Ubiquitination by TOPORS Regulates the Prostate Tumor Suppressor NKX3.1*

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The NKX3.1 gene located at 8p21.2 encodes a homeodomain-containing transcription factor that acts as a haploinsufficient tumor suppressor in prostate cancer. Diminished protein expression of NKX3.1 has been observed in prostate cancer precursors and carcinomas. TOPORS is a ubiquitously expressed E3 ubiquitin ligase that can ubiquitinate tumor suppressor p53. Here we report interaction between NKX3.1 and TOPORS. NKX3.1 can be ubiquitinated by TOPORS in vitro and in vivo, and overexpression of TOPORS leads to NKX3.1 proteasomal degradation in prostate cancer cells. Conversely, small interfering RNA-mediated knockdown of TOPORS leads to an increased steady-state level and prolonged half-life of NKX3.1. These data establish TOPORS as a negative regulator of NKX3.1 and implicate TOPORS in prostate cancer progression.

Prostate cancer is the second leading cause of cancer deaths and the most frequently diagnosed malignancy in American men (1). Prostate carcinomas are considered to arise from cancer precursors including prostatic intraepithelial neoplasia (PIN)2 and proliferative inflammatory atrophy (2). Molecular alterations, including hereditary and somatic gene mutations, gene deletions, gene amplification, chromosomal rearrangements, as well as epigenetic changes, have been implicated in prostate cancer initiation and progression (3).

The NKX3.1 gene has been studied extensively over the past decade for its roles in prostate development and carcinogenesis (4). The expression of the mouse Nkx3.1 gene is androgen-dependent and is restricted largely to prostate epithelial cells in adults (5–7). Deletion of Nkx3.1 by gene targeting leads to prostate ductal morphological defects, as well as prostatic dysplasia and hyperplasia that resembles human PIN (8–10). Interestingly, heterozygous Nkx3.1 mice also develop hyperplasia and PIN-like lesions (8). The human NKX3.1 gene maps to 8p21.2 within a region where loss of heterozygosity occurs in PIN and is common in prostate carcinomas; however, no mutations have been found in the coding region of the NKX3.1 allele remaining (11, 12). In light of these observations, NKX3.1 has been proposed to function as a haploinsufficient tumor suppressor. In support of a dose-dependent growth regulatory function of NKX3.1, reduced but not complete loss of NKX3.1 protein expression is now well documented in most human prostate cancer samples in a manner inversely correlated with Gleason score (13) and also with disease progression (14). Diminished NKX3.1 expression is thought to be an early event in prostate carcinogenesis, and reduced NKX3.1 immunostaining was observed in most proliferative inflammatory atrophy and PIN lesions analyzed (13). The diminished NKX3.1 expression may be partly attributed to selective CpG methylation of the NKX3.1 promoter in some prostate cancer cases (15). Nevertheless, quantitative analyses of mRNA and protein levels of NKX3.1 in prostate carcinoma lesions revealed a lack of concordance between mRNA and protein levels (13). In particular, decreased protein accumulation was found in four of six cases with a normal or increased mRNA level, suggesting post-transcriptional regulation of NKX3.1 occurs in a significant percentage of prostate carcinomas (13). It appears that NKX3.1 protein levels are regulated by multiple mechanisms in normal prostate, prostate cancer precursors, and carcinomas (13).

To gain further insights into the regulation of NKX3.1, we have studied its post-translational modification. NKX3.1 is known to be phosphorylated by protein kinase C, and this modification regulates DNA binding activity (16). We previously reported that the stability of NKX3.1 protein is regulated by protein kinase CK2 in LNCaP cells, and CK2 phosphorylation prevents ubiquitin-mediated NKX3.1 degradation by the 26S proteasome (17). Ubiquitin is an 8-kDa small-molecule modifier that can be covalently attached to lysine residues of a substrate protein. Ubiquitination is catalyzed by three enzymes working in a cascade: ubiquitin-activating enzyme E1, ubiquitin carrier enzyme E2, and ubiquitin-protein isopeptide ligase E3. Ubiquitin itself contains 7 lysine residues, and the ubiquitin chain can be lengthened by the E2 and E3, leading to polyubiquitination of a substrate protein. Typically, a minimum of four sequentially attached ubiquitin moieties allows the target pro-

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2 The abbreviations used are: PIN, prostatic intraepithelial neoplasia; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GST, glutathione S-transferase; GFP, green fluorescent protein; Ni-NTA, nickel-nitritriacetic acid; siRNA, small interfering RNA; HA, hemagglutinin; PML, promyelocytic leukemia.
tein to be recognized and degraded by the 26S proteasome (18). Although the most recognized function of ubiquitination remains as a signal for protein degradation, protein ubiquitination has recently been realized to regulate diverse protein functions and cellular processes, including the cell cycle, DNA repair, transcription, endocytosis, and sorting (19).

As the specificity of ubiquitination is largely conferred by the recognition of substrates by E3, we attempted to identify candidate ubiquitin E3 ligases for NKX3.1 using a “guilt by association” strategy. NKX3.1 has recently been demonstrated to interact with topoisomerase I and enhance topoisomerase I DNA-unwinding activity (20). Topoisomerase I is thought to interact with the RING domain-containing protein TOPORS (21), which is known to interact with the tumor suppressor p53 (22) and later was found to ubiquitinate p53 (23). TOPORS is widely expressed in various tissues including the prostate (24).

Although the most recognized function of ubiquitination is to mark proteins for degradation by the 26S proteasome (18), TOPORS has been shown to ubiquitinate p53 (23). TOPORS is a known E3 ubiquitin ligase for the transcription factor p53 (23) and Hairy (29), we sought to determine whether TOPORS can interact with NKX3.1, GST-pulldown assays were conducted. Recombinant GST-tagged TOPORS was purified and immobilized on glutathione beads as bait (Fig. 1A). Recombinant His-tagged NKX3.1 protein was specifically captured by GST-TOPORS in the pulldown assay (Fig. 1B). These data demonstrate that NKX3.1 can interact directly with TOPORS in vitro.

Because TOPORS is a known E3 ubiquitin ligase for the transcription factors p53 (23) and Hairy (29), we sought to determine whether TOPORS can also ubiquitinate NKX3.1. In the presence of ubiquitin, E1, and E2 (Ubch5a) enzymes, TOPORS induced the appearance of higher molecular weight species of NKX3.1 (Fig. 1C), indicating TOPORS functions as a ubiquitin...
TOPORS Ubiquitinates NKX3.1

![Image of a Coomassie-stained gel and a Western blot](https://example.com/image)

**FIGURE 1.** TOPORS interacts with and ubiquitinates NKX3.1. *A,* Coomassie staining of purified recombinant GST and GST-TOPORS. *B,* Western blot (WB) of GST-pulldown assay using anti-NKX3.1 antibodies. Equimolar amounts of GST and GST-TOPORS immobilized on glutathione beads were incubated with purified His-tagged recombinant NKX3.1, washed, and eluted with SDS-PAGE buffer. *C,* TOPORS ubiquitinates NKX3.1 in *vitro.* *In vitro* ubiquitination assays were conducted using recombinant E1, E2 (UbcH5a), E3 (TOPORS), ubiquitin (Ub), and NKX3.1. A Western blot using anti-NKX3.1 antibodies is shown. *D,* TOPORS ubiquitinates NKX3.1 in *vitro.* *In vitro* ubiquitination reactions with a constant amount of NKX3.1 were carried out with 5-fold dilutions of TOPORS, either in the presence of wild-type ubiquitin or no-lysine ubiquitin. Asterisk, background band copurified with GST-TOPORS and recognized by anti-NKX3.1 antibodies.

### Results

#### Monoubiquitination of NKX3.1

- NKX3.1 is a 234-amino acid protein with the homeodomain spanning residues 124–184 (Fig. 2A).
- To study the domains in NKX3.1 responsible for TOPORS interaction, His- or HA-tagged NKX3.1 truncation fragments (Fig. 2A) were generated and used for GST-pulldown assays (Fig. 2, B and C). The HA-tagged full-length NKX3.1, fragments 1–183, and 124–234 could be efficiently translated *in vitro* (Fig. 2E); however, the *in vitro* translation efficiency for HA-tagged 1–123 and 124–183 fragments was too low to obtain comparable materials for GST-pulldown assays (data not shown). Thus bacteria-purified recombinant His-tagged NKX3.1 (residues 1–123) was used, and HA-NKX3.1 (residues 124–183) was excluded from the GST-pulldown analyses.
- The N terminus alone (Fig. 2B, construct *b* (1–123)) could interact with TOPORS, although the interaction was weaker than with full-length NKX3.1. Both the N terminus plus homeodomain (Fig. 2C, construct *c*; 1–183) and the homeodomain plus C terminus (Fig. 2C, construct *d*; 124–234) readily interacted with TOPORS.
- In the *in vitro* ubiquitination assays, the N terminus alone was not ubiquitinates by TOPORS, suggesting that the N-terminal lysines are not likely to be ubiquitin acceptors.
- In contrast, all other constructs containing the homeodomain were readily ubiquitinates (Fig. 2C). The homeodomain alone was ubiquitinates by TOPORS, although at a lower efficiency compared with other constructs (Fig. 2C). This may be due to the fact that the *in vitro* translation efficiency for the homeodomain alone construct is relatively low, or alternatively, because the N or C termini may stabilize the interaction with TOPORS.

#### TOPORS Targets NKX3.1 for Degradation in Cells

- To study the effect of TOPORS on NKX3.1 in *vivo,* an NKX3.1-negative cell line (NIH/3T3) was transfected with an NKX3.1 expression construct along with increasing amounts of a GFP-tagged TOPORS expression construct. Overexpression of TOPORS resulted in a reduction of the steady-state levels of exogenous NKX3.1 in a dose-dependent manner (Fig. 3A). The level of endogenous NKX3.1 in LNCaP cells was also quantified and shown to be consistently down-regulated (~50%) by TOPORS overexpression, and the effect was partially reversed by the proteasomal inhibitor MG132 (Fig. 3B). Considering that the transfection efficiency of LNCaP cells is less than 100%, the down-regulation of NKX3.1 by TOPORS overexpression measured by Western blot analysis is likely to be an underestimate.
- To examine whether the effect of down-regulation of NKX3.1 by TOPORS is mediated by ubiquitination in cells, LNCaP cells were cotransfected with NKX3.1, His-ubiquitin, and TOPORS expression vectors and treated with MG132. Ubiquitinates
NKX3.1 was then analyzed by Western blotting after ubiquitinated proteins were pulled down using Ni-NTA beads. TOPORS led to an increase in the accumulation of ubiquitinated forms of NKX3.1 in vivo (Fig. 3C). The down-regulation of NKX3.1 by GFP-TOPORS was also confirmed by indirect immunofluorescence microscopy using anti-NKX3.1 antibodies. Under standard culture conditions, NKX3.1 showed nuclear staining with varying intensity among LNCaP cells (Fig. 4A). However, NKX3.1 staining was undetectable in all GFP-TOPORS positive cells (Fig. 4A and data not shown). Interestingly, NKX3.1 was found to colocalize with GFP-TOPORS in distinct nuclear bodies upon the addition of the proteasomal inhibitor MG132 (Fig. 4B).

**FIGURE 2.** The mapping of domains in NKX3.1 that mediate interaction with TOPORS. A, diagram of NKX3.1 deletion constructs. Locations of the 14 lysine (K) residues are indicated. The constructs are either HA- or His6-tagged at the N terminus. HD, homeodomain. B, Western blot analysis of the GST-pulldown assay of the recombinant full-length NKX3.1 and 1–123 fragment using anti-NKX3.1 antibodies. The rabbit anti-NKX3.1 antibodies were derived using the 1–123 fragment as an antigen. C, Western blot analysis of the GST-pulldown assay of in vitro translated HA-tagged NKX3.1 fragments using anti-HA antibodies. D, in vitro ubiquitination assay of NKX3.1 constructs analyzed using anti-NKX3.1 antibodies. UbKO, no-lysine ubiquitin. E, in vitro ubiquitination assay of NKX3.1 deletion constructs analyzed using anti-HA antibodies. No-lysine ubiquitin was used in the reaction.

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**Knockdown of TOPORS in LNCaP Cells Stabilizes NKX3.1**—To study the effect of endogenous TOPORS on NKX3.1 in cells, an siRNA approach was used to knock down TOPORS in LNCaP cells. Transfection of TOPORS siRNA resulted in over 90% reduction of TOPORS mRNA analyzed by quantitative real-time PCR (Fig. 5A). Western blot analyses of parallel samples demonstrated that the NKX3.1 steady-state level increased 2.6-fold (Student’s t test, p = 0.002) in the presence of TOPORS siRNA (Fig. 5B). As a control, the protein level of the known TOPORS substrate, p53, was also analyzed and shown to be similarly increased (Fig. 5B). To further confirm that the negative regulation of NKX3.1 by TOPORS is indeed mediated by protein degradation, we examined the rate of degradation of NKX3.1. After TOPORS siRNA transfection, protein synthesis was inhibited using cycloheximide treatment, and the levels of NKX3.1 and p53 were then analyzed at the indicated times. The half-life of NKX3.1 was increased 1.5-fold (Student’s t test, p = 0.004) in TOPORS siRNA-treated samples (Fig. 5, C and D). These data together demonstrate that TOPORS negatively regulates NKX3.1 in LNCaP cells.

**DISCUSSION**

Deregulation of the ubiquitin-proteasome system has been linked to the genesis of multiple forms of human cancer (30, 31). Here we have reported the interaction of the prostate-specific tumor suppressor NKX3.1 with the ubiquitously expressed E3 ubiquitin ligase TOPORS. Overall, our data support a negative regulatory role for TOPORS on NKX3.1 in prostate cancer cells through the ubiquitin-proteasome pathway.

We have demonstrated that TOPORS and NKX3.1 directly interact in vitro and that the homeodomain of NKX3.1 is essential for the interaction (Fig. 2). The homeodomain of NKX3.1 is known to be involved in mediating the interaction with multiple protein partners, including prostate-derived Ets factor (25), serum response factor (32), Sp proteins (33), and topoisomerase I (20). The colocalization of NKX3.1 and GFP-TOPORS after MG132 treatment further supports their interaction in vivo. Interestingly, NKX3.1 has been reported to colocalize with topoisomerase I in nuclear speckles when LNCaP cells were cultured in the presence of the androgen analog R1881 (20). Thus it is possible that endogenous NKX3.1, topoisomerase I, and TOPORS may colocalize in the nuclear bodies. However, using a different antibody, we did not observe NKX3.1 localization in nuclear speckles without MG132 treatment under our culture conditions. TOPORS is known to be associated with PML nuclear bodies (27), and nuclear proteasomal degradation foci partially overlap with PML bodies (34); thus our data suggest that TOPORS may actively degrade a pool of NKX3.1 protein in the PML bodies.
TOPORS Ubiquitates NKX3.1

Using no-lysine-containing ubiquitin in the in vitro reaction, we estimate that TOPORS may ubiquitinate as many as 10 of the 14 lysine residues in NKX3.1 (Fig. 1D). Given that 5 lysines lie outside of the homeodomain, these data suggest that at least 5 of the lysine residues within the homeodomain could be ubiquitinated. Indeed, TOPORS can ubiquitinate the homeodomain alone (Fig. 2E). Despite considerable effort, consensus motifs for ubiquitination have not been defined (35). However, analyses of known ubiquitin acceptor sites in yeast demonstrated that loops and helices are preferred structures harboring ubiquitination sites (36). The homeodomain is comprised of three α helices connected by short loops. The NKX3.1 homeodomain may serve as a paradigm to dissect structural features that mediate E3 ligase-substrate interactions. Our observations raise the possibility that TOPORS may be able to ubiquitinate other homeoproteins, although their interaction in vivo could be further regulated by other means, for example by cellular localization and other protein-protein interactions. Ubiquitination of NKX3.1 in the homeodomain by TOPORS could affect the interaction of NKX3.1 with DNA or with other proteins. However, the exact sites of ubiquitination and their functional significance in vivo require further study. Determination of ubiquitination sites could be achieved by mass spectrometric analysis of ubiquitinated NKX3.1 or by site-directed mutagenesis.

Given the established tumor suppressor function of NKX3.1, the negative regulation of NKX3.1 by TOPORS suggests that TOPORS may be proto-oncogenic in prostate cancer. However, TOPORS maps to chromosome 9p21 and is located between microsatellite markers D9S270 and D9S273, which show loss of heterozygosity in 21.8 and 13.8% of prostate cancer samples examined, respectively (37). In addition, TOPORS protein has been shown to be diminished in colon cancer, where it may have tumor suppressor function (24). TOPORS has also been implicated as a tumor suppressor in human gliomas and non-small cell lung cancers (38, 39). However, anti-TOPORS antibodies capable of detecting expression in situ have not been described. To obtain more insight regarding TOPORS expression in human cancers, we queried a publicly available cDNA microarray data base, Oncomine (40). Queries in Oncomine showed that TOPORS mRNA is down-regulated in seminomas and ovarian cancer; however, TOPORS mRNA is overexpressed in bladder, myeloma, endometrial, prostate, and salivary gland tumors, as well as in carcinoid and small cell lung cancers at a 0.01 \( p \) value threshold. It is possible that TOPORS may be both oncogenic and tumor-suppressive, depending on the cellular context. It is also possible that the effects of TOPORS on a particular substrate may vary depending on the level of TOPORS, in a manner analogous to the MDM2-p53 relationship (41). Although robust TOPORS expression leads to loss of NKX3.1, lower levels could have a different functional outcome. In addition, TOPORS also has SUMO (small ubiquitin-like modifier) E3 ligase activity (42, 43), and sumoylation is known to regulate transcription factor activity and localization (44, 45).
In summary, we established the biochemical interaction between the prostate tumor suppressor NKX3.1 and the ubiquitin E3 ligase TOPORS. We also demonstrated a negative regulatory role of TOPORS on NKX3.1 in the prostate cancer cell line LNCaP cells. Increasing evidence points to NKX3.1 as a haploinsufficient cell growth/differentiation regulator. cDNA profiling from tissues of Nkx3.1 heterozygous and homozygous knock-out mice indicates that NKX3.1 regulates a class of genes in a dosage-sensitive manner (46). Overexpression of NKX3.1 in prostate tumor cells decreases proliferation (47), and more recently, the restoration of NKX3.1 by retroviral transduction in Pten−/− prostate epithelial cells was shown to lead to decreased cell proliferation and increased cell death and to block Pten loss-induced tumor initiation in vivo (48). In light of these observations, it appears that restoring NKX3.1 protein levels, perhaps even partially, may be a viable therapeutic strategy. A clear understanding of the cellular mechanisms, including degradation pathways, that modulate NKX3.1 could facilitate the design of these approaches. The recognition of TOPORS as a mediator of NKX3.1 degradation is an important first step in this direction.

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FIGURE 5. Knockdown of TOPORS stabilizes NKX3.1 and p53 in LNCaP cells. A, real-time PCR analysis of TOPORS mRNA. B, steady-state levels of NKX3.1 and p53 were increased in TOPORS siRNA-transfected cells. C, TOPORS siRNA increased half-life of NKX3.1 and p53. NKX3.1 and p53 levels were analyzed by Western blotting after cycloheximide (CHX) chase. The half-life values of NKX3.1 and p53 calculated from graphs in D are indicated. D, semi-log plot of quantification in C using ImageQuant. The protein levels were normalized to β-actin levels, and the relative values were compared with the no-cycloheximide-treated samples. Representative blots from three independent experiments are shown.
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