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Inflammatory Cytokines Induce Phosphorylation and Ubiquitination of Prostate Suppressor Protein NKX3.1

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Abstract

Inflammation of the prostate is a risk factor for the development of prostate cancer. In the aging prostate regions of inflammatory atrophy are foci for prostate epithelial cell transformation. Expression of the suppressor protein NKX3.1 is reduced in regions of inflammatory atrophy and in preinvasive prostate cancer. Inflammatory cytokines TNF-α and IL-1β accelerate NKX3.1 protein loss by inducing rapid ubiquitination and proteasomal degradation. The effect of TNF-α is mediated via the C-terminal domain of NKX3.1 where phosphorylation of serine 196 is critical for cytokine-induced degradation. Mutation of serine 196 to alanine abrogates phosphorylation at that site and the effect of TNF-α on NKX3.1 ubiquitination and protein loss. This is in contrast to control of steady state NKX3.1 turnover which is mediated by serine 185. Mutation of serine 185 to alanine increases NKX3.1 steady-state turnover by inhibiting ubiquitination and doubling the protein half-life. A third C-terminal serine at position 195 has a modulating effect on both steady state protein turnover and on ubiquitination induced by TNF-α. Thus, cellular levels of the NKX3.1 tumour suppressor are affected by inflammatory cytokines that target C-terminal serine residues to activate ubiquitination and protein degradation. Our data suggest that strategies to inhibit inflammation or to inhibit effector kinases may be useful approaches to prostate cancer prevention.

Inflammation has been implicated as a carcinogenic insult in a number of human cancers. For example, transformation of human prostate epithelial cells occurs adjacent to foci of inflammatory atrophy. Inflammation causes the generation of reactive oxygen species that increase the risk of oxidative damage of DNA and generation of mutations (1). Inflammation of the prostate is a risk factor for the development of prostate cancer (2,3). One of the earliest events in prostate cellular transformation is reduced expression of the haploinsufficient prostate specific suppressor protein, NKX3.1. The *NKX3.1* gene is subject to loss at chromosome 8p21 and/or methylation (4). Intracellular levels of the NKX3.1 protein are reduced in prostate intraepithelial neoplasia, a noninvasive precursor to prostate cancer (4) and in regions of inflammatory atrophy that are precursors to malignant transformation in the prostate (5). Control of NKX3.1 protein levels are under the influence of many factors including those that result in N-terminal threonine phosphorylation that results in prolongation of protein half-life (6). This paper addresses the mechanism by which NKX3.1 is reduced in regions of prostatic inflammation.

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Statement of Competing Interests and Author Contributions
The authors declare no competing interests. Mark Markowski and Cai Bowen both contributed data to this report. Edward Gelmann directed the research and wrote the paper.

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The prostate-specific homeodomain protein NKX3.1 is expressed in the adult almost exclusively in the nuclei of luminal prostate epithelial cells (7). Gene targeting studies in mice have shown that haploinsufficiency of *Nkx3.1* is semidominant since *Nkx3.1*$^{+/-}$ mice develop prostatic dysplasia with longer latency than *Nkx3.1*$^{-/-}$ mice and loss of a single allele cooperates with *Pten* loss to accelerate the development and increase the severity of prostate cancer (8, 9). That prostate epithelial cells are subjected to a dose-response of Nkx3.1 protein levels is underscored by proportionately altered expression of downstream Nkx3.1 transcription targets in *Nkx3.1*$^{+/-}$ mice (10). In human prostate cancer NKX3.1 expression is reduced in primary disease (4) and completely abrogated in most metastatic foci, suggesting a continued selection for loss of the protein during prostate cancer progression (7).

We show here that inflammatory cytokines TNF-α and IL-1β accelerate NKX3.1 protein loss by inducing rapid ubiquitination and proteasomal degradation. The C-terminal domain distal to the homeodomain of NKX3.1 is not the site of ubiquitination, but is targeted at specific serine residues, presumptive phosphorylation sites, to mediate either steady-state or cytokine-mediated protein degradation.

Materials and Methods

Cell culture

LNCaP and PC-3 cells were routinely cultured in Improved Minimal Essential Medium (IMEM) supplemented with 5% Fetal Bovine Serum (FBS) (Life Technologies, Gaithersburg, MD) at 37°C in 5% CO_2. LNCaP cells were treated with 40 ng/ml TNF-α (Roche Diagnostics, Indianapolis, IN), 40 ng/ml IL-1β. (Leinco Technologies, St. Louis, MO), and 40 ng/ml IL-6 (Sigma Chemical Co., St. Louis, MO). Bortezomib was a gift of Millennium Pharmaceuticals (Cambridge, MA). To determine relative NKX3.1 stability, cells were treated with either 10 μM cycloheximide (Sigma Chemical Co.) for 1 hour except where indicated or 100 nM bortezomib for 6 hours. When testing the effect of TNF-α on exogenous NKX3.1 levels, LNCaP cells were treated with 100 μM cycloheximide for 1 hour prior to the addition of 40 ng/ml TNF-α unless otherwise stated.

Plasmid construction and expression

Full length NKX3.1 and NKX3.1(1−183) cDNA were cloned into the mammalian expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA), as previously described (11). Mammalian expression vectors were engineered to express full length NKX3.1 and COOH-terminal deletion mutants with an NH_2-terminal MYC epitope. Sequential NKX3.1 deletion mutant cDNA was amplified by PCR, excised on a 1% agarose gel, and purified using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Isolated NKX3.1 cDNA was ligated into a cloning vector using a TOPO-TA cloning kit (Invitrogen). NKX3.1 cDNA was digested with *EcoRI* and *XhoI* for directional cloning into pCMV-MYC (Clontech, Mountain View, CA). All point mutations in *NKX3.1* were generated using a Quickchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. All constructs were fully sequenced to confirm the presence of the target mutations and ensure that no additional mutations were generated. Twenty four hours prior to transfection, 750,000 LNCaP and PC-3 cells were plated in FBS-IMEM. Cells were transfected overnight with 500 ng MYC-NKX3.1 expression plasmid or MYC empty vector, 500 ng green fluorescent protein (GFP) expression plasmid, or 1.5 μg of NKX3.1 expression plasmid or pcDNA3 plasmid using lipofectAMINE Reagent (Invitrogen). The transfection media was replaced with FBS-IMEM for 24 hours prior to treatment.

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NKX3.1 ubiquitination assay

To assess the ubiquitination of exogenous NKX3.1, a His Bind Purification Kit was used (Novagen, Madison, WI). 2×10^6 LNCaP cells were transfected overnight with 2 μg MYC-NKX3.1 or MYC-empty expression plasmid and 3 μg of a histidine-tagged ubiquitin (His-Ub) expression plasmid (gift of Dirk Bohmann, University of Rochester). Cells were pretreated for 1 h with 100 nM bortezomib followed by a 6 h treatment with 40 ng/ml TNF-α. Cells were harvested in cold PBS and resuspended in 1X Binding Buffer (diluted to volume with 6 M Urea). Samples were sonicated and centrifuged for 30 min at 14,000 × g at 4°C. The supernatant was incubated with 50 μl precharged Ni^{2+} agarose bead slurry for 3 h at room temperature with rotation. Samples were washed with 1X Binding Buffer (6 M Urea) followed by 1X Wash Buffer (6 M Urea). Ubiquitinated proteins were eluded from the Ni^{2+} agarose beads with 4X SDS sample buffer and resolved on a 4−20% SDS- polyacrylamide gel. Ubiquitinated NKX3.1 was detected using a mouse monoclonal anti-MYC antibody (1:1000) (Santa Cruz Biotechnologies, Santa Cruz, CA).

Immunoprecipitation and immunoblotting

LNCaP cells transfected with MYC-tagged NKX3.1 with lipofectamine 2000 (Invitrogen) were pretreated with CHX (100 μM) for 15 min and then exposed to 40 ng/ml TNF-α 15 min. An aliquot of cell extract was treated with calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Ipswich, MA). Cells were harvested for immunoprecipitation with polyclonal anti-MYC antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by western blotting with monoclonal anti-MYC antibody (Santa Cruz) or anti-phosphoserine antibody (Sigma-Aldrich, St. Louis, MO).

Results

Cellular NKX3.1 protein levels are critical for maintenance of the prostate epithelial phenotype. We therefore characterized intracellular protein turnover to determine the half-life of NKX3.1. In PC-3 prostate cancer cells exogenously expressed NKX3.1 has a half-life of approximately one hour (Fig 1A). Members of the NK family of homeodomain proteins such as Nkx2.5 and Nkx3.1 have been shown to have increased levels of expression and of protein activity after removal of the peptide domain that lies C-terminal to the homeodomain (12,13). Therefore we measured the half-life of a C-terminal truncated NKX3.1 lacking the 51 amino acids downstream from the homeodomain and observed a prolonged half life (Fig 1A). LNCaP prostate cancer cells are one of the few cell lines that express endogenous NKX3.1. In LNCaP cells exogenous MYC-tagged NKX3.1 has a half life of approximately 60 min whereas the C-terminal truncated protein had a half life of nearly 4 hours (Fig 1B). We also examined turnover of endogenous NKX3.1 in LNCaP cells by treating cells with bortezomib, a reversible proteasome inhibitor that prolonged half-life of endogenous NKX3.1, but had no effect on the level of exogenous C-terminal truncated protein (Figure 1C). Bortezomib also blocked turnover of exogenous NKX3.1 expressed in PC-3 prostate cancer cells. Moreover, in the presence of bortezomib higher molecular weight moieties of NKX3.1 were seen that represented polyubiquitinated NKX3.1 accumulating in PC-3 cells. Under the same conditions no ubiquitination of NKX3.1(1−183) was seen (Figure 1D).

We had observed that exposure of LNCaP cells to the inflammatory cytokine TNF-α caused rapid loss of NKX3.1. Similar loss of NKX3.1 was seen when cells were exposed to another inflammatory cytokine IL-1β (Fig 2A). In contrast, no effect on NKX3.1 levels was seen in response to the proliferative cytokine IL-6. LNCaP cells are known to express IL-6 receptors and respond to IL-6 (14). A MYC-tagged NKX3.1 fusion protein had a half-life similar to endogenous NKX3.1 in LNCaP cells treated with cycloheximide indicating that both endogenous and exogenous MYC-tagged NKX3.1 were subjected to similar mechanisms of

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protein turnover. TNF-α accelerated degradation of full-length MYC-tagged protein. In contrast, truncation of the C-terminal domain prolonged protein half-life and conferred resistance to the effect of TNF-α on protein loss (Fig 2B). The MYC-tagged NKX3.1 fusion protein was ubiquitinated in response to TNF-α, but the C-terminal truncated protein was resistant to ubiquitination (Fig 2C). Polyubiquitination most commonly occurs at lysine residues. The C-terminal domain of NKX3.1 has lysines at positions 193 and 201. Mutation of either or both lysine residues to arginines had no detectable effect either on steady-state turnover or on TNF-α-induced degradation of NKX3.1 (data not shown). TNF-α causes apoptosis of LNCaP cells, but the effect is not seen until more than 48 hr after exposure to the cytokine (15). To determine whether caspase activation contributed to NKX3.1 turnover after exposure to TNF-α we treated cells with the pancaspase inhibitor zVAD-FMK and saw no effect on the degradation of NKX3.1 within 24 hrs of exposure to 40 ng/ml TNF-α (data not shown).

To determine what region of C-terminal domain influenced NKX3.1 stability we engineered a series of MYC-tagged deletion constructs whose stability were tested in LNCaP cells. Deletion at amino acids 216, 208, or 200 had no effect on steady-state turnover of NKX3.1. Deletion at amino acid 192 prolonged half-life to a lesser degree than seen with deletion to amino acid 183 (Figure 3A). The NKX3.1 constructs truncated at amino acids 192 and 183 were also less sensitive to the effect of bortezomib on protein turnover (Fig 3B). Truncation at amino acid 192 resulted in an increased protein half-life and abrogation of the effect of TNF-α (Fig 3C top). MYC-NKX3.1 truncation extended to amino acid 183 caused further increase in protein half-life.

Phosphorylation of a number of proteins, for example IκB and β-catenin, is known to mediate association with ubiquitin ligase and subsequent proteasomal degradation, Computer-based analysis of the C-terminal 51 amino acids of NKX3.1 contain three potential phosphorylation sites, all proximal to amino acid 200, at serines 185, 195, and 196[1] (16). Each of these serines was individually mutated to an alanine residue to abrogate each putative phosphorylation site. In addition we made compound serine→alanine mutant constructs. The serine mutants were tested both for protein turnover and for sensitivity to TNF-α (Fig 3C bottom). Mutation of serine 185 doubled the half-life of NKX3.1 and also increased protein half-life after TNF-α exposure from 25 to 40 minutes. However, NKX3.1(S185A) retained sensitivity to TNF-α, suggesting that serine 185 had a major influence on protein degradation, but was not targeted by TNF-α. Mutation of either serine 195 or serine 196 prolonged protein half-life. The serine 195 mutation attenuated and the serine 196 mutation abrogated the effect of TNF-α on protein degradation since there was no change in the half-life of NKX3.1(S196A) after exposure to TNF-α. Mutation of both serines 195 and 196 enhanced the protein half-life and resistance to TNF-α more than the effect of the serine 196 mutant alone. The compound mutant with altered serine 185 and serine 195 showed a half-life of 110 min, but retained an effect of TNF-α. In contrast the serine 185, 196 compound mutant had a prolonged protein half-life and essentially no TNF-α sensitivity. Lastly, simultaneous mutation of serines 185, 195, and 196 resulted in a protein with no sensitivity to TNF-α and a half-life similar to the NKX3.1(1−183) construct. Thus the effect of C-terminal truncation on protein turnover and TNF-α sensitivity was recapitulated by mutation of three serines. To determine whether any of the serine mutations affected nuclear localization of NKX3.1 and thereby affected protein turnover we expressed each MYC-tagged NKX3.1 construct in LNCaP cells and determined subcellular localization by immunohistochemistry. We observed that all MYC-tagged serine→alanine constructs localized to the nucleus of LNCaP cells (data not shown).

[1](http://www.cbs.dtu.dk/services/NetPhos/)

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To demonstrate directly that the C-terminus of NKX3.1 was the target for TNF-α-induced phosphorylation, we performed immunodetection of phosphoserine on MYC-tagged NKX3.1. LNCaP cells were transfected with a MYC-NKX3.1 expression vector and pretreated with cycloheximide prior to 15 min exposure to TNF-α. As shown in the left panel of Figure 4A, exposure to TNF-α induced the presence of phosphoserine residues that were sensitive to treatment with CIP (right panel Figure 4A). TNF-α induced serine phosphorylation only on the C-terminus as shown in Figure 4B since C- terminal truncation abolished immunodetection with anti-phosphoserine antibody. In contrast, deletion of the N-terminal domain upstream from the homeodomain did not affect TNF-α-induced serine phosphorylation. Mutation of serine 196 to alanine specifically abrogated TNF-α-induced serine phosphorylation. The effect was not changed by concurrent mutation of serine 195 to alanine (Figure 4C). We were also able to show that in the presence of cycloheximide alone mutation of serine 185 to alanine diminished detection with the anti-phosphoserine antibody whereas mutation of serines 195 and 196 or C-terminal truncation to amino acid 192 had no effect on NKX3.1 serine phosphorylation in the presence of cycloheximide.

Lastly, the C-terminal serine mutations were also found to decrease the polyubiquitination of NKX3.1 in the presence of bortezomib and in response to TNF-α (Fig 5). Thus the C-terminal serines determined both ubiquitination and protein loss after exposure of LNCaP cells to TNF-α.

Discussion

Our data strongly suggest that C-terminal phosphorylation was the biochemical signal that initiated ubiquitination and protein degradation both for purposes of NKX3.1 turnover and for degradation in response to TNF-α and perhaps other inflammatory cytokines. Experiments with NKX3.1 mutants demonstrated that serine 185 was targeted for steady-state protein turnover and serine 196 was targeted by TNF-α. The data suggest that serine 195 had a modulating effect on the effects mediated by both serine 185 and serine 196, further augmenting both steady-state turnover and TNF-α-induced degradation. However, phosphorylation at serine 196 appears to be the primary signal for TNF-α-induced degradation of NKX3.1.

Our data demonstrated how inflammation may enhance degradation of a short-lived suppressor protein in prostate epithelial cells. These data provide a mechanism for direct and specific degradation of a suppressor protein by inflammatory cytokines. The data also suggest that anti-inflammatory agents may have activity in prostate cancer prevention. A trial of refecoxib for prostate cancer prevention was closed early when the drug was withdrawn from the market (17). The role of anti-inflammatory agents in prostate cancer prevention remains to be shown. Prostate cancer prevention or treatment might be approached by inhibition of NKX3.1 ubiquitination. Recently NKX3.1 has been shown to be a target of at least one ubiquitin E3 ligase, TOPORS (18). Interestingly TOPORS is a nuclear protein that associates with PML bodies and also targets p53 for degradation (19,20). Since we have shown that NKX3.1 ubiquitination is mediated by phosphorylation, it is conceivable that identification of the kinase that targets the NKX3.1 C-terminal domain may be a future target for a prostate cancer prevention or treatment strategy. Moreover, the activation of TNF-α receptor is affected by drugs like etanercept that interfere with ligand availability in the treatment of inflammatory diseases. Thus there are therapeutic agents available to test the effects of TNF-α inhibition on NKX3.1 expression and prostate epithelial cell transformation. The control of NKX3.1 levels is complex and is also under control of kinase that target N-terminal threonines. In contrast to the effect of phosphorylation at the C-terminal domain, phosphorylation of the N-terminal threonines prolongs NKX3.1 protein half-life (6).

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Prostate inflammation and inflammatory atrophy can occur without subsequent development of cancer and predispose prostate epithelial cells to malignant transformation. We propose that prostatic inflammation results in reduced intracellular levels of NKX3.1 and thereby predisposes cells to oncogenic transformation. Our unpublished data show that NKX3.1 can mediate proliferative signals via IGF-1 receptor (Mulhbradt et al, in preparation). Thus reducing NKX3.1 levels may enhance IGF-1-driven cell growth. NKX3.1 also mediates binding of topoisomerase I to DNA (21). In prostate epithelial cells this interaction influences the efficiency of DNA repair and therefore loss of NKX3.1 expression may predispose cells to DNA damage and oncogenic mutations (our unpublished data). We propose that prostate epithelial cell proliferation and transformation are favoured by loss of NKX3.1 and therefore selection of clones with *NKX3.1* genetic loss or methylation provide an irreversible growth advantage that frequently represents the first steps in prostate carcinogenesis. In light of this, efforts to prolong NKX3.1 protein half-life are a logical strategy for prostate cancer prevention.

Acknowledgements

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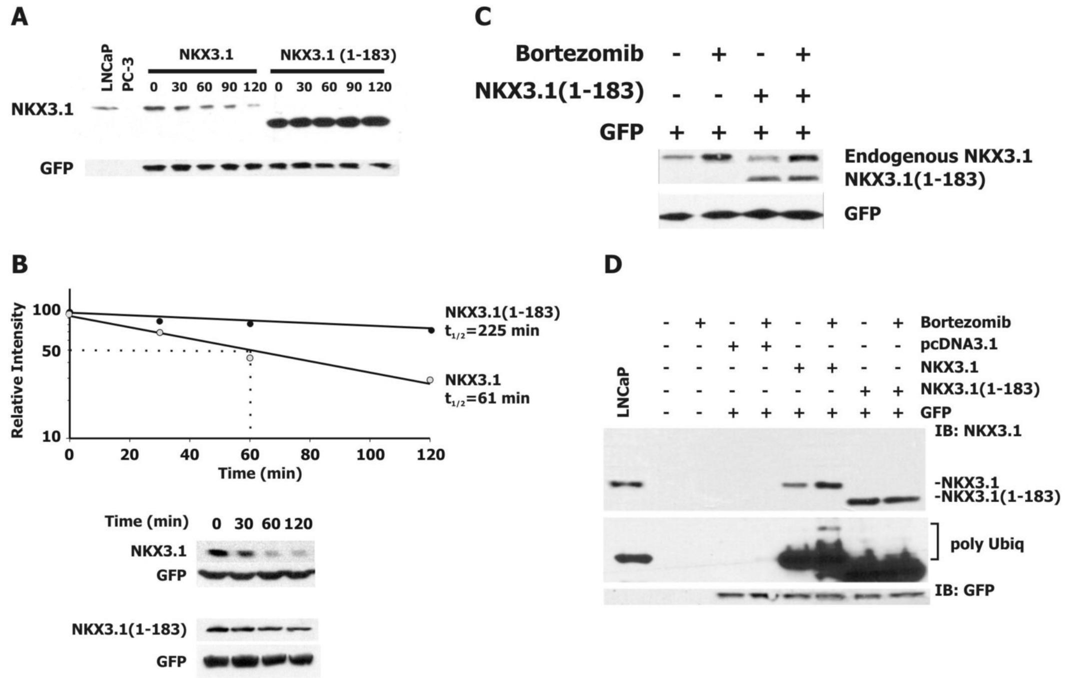

Figure 1.

C-terminus of NKX3.1 affects protein stability. A. PC-3 cells transfected with expression plasmids pcDNA-NKX3.1, or pcDNA3.1-NKX3.1(1−183) plasmids were exposed to 50 μM cycloheximide for 2 hr. NKX3.1 levels were assessed by western blotting with anit-NKX3.1 antibody 2 h post treatment. Transfection efficiency was monitored by cotransfection with a GFP expression plasmid. B. NKX3.1 and NKX3.1(1−183) levels were quantitated in LNCaP cells 2 h after treatment with cycloheximide. C. LNCaP cells were transfected with pcDNA3 or pcDNA3.1[NKX3.1(1−183)] and treated with 100 nM bortezomib for 6 h. Both endogenous and exogenous NKX3.1 levels were determined through western blot analysis with anti-NKX3.1 antibody. D. NKX3.1 and NKX3.1(1−183) expression in PC-3 cells was analyzed 6 hr after exposure to bortezomib. The top panel shows a short exposure and the bottom panel a long exposure of the same blot. The higher molecular weight bands, in this blot detected by NKX3.1 antibody, are consistent with polyubiquitinated NKX3.1.

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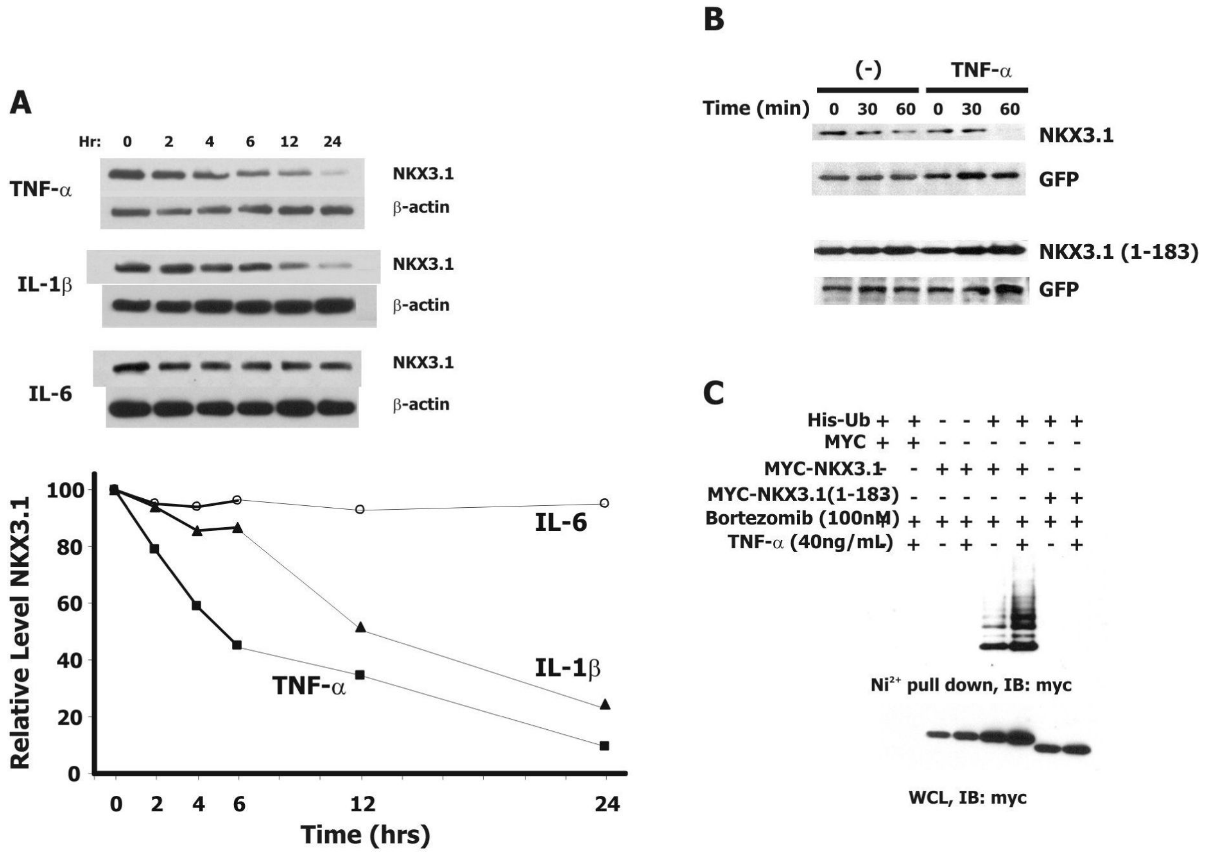

Figure 2.

TNF-α and IL-1β induce NKX3.1 degradation. A. Endogenous NKX3.1 levels in LNCaP cells after exposure to 40 ng/ml TNF-α , IL-1β or IL-6. Relative levels of NKX3.1 were normalized to β-actin levels and quantitated in the graph. B. TNF- is shown to accelerate NKX3.1 protein turnover, but have no effect on NKX3.1(1−183). MYC-tagged NKX3.1 expression plasmids were transfected into LNCaP cells. Cells were treated with 100 μM cycloheximide for 1 hour and then exposed to 40 ng/ml TNF-α. C. The effect of TNF-α on ubiquitination of MYC-tagged NKX3.1 and NKX3.1(1−183) in LNCaP is shown. Cells were transfected with MYC-tagged NKX3.1 expression plasmids and with a polyhistidine-tagged ubiquitination expression plasmid. Cells were treated for 1 hr with bortezomib and then for 6 hr with TNF-α. Cells lysates were exposed to Ni^{2+}-charged agarose beads and then subjected to western blotting with anti-MYC antibody. Input levels of NKX3.1 were determined by western blot analysis of each total cellular lysate prior to the addition of Ni^{2+} beads.

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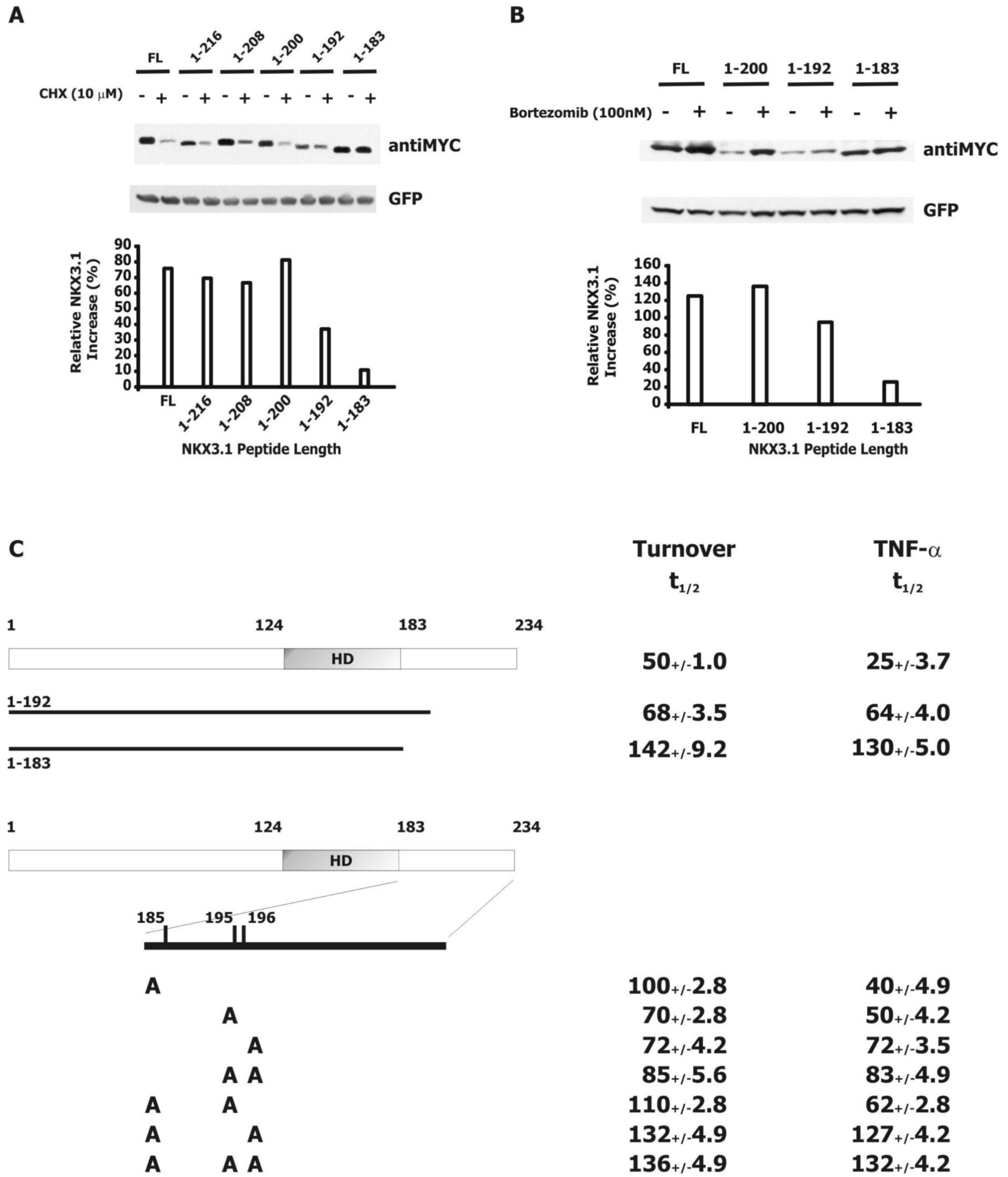

Figure 3.

Determinants of NKX3.1 steady state and TNF-α-induced turnover. A. C-terminal truncation mutants of NKX3.1 were expressed as MYC-tagged constructs and cotransfected with a GFP expression plasmid. Cells were treated with cycloheximide for 1 hr and processed for western blotting with anti-MYC antibody. FL=full-length. B. The effect of bortezomib on levels of full length and C-terminal truncated MYC-tagged NKX3.1 expression constructs was assayed by western blot. FL=full-length. C. The MYC-tagged NKX3.1 deletion constructs and point-mutants were analyzed for half-life after 0, 30, 60 and 120 min of exposure to cycloheximide or to cycloheximide + 40 ng/ml TNF-α. NKX3.1 proteins were detected by western blotting. Maps of the mutant constructs are shown at the left. Protein half-lives during turnover or after

exposure to TNF-α are shown at the right. At least three separate determinations were done for each value. Mean ± standard deviations are shown for each half-life.

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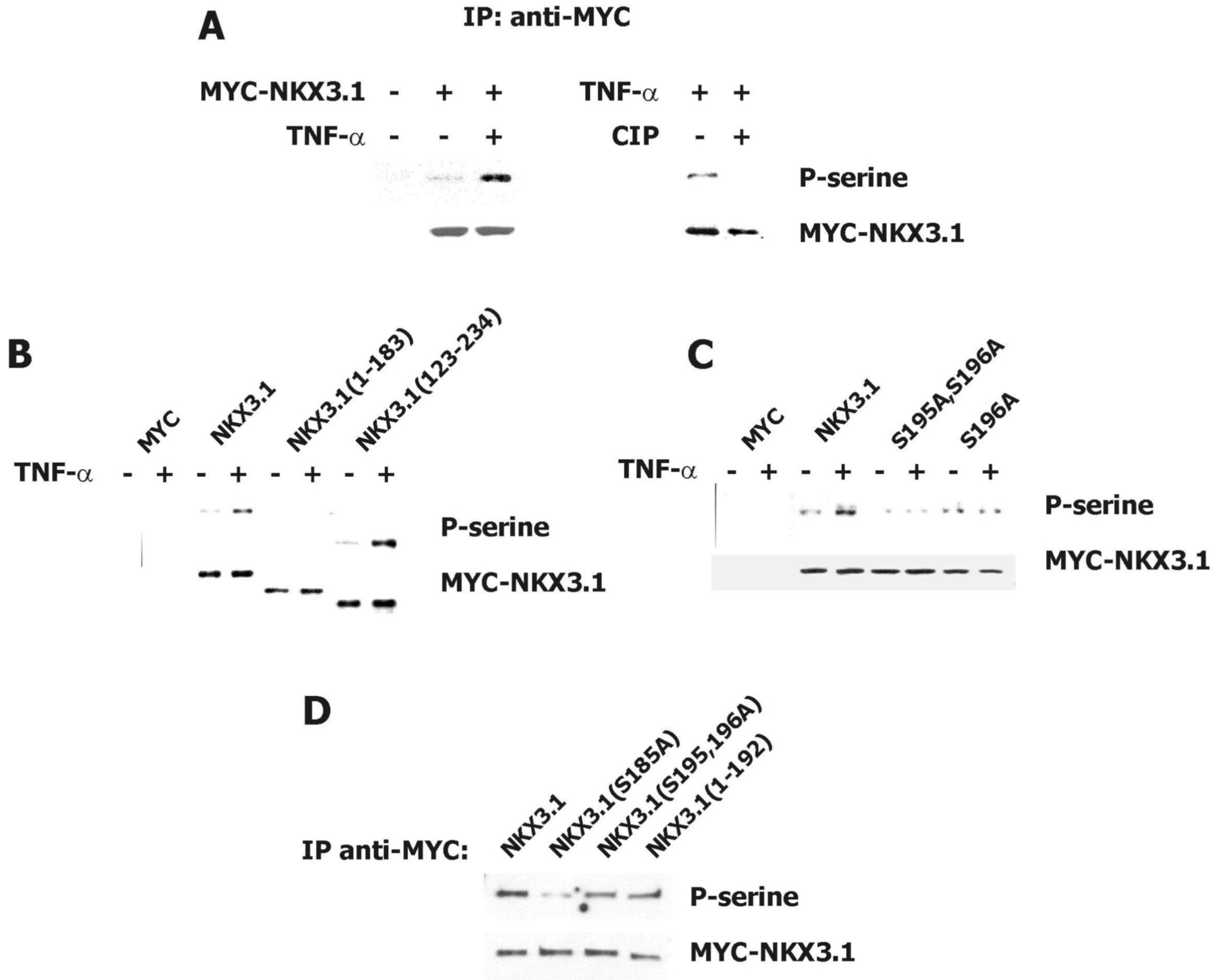

Figure 4.

TNF-α induces phosphorylation of NKX3.1. A. TNF-α induces NKX3.1 phosphorylation. LNCaP cells, transfected with MYC-tagged NKX3.1 were pretreated with 100 μM cycloheximide for 15 min and then exposed to 40 ng/ml TNF-α for 15 min. In the right panel blotting was done after one aliquot was treated with CIP. Cells were harvested for immunoprecipitation with polyclonal anti-MYC antibody followed by western blotting with either monoclonal anti-MYC or anti-phosphoserine antibody. In B. the effect of C-terminal truncation and in C. S196 and S195 mutations abolish NKX3.1 phosphorylation induced by TNF-α. D. MYC-tagged NKX3.1 constructs as indicated were expressed in LNCaP cells subjected to CHX treatment for 30 min. Immunoprecipitation was with anti-MYC antibody and immunoblotting was done with either anti-phosphoserine antibody or anti-MYC antibody.

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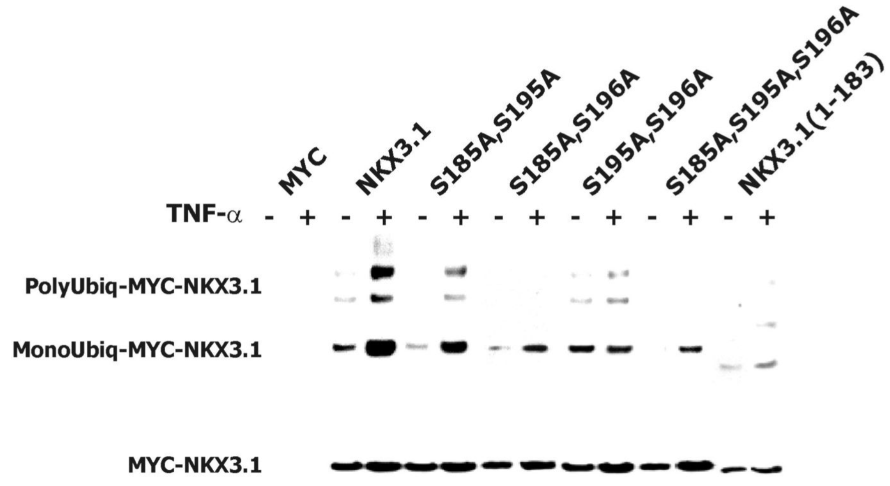

Figure 5.

Determinants of NKX3.1 ubiquitination in steady state turnover and after exposure to TNF-α. Ubiquitination of wild type and mutant MYC-tagged NKX3.1 after cells were treated with bortezomib with or without subsequent exposure to TNF-α was analyzed in LNCaP cell extracts. An expression vector for polyhistidine-tagged ubiquitin was cotransfected into the LNCaP cells. Ubiquitinated proteins were pulled downed by Ni^{2+} beads and analyzed by western blotting using an anti-MYC antibody.