damaging agents. We
found that both NKX3.1 and Topo I acutely altered their subcellular
localization in response to both γ-irradiation and mitomycin C.
The subcellular location of Topo I has been generally found in the
nucleus with additional punctate distribution in the nucleo-
plasm (37, 38). The nucleolus is a nuclear nonmembrane protein
structure, and its formation is cell cycle and cell growth dependent
(39). The subcellular distribution of Topo I can depend on both cell
type and growth conditions. For example, in CEM human leukemia
cells, Topo I is seen predominantly in the nucleoli (38). In A431
human epidermoid carcinoma cells and A549 human non–small
lung cancer cells, Topo I is detected both in the nucleoplasm
and the nucleoli (43, 44). Cell cycle phase also influences the
subcellular localization of Topo I (43, 45). Lastly, both nutrient
depression and cytotoxic agents can affect the nucleolar
localization of Topo I (38, 46).

In one of our experiments (Fig. 9B), we expressed EGFP-Topo I in
LNCaP cells by transient transfection of pTI-2 and found Topo I
exclusively in the nucleus at interphase as was reported previously
(46). About 5% to 10% of the transfected cells had nucleolar
localization of Topo I. NKX3.1 was seen to have a punctate
distribution in nucleus. Some cells had nucleolar localization of
NKX3.1. Colocalization of NKX3.1 and Topo I was detected with
both EGFP-Topo I and double-antibody staining. After exposure
either to irradiation or to mitomycin C, both Topo I and NKX3.1
delocalized to be more homogenously distributed, consistent with
the notion that the two proteins interacted during the response to
DNA-damaging agents.

Topo I is involved in a variety of processes affecting DNA,
including replication, transcription, and recombination (20, 47).
Topo I can also affect DNA repair efficiency. For example, Topo I
is induced to bind p53 by UV-induced DNA damage (22, 23, 48).
The tumor suppressor p53 is known to activate Topo I by binding to
the enzyme (49, 50). Both WT and mutant forms of p53 are equally
effective. A role for Topo I in DNA repair has not been well defined
but is proposed, in part, because of the interaction with p53 (48).
Topo I binding to DNA is also favored at sites affected by oxidative
damage, suggesting a possible role in DNA repair (51).

**Figure 9.** Colocalization of NKX3.1 and Topo I. A, LNCaP
prostate cancer cells grown in the presence of 1 nmol/L
R1881 were subjected to 20 Gy irradiation and fixed with
formalin at the indicated times. Indirect fluorescence assay
was carried out by staining with murine anti-NKX3.1
antibody and rabbit anti–Topo I antibodies (1:1,000).
The rabbit secondary antibody was detected by
fluorescence conjugated anti rabbit secondary antibody
(1:1,000). The mouse anti-NKX3.1 antibody was detected
using antimouse biotinylated secondary antibody (1:200)
and the signal was revealed with Texas red avidin. B, pTI-2
plasmid (pEGFP-Topo I–transfected LNCaP cells grown
on coverslips were treated with 10 μg/mL mitomycin C
and fixed with cold methanol at the indicated times. Cells were
probed with mouse anti-human NKX3.1 antibody. Signal
was revealed by (1:500) Alexa Fluoro 568 antimonochrome
antibody. Expression of the proteins was examined by
Olympus IX-70 laser scanning confocal microscope.
Activation of Topo-I is also affected by another tumor suppressor protein, p14ARF, which interacts with Topo-I via the NH2 terminus and activate its DNA relaxation activity (24, 52). There may be a common mechanism underlying the role that Topo-I plays in tumor suppression by these other proteins yet to be identified. Interestingly, Topo-I is also bound by oncogenic proteins, such as SV40 T antigen (53, 54). Topo-I also interacts with the Werner helicase, a member of the RecQ family of helicases that play a role in genomic integrity (55).

References

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