ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 4, 2011

DOI: 10.1089/ars.2010.3243

Oxidative Stress-Dependent Regulation of Forkhead Box O4 Activity by Nemo-Like Kinase

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Abstract

Forkhead box O (FOXO) transcription factors are involved in various cellular processes, including cell proliferation, stress resistance, metabolism, and longevity. Regulation of FOXO transcriptional activity occurs mainly through a variety of post-translational modifications, including phosphorylation, acetylation, and ubiquitination. Here we describe nemo-like kinase (NLK) as a novel regulator of FOXOs. NLK binds to and phosphorylates FOXO1, FOXO3a, and FOXO4 on multiple residues. NLK acts as a negative regulator of FOXO transcriptional activity. For FOXO4 we show that NLK-mediated loss of FOXO4 activity co-occurs with inhibition of FOXO4 monoubiquitination. Previously, we have shown that oxidative stress-induced monoubiquitination of FOXO4 stimulates its transactivation, which leads to activation of an antioxidant defensive program. Conversely, NLK-dependent inhibition of FOXO4 activity can provide a means to downregulate this defensive program, when oxidative stress reaches a level beyond which repair is no longer feasible and cells need to undergo apoptosis. *Antioxid. Redox Signal*. 14, 563–578.

Introduction

The family of Forkhead box O (FOXO) transcription factors consists of four members, FOXO1, FOXO3a, FOXO4, and FOXO6 (25). Of these, FOXO6 expression appears restricted to the brain, whereas all others are ubiquitously expressed although at varying levels (23). Regulation of FOXO activity occurs predominantly through two signaling pathways (7, 17). First, FOXO activity is controlled by phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also known as Akt) signaling, and direct PKB-mediated phosphorylation inhibits FOXO activity by inducing a relocalization of FOXO from the nucleus to the cytosol (5, 6, 29). Second, an increased level of cellular oxidative stress results in FOXO activation and this is primarily mediated by direct phosphorylation by c-Jun-terminal kinase (JNK). However, the pathway(s) leading to JNK activation with respect to FOXO activation is largely unknown, and for FOXO4 we have obtained evidence that this at least requires the small GTPase Ral (9, 13). Importantly, regulation through PKB and JNK is evolutionary conserved. In *Caenorhabditis elegans*, the FOXO ortholog DAF-16 is required for lifespan determination through both the PI3K/PKB and the JNK pathway. Whereas PI3K/PKB activity shortens lifespan, JNK activity lengthens lifespan, and both require DAF-16 (26, 34). Despite the general picture, many details of FOXO regulation are still lacking, and especially regulation by oxidative stress appears highly complex and involves, besides JNK-mediated phosphorylation, many other post-translational modifications, including mono- and polyubiquitination, acetylation, and methylation (8, 45). Increased oxidative stress also induces binding of accessory proteins and we have described an evolutionary conserved role for binding of β-catenin to FOXO/DAF-16 in oxidative stress control (12). The oxidative stress-induced binding of β-catenin to FOXO4 induces its transcriptional activity. In agreement with this, loss of the BAR-1, the *C. elegans* ortholog of β-catenin reduces the activity of DAF-16 in dauer formation and lifespan. On the other hand, under normal physiological conditions binding between β-catenin and FOXO4 is reduced (19). This allows β-catenin to form a complex with members of the lymphoid enhancer factor-1/T-cell factor (LEF-1/TCF) transcription factor family, which promotes their activity, leading to expression of its targets genes. The binding of β-catenin to TCF is additionally regulated by Wnt signaling, which is involved in regulation of various developmental processes (1, 11). Finally, elevated levels of β-catenin leading to the constitutive activation of TCF-mediated transcription are associated with carcinogenesis (38). Therefore, it is important to tightly regulate the activity of the β-catenin/TCF complex. Activation of a mitogen-activated protein kinase (MAPK) pathway involving transforming growth factor β-activated kinase 1 (TAK1) and nemo-like kinase (NLK) leads to suppression of Wnt signaling (22). TAK1 in complex with TAK1-binding protein 1 (TAB1) activates

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NLK, which subsequently binds to and phosphorylates TCF. NLK-mediated phosphorylation of TCF impairs its ability to bind DNA and thereby decreases its transcriptional activity (21). Additionally, NLK has been reported to stimulate ubiquitination of TCF by recruiting the E3 ligase NLK-associated ring finger protein (NARF), which targets TCF for degradation (48). Moreover, it has been postulated that the TAK1-NLK pathway is activated directly by Wnt signaling and functions as a negative feedback mechanism (24, 40).

Besides modulating TCF function, NLK is emerging as a regulator of many different transcription factors, including peroxisome proliferator-activated receptor-γ (PPAR-γ), myb proteins, and Notch1 (20, 24, 30, 41). Surprisingly, the mechanism by which NLK controls the activity of this diverse set of transcription factors is highly versatile. For example, even though NLK has been reported to phosphorylate all three members of the mammalian Myb protein family, which results in suppression of their transcriptional activity, the molecular mechanism of this repression seems to be dissimilar for each Myb protein. NLK-directed phosphorylation of c-Myb in response to Wnt signaling leads to proteasomal degradation of c-Myb (24). In contrast, phosphorylation of a-Myb does not affect its stability but prevents association of a-Myb with its coactivator CREB-binding protein (CBP). Additionally, ectopic expression of NLK has been associated with elevated levels of histone methylation, which might indirectly suppress activity of a-Myb (30). Similarly, NLK inhibits transactivation of PPAR-γ by promoting histone-inactivating modifications. However, in this case NLK does not directly act on PPAR-γ itself, but after being activated in response to Wnt-5a signaling NLK phosphorylates a histone methyltransferase, SET domain bifurcated 1 (SETDB1). This is driving the recruitment of the corepressor complex to the PPAR-γ-specific promoter regions, leading to an increase in histone H3-K9 methylation and blocking expression of PPAR-γ target genes (41).

Because of the observations described above, we set out to investigate whether NLK could regulate FOXO function. Here we provide evidence that NLK inhibits FOXO transcriptional activity. NLK binds to FOXO and this binding is increased by elevated cellular oxidative stress and consequently FOXO is phosphorylated by NLK on a large number of sites both *in vitro* and *in vivo*. Finally, we show that NLK mediates FOXO4 repression by inhibiting the monoubiquitination of FOXO4. Taken together, our data reveal NLK as a novel regulator of FOXO function.

Materials and Methods

Cell culture and transfections

All cell lines except DLD1 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 2 m*M* L-glutamine. DLD1 cells were maintained in RPMI medium 1640 supplemented like described above. Pin1$^{-/-}$ mouse embryonic fibroblast (MEFs) were a kind gift from Dr. P. van der Sluijs. Mdm2$^{-/-}$p53$^{-/-}$ MEFs were a kind gift from Dr. A.G. Jochemsen. In all experiments, cells were cultured in the presence of 10% fetal bovine serum. HEK293T and DLD1 cells were transiently transfected using FuGENE6 reagent according to the suggestions of the manufacturer (Roche Applied Science). Mdm2$^{-/-}$p53$^{-/-}$ MEFs and Pin1$^{-/-}$ MEFs were transiently transfected using Effectene according to the protocol of the manufacturer (Qiagen). Total amounts of DNA were equalized using pcDNA4-TO. siRNA oligomers were transfected using HiPerFect according to the suggestions of the manufacturer (Qiagen). The following compounds were purchased: cycloheximide and MG132 (Sigma and Biomol, respectively).

Plasmids, oligomers, and recombinant proteins

pMT2-HA-FOXO4, GLOFLAG3-Flag-FOXO4, HA-FOXO1, HA-FOXO3a, pBabe-puro, His-Ubiquitin, GloMyc-ubiquitin-specific peptidase 7 (USP7) (46), Flag-murine double minute 2 (Mdm2), Flag-peptidyl-prolyl isomerase 1 (Pin1) CMV-HA-p27^{kip1} (3), and pcDNA3-Flag-β-catenin (12) have been described previously. 6xDBE and p27^{kip1} Luciferase have been described before (13). pRL-Tk (Tk *Renilla* luciferase) was purchased from Promega. Flag-NLK and Flag-NLK^{K155M} were a kind gift from Dr. L. Smit (40). myc-TAK1 and myc-TAK1^{D175A} were kindly provided by Dr. P. Cohen. pcDNA4-TO-StrepTag-NLK was generated by inserting phosphorylated oligonucleotides containing the *Bam*HI-StrepTag-*Eco*RV sequence into *Bam*HI/*Eco*RV-digested pcDNA4-TO vector (Invitrogen) generating pcDNA4-TO-StrepTag. Subsequently, the NLK coding sequence was amplified using forward EcoRV primer 5′-CAGTCCAGTGAGATATCGCCGCGGCTTACAATGGC-3′ and reverse XhoI primer 5′-GTGACTATCACTCGAGTCAT CACTCCCACACCAGAG-3′, and was cloned into *Eco*RV/*Xho*I-digested pcDNA4-TO-StrepTag generating pcDNA4-TO-StrepTag-NLK. GLOFLAG3-Flag-FOXO4Δ8 was generated by site-directed mutagenesis according to the QuikChange Site-Directed Mutagenesis Kit protocol (Stratagene). Nontargeting siRNA (siCTR), siRNA against NLK (NLK #2, sense sequence 5′-GAAGTTGTTACTCAGTATTAT-dTdT-3′; NLK #3, sense sequence 5′-CTCCAACCTCCACACATTGAC-dTdT-3′) and siRNA against USP7 described previously (46) were purchased from Dharmacon RNA. Recombinant proteins were purchased: His$_6$-NLK (Millipore), E1 (UBE1), E2 (UbcH5b), His$_6$-Mdm2, and His$_6$-Ubiquitin (BostonBiochem). Glutathione *S*-transferase (GST) or GST–FOXO4 fusion protein were expressed in Bl21DE3 Rosetta cells and purified by binding to glutathione-agarose beads.

Antibodies

Monoclonal antibodies. 12CA5 anti-HA and 9E10 anti-myc were produced in-house using a hybridoma cell line. The antibody against FOXO4 (834) has been described previously (32). The following antibodies were purchased: NLK-H100, NLK-B5, GST-B14, and Mdm2-SMP14 (Santa Cruz Biotechnology); p27^{kip1} (BD Biosciences); Acetylated-Lysine Antibody #9441 (Cell Signaling); USP7-BL851 (Bethyl Laboratories); tubulin and Flag-M2 (Sigma).

Cell lysates and western blot analysis

When preparing the total lysate samples, cells were washed with ice-cold phosphate-buffered saline and harvested in Laemmli sample buffer. Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Perkin-Elmer). Western blot analysis was performed under standard conditions, using the indicated antibodies.

FOXO4 activity assays

To determine expression of endogenous $p27^{kip1}$, HEK293T cells were transfected with empty vector or HA-FOXO4 together with pBabe-puro. Each of the constructs encoding Flag-NLK, Myc-TAK1, and Flag-p38 was cotransfected and cells were left for 24 h. To select for transfected cells, puromycin was added to the culture medium to a final concentration of 2 μg/ml for 48 h. When indicated, the cells were additionally treated with 5 μM MG132 for a 12-h period before harvesting. Cells were lysed in Laemmli sample buffer. The cell debris was pelleted by centrifugation and the supernatant was used for western blot analysis.

For luciferase assays DLD1 cells were transfected with either reporter construct bearing six canonical FOXO binding sites (6xDBE-luciferase) or human $p27^{kip1}$-promoter linked to luciferase (p27GL-1609) and the additional constructs were cotransfected as indicated. Luciferase counts were normalized using TK-*Renilla* luciferase. Luciferase levels were measured 48 h after transfection employing a luminometer in combination with a dual luciferase assay kit according to the instructions of the manufacturer (Promega). All experiments were performed in triplicate.

Coimmunoprecipitations

Before the coimmunoprecipitations, cells were treated with the optimized concentration of 200 μM of hydrogen peroxide (H_2O_2) (data not shown) in the presence of serum for the indicated duration. Cells were lysed in lysis buffer containing 50 m*M* Tris–HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 10 m*M* EDTA, 150 m*M* NaCl, phosphatase, and protease inhibitors and centrifuged at 20,800×*g* for 10 min at 4°C. The cleared lysates were incubated with 5 μl solid anti-Flag M2 affinity beads for 2 h at 4°C. Beads were washed four times with lysis buffer, and proteins bound to the beads were eluted in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting as described above using the indicated antibodies.

For the endogenous coimmunoprecipitations, protein complexes within the cells were *in vivo* cross-linked by adding dithiobis(succinimidyl) propionate (Pierce) to the medium to a final concentration of 2.5 m*M* for 20 min at 37°C. The cross-linking reaction was quenched by adding Tris-HCl pH 7.5 to final concentration of 20 m*M* for 15 min at room temperature. Endogenous coimmunoprecipitations were performed using FOXO4 (834) antibodies.

In vitro *kinase assay*

GST or GST-FOXO4 were incubated with recombinant His-NLK in ATP buffer containing 30 m*M* Tris-HCl (pH 7.4), 15 m*M* $MgCl_2$, 1 m*M* EGTA, 100 μg/ml bovine serum albumin, 200 μM DTT, 1 m*M* ATP, and 5 μCi of [γ-^{32}P]ATP, at 30°C for 15 min. The reaction was terminated by adding 5×concentrated Laemmli sample buffer and heating the samples for 5 min at 95°C.

For *in vitro* kinase assays using exogenously expressed proteins, HEK293T cells were transfected with Flag-FOXO4 or Flag-NLK, respectively. The lysates were prepared and immunoprecipitated as described for the coimmunoprecipitation assay. The immunoprecipitations were subsequently washed three times with lysis buffer and then two times with kinase buffer containing 30 m*M* Tris-HCl (pH 7.4), 15 m*M* $MgCl_2$, 1 m*M* EGTA, and 100 μg/ml bovine serum albumin. Flag-FOXO4 proteins coupled to the beads were eluted with 200 μg/ml 3×Flag-peptide (Sigma). Next, the FOXO4-containing eluates were incubated with Flag-NLK that was coupled to the beads, in ATP buffer for 15 min at 30°C. Phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography.

In vivo *ubiquitination assay*

The monoubiquitination assay was performed as described previously (46). Shortly, cells were transfected with the indicated constructs. When indicated, 5 μM MG132 was added to the cultures for 12 h before harvesting the cells. Forty-eight hours after transfection, cells were treated as indicated and lysed in urea lysis buffer containing 8 *M* urea, 10 m*M* of Tris-HCl (pH 8.0), 100 m*M* of Na_2HPO_4/NaH_2PO_4 (pH 8.0), 0.2% Triton X-100, 5 m*M* of NEM, and protease inhibitors. Ubiquitinated proteins were precipitated using 10 μl solid nickel-nitrilotriacetic acid (Ni-NTA) agarose (Sigma) and analyzed by SDS-PAGE followed by immunoblotting.

In vitro *ubiquitination assay*

Purified GST-FOXO4 (1.2 μg) was mixed with 275 ng UBE1, 85 ng UbcH5b, 400 ng His_6-Mdm2, and 10 ng His_6-Ubiquitin in a final volume of 40 μl reaction buffer containing 25 m*M* Tris-HCl pH 7.5, 60 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, and 2 m*M* ATP. The reaction was carried out for 120 min at 30°C. When indicated, 50 ng of His_6-NLK was added to the reaction. Ubiquitinated proteins were precipitated using 10 μl solid Ni-NTA agarose (Sigma) and analyzed by SDS-PAGE followed by immunoblotting. Additionally, when indicated, GST-FOXO4 or His-Mdm2 were prephosphorylated using His-NLK for 30 min at 30°C. In parallel, control reactions containing GST-FOXO4 or His-Mdm2 but lacking His-NLK were treated identically. Subsequently, E1, E2 and His-ubiquitin were added and the reaction was carried out as described above.

FOXO4 half-life assay

HEK293T cells were transfected with pBabe-puro plasmid and the indicated constructs. To select for transfected cells, puromycin was added to the culture medium to a final concentration of 2 μg/ml for 48 h. Subsequently, cells were treated with cycloheximide for indicated time points. Protein samples were analyzed by SDS-PAGE followed by immunoblotting. Relative FOXO4 protein expression levels were calculated using Odyssey application software (LI-COR).

Results

H_2O_2 increases the interaction between NLK and FOXOs

To determine possible regulation of FOXO by NLK, we first tested whether FOXO4 can interact with NLK. To this end Flag-NLK and HA-FOXO4 were ectopically overexpressed in HEK293T cells. After immunoprecipitation of Flag-NLK, we observed coimmunoprecipitation of FOXO4 and also in the reverse experiment we detected coimmunoprecipitation of NLK after immunoprecipitation of Flag-FOXO4, suggesting binding between NLK and FOXO4 (Fig. 1A, B and

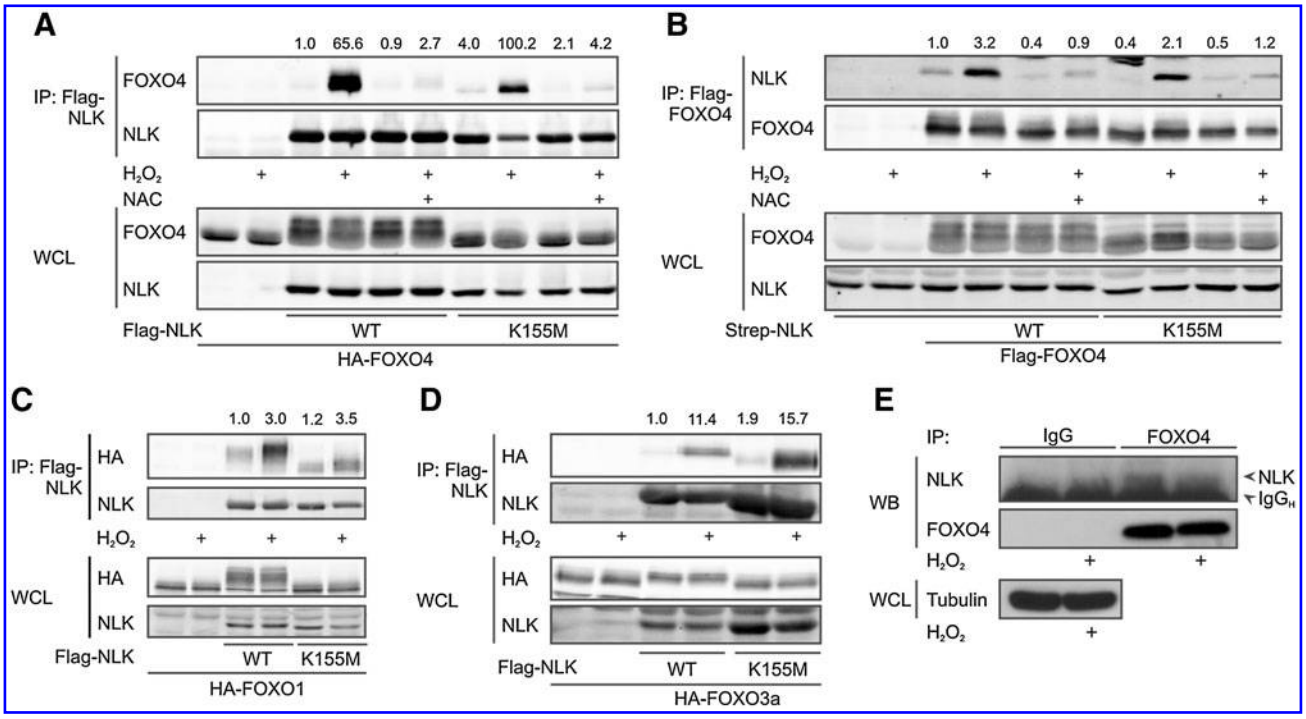


FIG. 1. H_2O_2 induces interaction between NLK and FOXOs. (A, B) NLK binds to FOXO4. HEK293T cells were transfected with the indicated constructs. Cells were treated as indicated with 4 m*M* NAC for 24 h followed by 200 μm H_2O_2 for 15 min. Cell lysates were subjected to immunoprecipitation using Flag-M2 affinity beads. Immunoprecipitated complexes were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(C, D)** NLK binds to FOXO1 and FOXO3a. Experiments were performed essentially as in **(A, B)** using the indicated constructs. **(E)** NLK binds to FOXO4 under endogenous conditions. Cells were treated as indicated with 200 μm H_2O_2 for 30 min, followed by *in vivo* cross-linking using dithiobis (succinimidyl) propionate (DSP). Cell lysates were subjected to immunoprecipitation using FOXO4 antibodies. Immunoprecipitated complexes were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(A, C, D)** Relative amounts of FOXOs bound to NLK for individual experiments are indicated. The protein levels were quantified using Odyssey application software (LI-COR). The levels of coimmunoprecipitated FOXOs were normalized to their total expression levels and the ratios between FOXOs and NLK were calculated. The amounts of FOXOs bound to NLK in the absence of H_2O_2 treatment were set to 1. **(B)** Relative amounts of NLK bound to FOXO4 are indicated and quantified as described above. FOXO, Forkhead box O; H_2O_2, hydrogen peroxide; NAC, N-acetyl cysteine; NLK, nemo-like kinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Supplementary Fig. S1A; Supplementary Data are available online at www.libertonline.com/ars). Similarly to FOXO4, we observed in coimmunoprecipitation assays that HA-FOXO1 and HA-FOXO3a interacted with Flag-NLK (Fig. 1C, D and Supplementary Fig. S1B, C). Moreover, H_2O_2 treatment of cells to increase the cellular level of reactive oxygen species (ROS) enhanced formation of this complex and this could be partially reversed upon preincubation of cells with the ROS scavenger N-acetyl cysteine (NAC) (Fig. 1A, B and Supplementary Fig. S1A).

However, interaction between FOXO4 and NLK did not depend on NLK activity since we observed also binding between kinase-dead NLK (NLK^{K155M}) and FOXO4.

Moreover, we were able to observe binding between FOXO4 and NLK under endogenous conditions (Fig. 1E).

Thus, NLK interacts with FOXOs independent of its kinase activity and this interaction is enhanced by peroxide treatment of cells.

NLK phosphorylates FOXO4

Simultaneous expression of FOXO4 and wild-type but not kinase-dead NLK resulted in the appearance of several forms of FOXO4 displaying reduced mobility on SDS-PAGE. This shift in the gel mobility of FOXO4 was abolished after treatment of immunocomplexes with λ-phosphatase, which indicated that NLK expression results in increased FOXO4 phosphorylation *in vivo* (Fig. 2A). To confirm direct phosphorylation of FOXO4 by NLK, we performed an *in vitro* kinase assay using Flag-FOXO4 and Flag-NLK that were both immunopurified from HEK293T cells and this further confirmed that NLK can directly phosphorylate FOXO4 (Fig. 2B). Essentially the same result was obtained when using commercially obtained recombinant His-NLK and bacterially expressed GST-FOXO4, but not GST alone, as a substrate in an *in vitro* kinase assay (Fig. 2C).

Taken together, these data indicate that NLK phosphorylates FOXO4 directly both *in vivo* and *in vitro*.

Because coexpression of NLK already results in an almost complete mobility shift of FOXO4 in the absence of peroxide treatment, we wished to address whether peroxide treatment affected NLK activity. To this end we performed an *in vitro* kinase assay on NLK precipitated from cells after various times of peroxide treatment (Fig. 2D). We did not observe any change of NLK activity measured *in vitro*, suggesting that increased ROS does not affect NLK activity. Thus, we con-

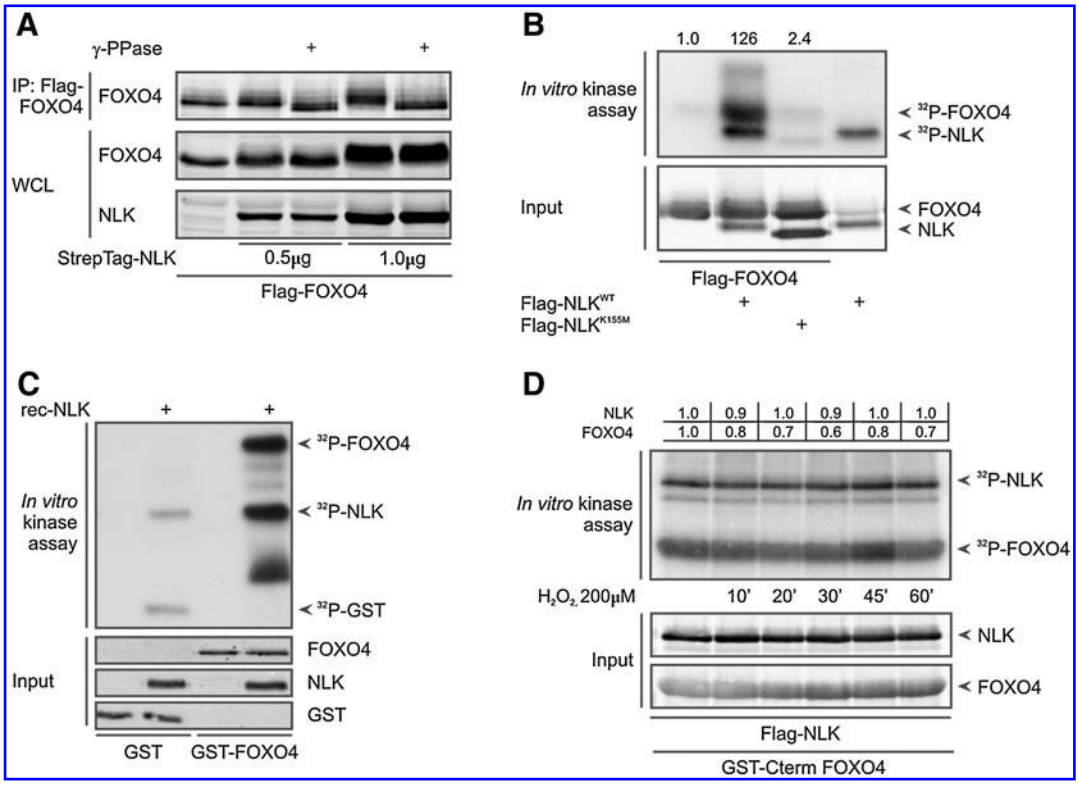


FIG. 2. NLK phosphorylates FOXO4 *in vitro* and *in vivo*. **(A)** NLK induces phosphorylation of FOXO4 *in vivo*. Flag-FOXO4 was immunopurified from HEK293T cells and treated with λ-PPase for 30 min as indicated. **(B)** NLK induces phosphorylation of FOXO4 *in vitro*. Flag-tagged FOXO4 and NLK were expressed in HEK293T cells and immunopurified. Aliquots of purified FOXO4 and NLK were subjected to an *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. Phosphorylation was measured after 15 min of incubation by autoradiography. The levels of purified protein were verified by immunoblotting using the indicated antibodies. **(C)** GST or GST-FOXO4 was incubated with His_6-NLK in the presence of [γ-^{32}P]ATP. Phosphorylation was measured as in **(A)**. The levels of recombinant proteins were verified by immunoblotting using the indicated antibodies. **(D)** H_2O_2 treatment has no effect on NLK activity. To assess the activity of NLK upon H_2O_2 treatment Flag-NLK was immunoprecipitated from HEK293T exposed to 200 μM of H_2O_2 for indicated durations. Purified NLK was incubated with [γ-^{32}P]ATP and bacterially purified C-terminal fragment of FOXO4. The phosphorylation of proteins was measured as in **(A)**. Additionally, in **(B, D)** levels of phosphorylated FOXO4 and/or NLK for individual experiments are indicated. Intensities of individual bands were quantified as in Figure 1 and the ratios between the total protein levels and the levels of phosphorylated proteins were calculated. The basal phosphorylation of FOXO4 and NLK was set to 1. GST, glutathione *S*-transferase; λ-PPase, λ-phosphatase.

clude that increased ROS stabilizes binding between NLK and FOXO4 by altering the on-off rate of the interaction. Apparently, the on-off rate of the interaction is as such that after 48 h of transient expression NLK-mediated FOXO4 phosphorylation is complete. In agreement with this and because NLK activity is not regulated by peroxide, NAC treatment also does not affect NLK-mediated phosphorylation but only the apparent binding observed in coimmunoprecipitation.

NLK negatively regulates FOXO4 transcriptional activity

To analyze whether NLK can influence FOXO4 transcriptional activity, we first performed a luciferase reporter assay using the FOXO responsive 6xDBE-luciferase (data not shown) and p27-luciferase reporters (16, 31). In both cases ectopic expression of NLK decreased FOXO4-induced expression of luciferase in a dose-dependent manner, whereas much weaker or no inhibition was observed using the kinase-dead mutant of NLK (Fig. 3A).

To further explore the effect of NLK on FOXO activity, we analyzed the endogenous expression of $p27^{kip1}$, a well-established FOXO target gene. Protein levels of $p27^{kip1}$ were strongly induced by exogenous expression of FOXO4 and FOXO1. More importantly, in both cases the FOXO-dependent induction of $p27^{kip1}$ was blocked by coexpression of NLK. Again, this inhibition was dependent on kinase activity since the kinase-dead form of NLK could not inhibit FOXO induction of $p27^{kip1}$ protein expression as efficiently as WT-NLK (Fig. 3B–D and quantification in Fig. 3E). In accordance with these observations, targeted depletion of endogenous NLK in HEK293T cells using siRNA resulted in significant increase in $p27^{kip1}$ protein levels (Fig. 3F and Supplementary Fig. S2). Further, the NLK-driven reduction in $p27^{kip1}$ protein could not be rescued by treating the cells with proteasomal inhibitor MG-132, which indicates that NLK does not influence stability of $p27^{kip1}$ but rather regulates its expression at the transcriptional level (Fig. 3C, D).

Taken together, these data show that NLK expression suppresses the ability of FOXOs to transactivate transcription.

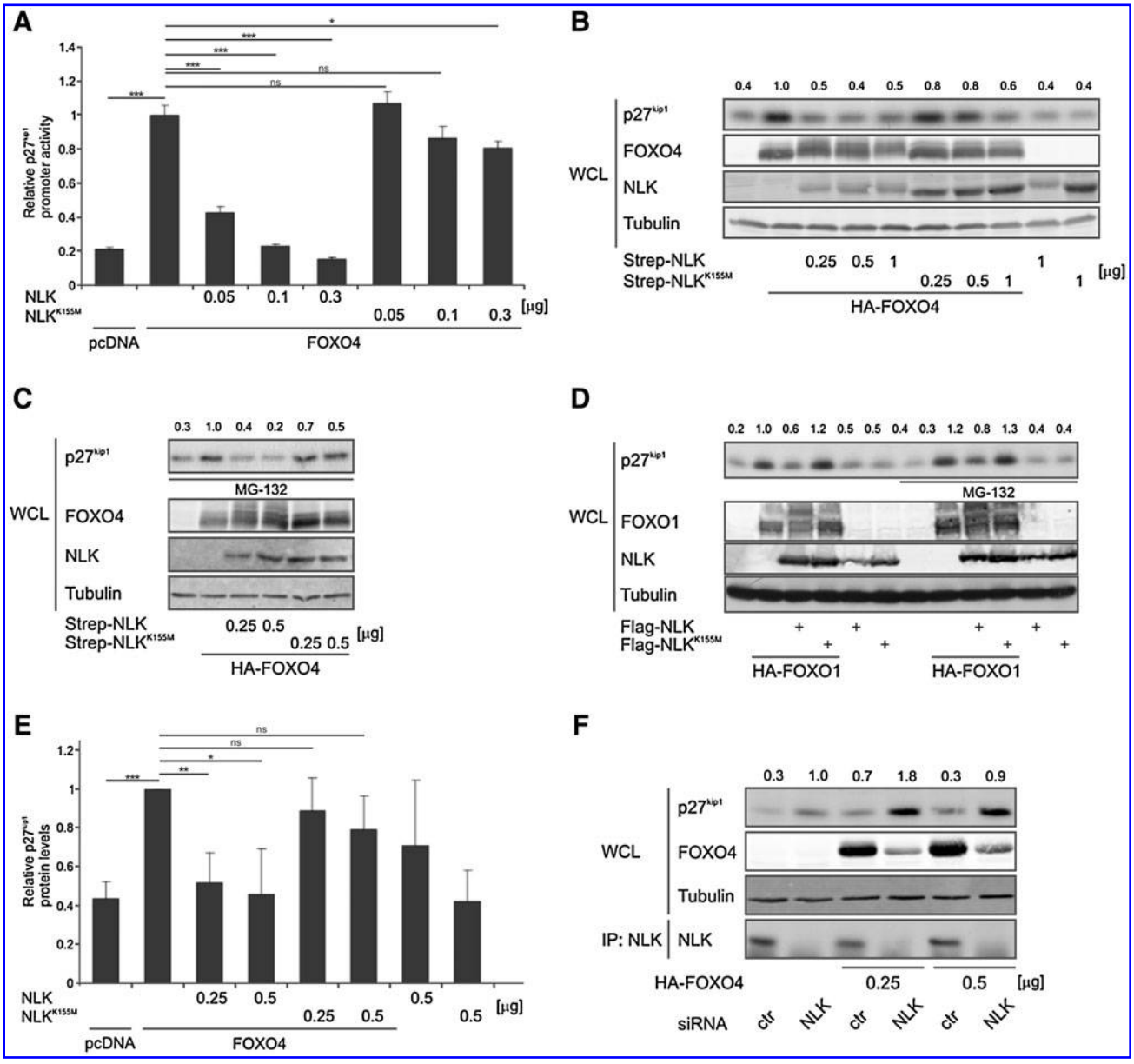


FIG. 3. NLK negatively regulates FOXO transcriptional activity. (A) NLK inhibits the transcriptional activity of FOXO4. Luciferase assay in DLD1 cells transfected with FOXO-responsive luciferase-reporters, Tk-*Renilla*, HA-FOXO4, and WT NLK or NLK^{K155M}. Data presented are normalized to FOXO4 activity in the absence of NLK set at 1. Error bars represent standard deviation; *p*-values were calculated by *t*-tests between indicated data points (experiments performed three times with three replicates in each experiment). $*p < 0.05$; $***p < 0.0001$; ns, not significant. Protein levels were confirmed by immunoblotting using the indicated antibodies. **(B–D)** Exogenous expression of NLK reduces $p27^{kip1}$ protein levels. Western blot analysis of $p27^{kip1}$ expression of puromycin-selected HEK293T cells expressing the indicated proteins. Cells were treated with 5 μM MG132 for 12 h when indicated. **(E)** $p27^{kip1}$ expression was quantified using Odyssey application software (LI-COR) and normalized to tubulin levels. The $p27^{kip1}$ level in the presence of FOXO4 was set to 1. Error bars represent standard deviation; *p*-values were calculated by *t*-tests between indicated data points (experiment performed three times); $*p < 0.05$; $**p < 0.01$; $***p < 0.0001$; ns, not significant. **(F)** Knockdown of NLK increases $p27^{kip1}$ protein levels. Western blot analysis of $p27^{kip1}$ expression of puromycin-selected HEK293T transfected with HA-FOXO4 and nontargeting siRNA or two combined siRNAs specific for NLK. To verify the efficiency of knockdown, endogenous NLK was immunoprecipitated. Additionally, in **(B–D, F)** relative $p27^{kip1}$ levels for individual experiments are indicated and quantified as in **(E)**. The $p27^{kip1}$ levels in the presence of FOXO4 or FOXO1 were set to 1.

NLK does not affect binding of FOXO4 to CBP or β-catenin

Next, we started to delineate the mechanism by which NLK could inhibit FOXO4 activity. As mentioned within the introduction, NLK regulates TCF activity by enhancing the dissociation of β-catenin/TCF complexes from the DNA, which leads to downregulation of TCF transcriptional activity. As we have shown that direct binding of β-catenin to FOXO4 after increased ROS positively regulates FOXO4 activity, we first

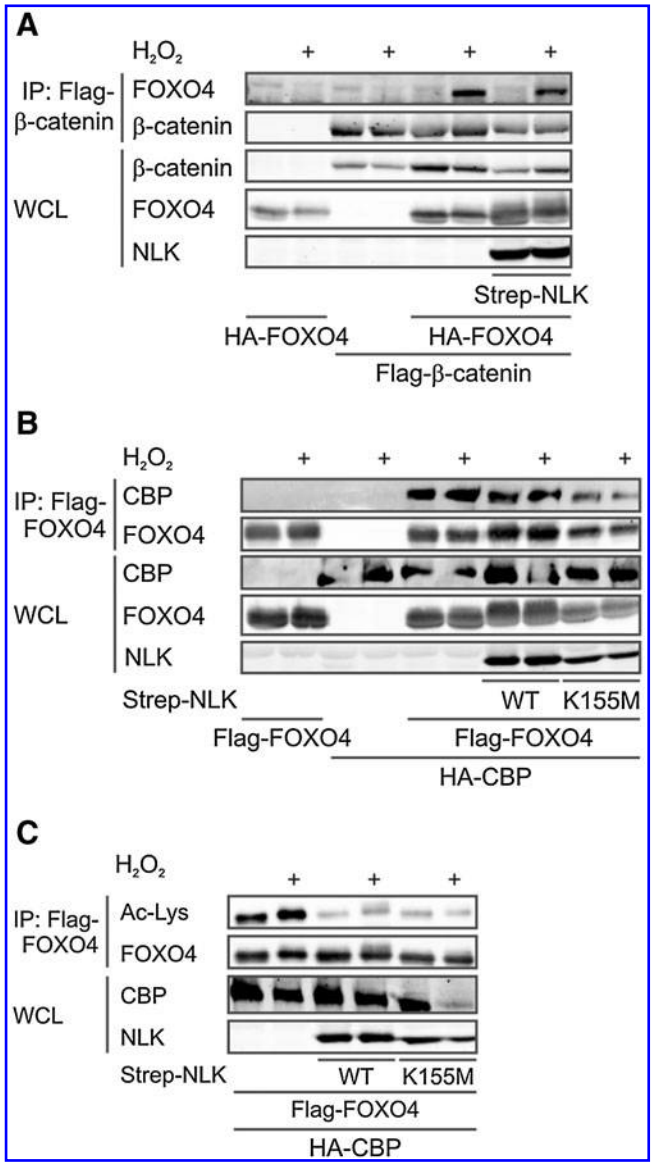


FIG. 4. NLK does not regulate FOXO4 binding to β-catenin or CBP. (A) FOXO4 binds to β-catenin in the presence of NLK. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-β-catenin was immunoprecipitated and binding of FOXO4 to β-catenin was analyzed by SDS-PAGE and immunoblotting. **(B)** FOXO4 binds to CBP in the presence of NLK. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-FOXO4 was immunoprecipitated and binding of CBP to FOXO4 was analyzed by SDS-PAGE and immunoblotting. **(C)** Both WT-NLK and NLK^{K155M} reduce acetylation of FOXO4. To assess the acetylation of FOXO4, Flag-FOXO4 was immunopurified from HEK293T cells transfected with indicated constructs followed by SDS-PAGE and immunoblotting using α-Acetyl-Lysine antibody. CBP, CREB-binding protein.

addressed whether NLK would affect the formation of the β-catenin/FOXO4 complex and by this would suppress its transactivation. However, upon coexpression of NLK, binding between FOXO4 and β-catenin did not significantly change, suggesting NLK to act independent of β-catenin (Fig. 4A).

NLK has been reported to reduce the activity of a-Myb by inhibiting the association of a-Myb with its coactivator CBP. Again, we and others have shown that CBP acetylates FOXO4, thereby reducing its activity (47). NLK-mediated loss of CBP-binding to FOXO4 would therefore unlikely represent a mechanism for NLK-mediated FOXO inhibition. However, CBP binding to FOXO also results in recruitment of CBP to histones, and CBP-mediated acetylation of histones is strongly associated with transcriptional activation (44). Thus, in this way inhibition of the CBP-FOXO4 interaction would indirectly affect the ability of FOXO4 to stimulate transcription. Again, as shown in Figure 4B, coexpression of NLK did not affect binding of CBP to FOXO4. Conversely, acetylation of FOXO4 was reduced by the presence of NLK but more importantly to the same extent by its kinase-dead mutant. We observed that both wild-type NLK and NLK^{K155M} were acetylated by CBP (data not shown). This may explain the decrease in CBP-mediated acetylation of FOXO4 as this could result from competition between substrates. By taking into consideration that acetylation inhibits FOXO4 activity and that NLK-mediated inhibition of FOXO4 transactivation strongly depends on the kinase activity of NLK, it is unlikely that the decrease in acetylation of FOXO4 will account for NLK-mediated repression. Taken together, NLK does not interfere with binding of FOXO4 to β-catenin and CBP. Moreover, the NLK-induced decrease in CBP-directed acetylation of FOXO4 does not require its kinase activity and thereby most likely does not account for NLK-mediated inhibition of FOXO-induced $p27^{kip1}$ levels.

NLK regulates FOXO4 monoubiquitination

In response to oxidative stress FOXO4 is rapidly monoubiquitinated and this results in upregulation of its transcriptional activity (46). Therefore, we decided to next investigate whether the NLK-induced suppression of FOXO4 activity was linked with changes in the ubiquitination status of FOXO4. To address this possibility, we performed an *in vivo* ubiquitination assay of FOXO4 as described previously (46) in the presence or absence of NLK. Expression of wild-type NLK but not its inactive mutant resulted in complete elimination of stress-induced FOXO4 monoubiquitination (Fig. 5A and Supplementary Fig. S3A). Moreover, NLK inhibited ubiquitination of FOXO4 in a concentration-dependent manner (Fig. 5B). This was not accompanied by a shift from mono- to enhanced poly-ubiquitination of FOXO4. In agreement with this, the protein half-life of FOXO4 was not affected by NLK coexpression (Fig. 5C). In addition, NLK-dependent inhibition of monoubiquitination was specific for FOXO4 since we did not observe any effect on ubiquitination of $p27^{kip1}$ and USP7 (Fig. 5D, Supplementary Fig. S3A, and data not shown). Thus, we conclude that NLK specifically inhibits monoubiquitination of FOXO4 after cellular oxidative stress and that this, in agreement with our previous studies, results in inhibition of FOXO4 transcriptional activity.

Phosphorylation of FOXO4 on S/T-P motifs is not required for NLK-mediated inhibition of FOXO4 monoubiquitination

Since we observed that NLK can phosphorylate FOXO4 *in vitro* and *in vivo*, we addressed whether NLK-dependent phosphorylation of FOXO4 is necessary for NLK-mediated

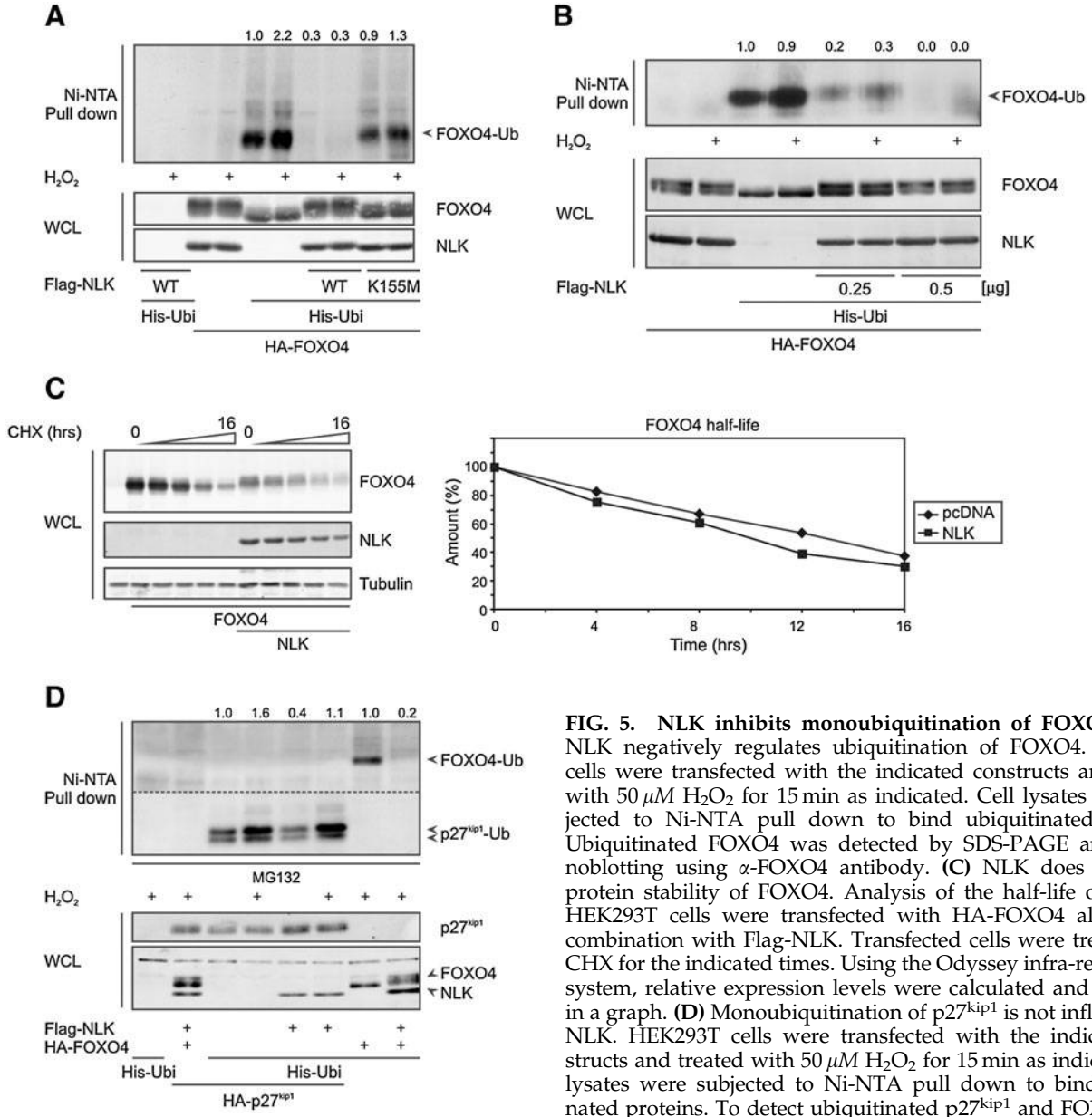


FIG. 5. NLK inhibits monoubiquitination of FOXO4. (A, B) NLK negatively regulates ubiquitination of FOXO4. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(C)** NLK does not affect protein stability of FOXO4. Analysis of the half-life of FOXO4. HEK293T cells were transfected with HA-FOXO4 alone or in combination with Flag-NLK. Transfected cells were treated with CHX for the indicated times. Using the Odyssey infra-red imaging system, relative expression levels were calculated and displayed in a graph. **(D)** Monoubiquitination of $p27^{kip1}$ is not influenced by NLK. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. To detect ubiquitinated $p27^{kip1}$ and FOXO4, SDS-PAGE and immunoblotting were performed using α-$p27^{kip1}$ and α-FOXO4 antibodies. Additionally, in **(A, B, D)** relative levels of ubiquitinated FOXO4 and/or $p27^{kip1}$ for individual experiments are indicated. The levels of ubiquitinated proteins were quantified as in Figure 1 and normalized to their total expression levels. The levels of FOXO4 and $p27^{kip1}$ ubiquitinated in the absence of NLK and H_2O_2 treatment were set to 1. CHX, cycloheximide.

inhibition of FOXO4 monoubiquitination and subsequent decrease in its transcriptional activity. Therefore, we first sought to determine which residues in FOXO4 are specifically phosphorylated by NLK. For that reason, we performed an *in vitro* kinase assay using bacterially expressed GST-FOXO4 and recombinant His-NLK. Subsequently, phosphorylated GST-FOXO4 was analyzed using liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) and we were able to identify 28 different phosphorylation sites on FOXO4 (Fig. 6A). Surprisingly, the identified phosphorylation sites did not adhere to a specific consensus sequence as suggested for NLK. NLK has been classified as a proline-directed MAPK, phosphorylating serine or threonine residues followed by proline (S/T-P motifs) (4). Yet, although we observed six out of the eight possible S/T-P sites of FOXO4 to be phosphorylated, we also observed S-Q (ATM consensus) and other types of phosphorylation sites. The reason for this apparent anomaly is at present unclear. LC-MS/MS analysis of Flag-FOXO4 extracted from cells after coexpression with NLK revealed phosphorylation of several SP/TP sites, including Ser237 and 268 and Thr447 and 451. However, protein coverage in these experiments was not 100%, so additional phosphorylation sites may have been missed. Nevertheless, considering that NLK was originally classified as an MAPK, we therefore decided to narrow our *in vivo* analysis on investigating the role of the six S/T-P phosphorylated sites in

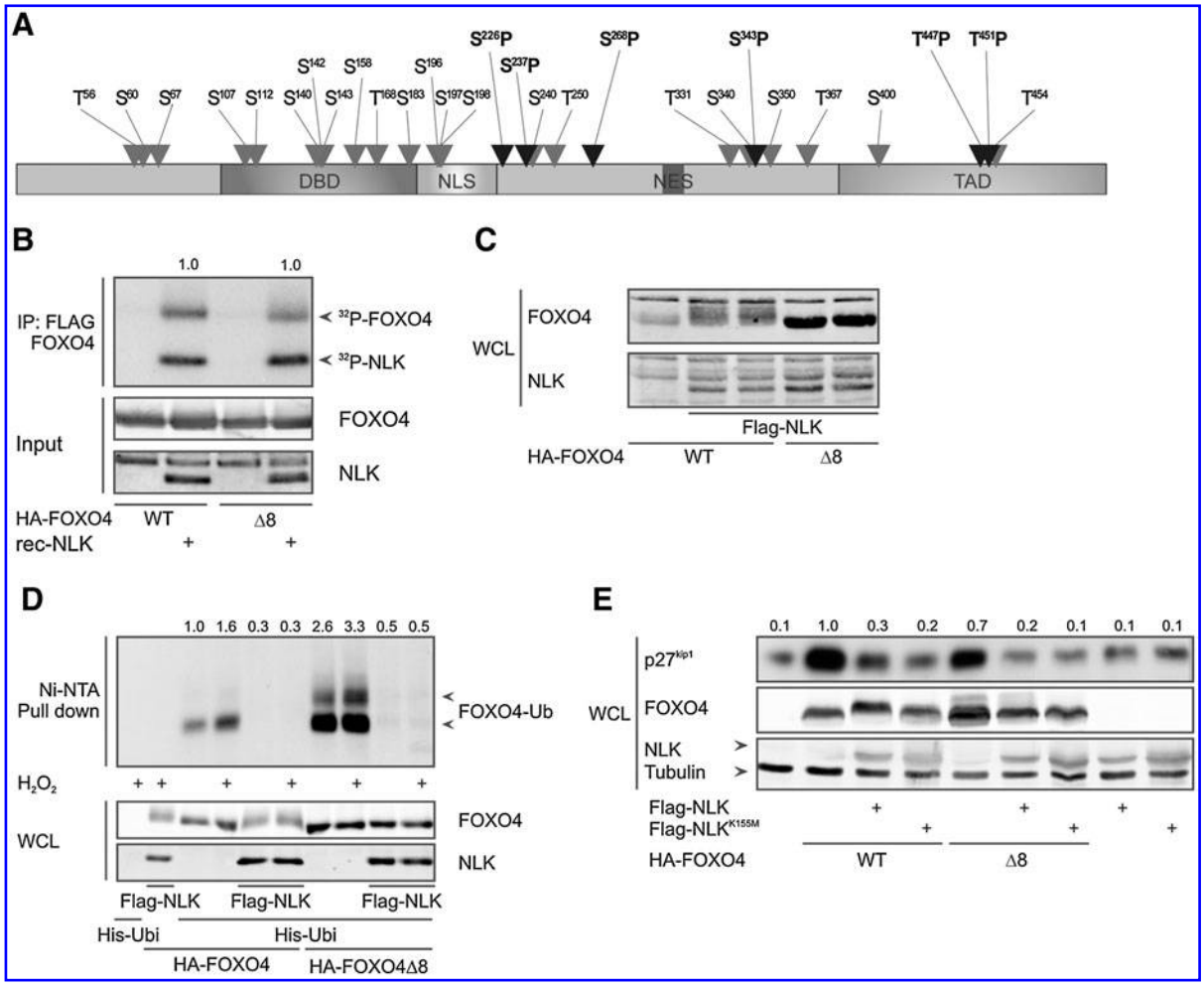


FIG. 6. NLK-directed phosphorylation of FOXO4 on S/T-P motifs is not required for inhibition of its monoubiquitination. **(A)** NLK phosphorylates FOXO4 on multiple S and T residues *in vitro*. Identification of NLK-specific phosphosites in FOXO4. GST-FOXO4 was incubated with His_6-NLK in the presence of ATP and analyzed using LC-MS/MS. The identified S/T-P sites are indicated by black triangles. **(B)** Effect of S/T-P motifs mutations on NLK-mediated phosphorylation *in vitro*. Flag-FOXO4 WT or 8A mutant (Δ8) was expressed in HEK293T cells and immunoprecipitated. Aliquots of purified FOXO4 were incubated with or without NLK in the presence of γ-P^{32}ATP. Phosphorylation was measured after 15 min. of incubation. Proteins levels were confirmed by SDS-PAGE and immunoblotting using the indicated antibodies. Additionally, relative levels of phosphorylated FOXO4 are indicated. Intensities of the individual bands were quantified as in Figure 2 and they were normalized to phosphorylation of WT-FOXO4 in presence of NLK was set to 1. **(C)** Effect of mutations of S/T-P motifs on the NLK-mediated shift in gel mobility of FOXO4. Total lysate of HEK293T cells expressing as indicated HA-FOXO4 WT and Δ8 and Flag-NLK were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(D)** NLK inhibits monoubiquitination of FOXO4Δ8. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. To detect ubiquitinated FOXO4 SDS-PAGE and immunoblotting was performed using α-FOXO4 antibody. Relative levels of ubiquitinated FOXO4 are indicated and quantified as in Figure 5. The level of ubiquitinated WT-FOXO4 in the absence of NLK and H_2O_2 treatment was set to 1. **(E)** NLK inhibits FOXO4Δ8-induced $p27^{kip1}$ expression. Total lysate of puromycin-selected HEK293T cells transfected with the indicated constructs were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. Relative levels of $p27^{kip1}$ normalized to tubulin levels are indicated. The $p27^{kip1}$ level in the presence of WT-FOXO4 was set to 1. DBD, DNA binding domain; NLS, nuclear localization signal; NES, nuclear export signal; TAD, transactivation domain.

the NLK-dependent shift in gel mobility of FOXO4. To this end, we substituted individual serine or threonine residues within S/T-P motifs of FOXO4 for alanine, and we observed that substitution of Ser^{268} with Ala^{268} in FOXO4 resulted in significant loss of reduced mobility (data not shown). This indicated that *in vivo* at least Ser^{268} is phosphorylated by NLK. However, NLK expression could still inhibit ubiquitination of the $Ser^{268}\rightarrow Ala^{268}$ mutant of FOXO4 to the same extent as wild-type FOXO4 (data not shown). Additionally, simultaneous mutation of all eight serine and threonine residues to alanine also had no effect on the efficiency of phosphorylation of FOXO4 by NLK in an *in vitro* kinase assay even though this mutant of FOXO4 (FOXO4Δ8) did not exhibit any shift in gel mobility (Fig. 6B, C). Consistent with the notion that NLK phosphorylated FOXO4Δ8 as efficiently as the wild-type FOXO4, mutation of these residues had no effect on

NLK-mediated inhibition of ubiquitination of FOXO4 (Fig. 6D and Supplementary Fig. S4A). Moreover, FOXO4Δ8 was still able to induce $p27^{kip1}$ and this induction was still reduced by ectopic overexpression of NLK (Fig. 6E and Supplementary Fig. S4B). Taken together, these data indicate that NLK-mediated phosphorylation of S/T-P in FOXO4 is unlikely to be required for inhibition of its monoubiquitination by NLK.

NLK-mediated inhibition of monoubiquitination occurs independently of Mdm2, USP7, or Pin1

To further understand at which level NLK regulates ubiquitination of FOXO4, we investigated whether NLK expression would affect the interaction between FOXO4 and its E3 ligase Mdm2 (2). Ectopic expression of NLK did not change binding between Mdm2 and FOXO4 (Fig. 7A). Moreover, NLK could still inhibit ubiquitination of FOXO4 in cells lacking Mdm2, suggesting that NLK does not block ubiquitination of FOXO4 through inhibition of Mdm2 activity (Fig. 7B and Supplementary Fig. S5A). To additionally support that NLK has no influence on Mdm2 activity, we also performed an *in vitro* ubiquitination assay for FOXO4. In this assay recombinant His-Mdm2 was able to ubiquitinate bacterially expressed GST-FOXO4 in the presence of recombinant E1 (UBE1), E2 (UbcH5b), and His-ubiquitin. However, as shown in Figure 7C, addition of recombinant NLK did not alter the ubiquitination pattern for FOXO4 under these conditions (lanes 4 and 5). Moreover, to enhance efficiency of NLK-directed phosphorylation and to enrich for the phosphorylated form of Mdm2 and FOXO4 (Mdm2-P and FOXO4-P), we pretreated both His-Mdm2 and GST-FOXO4 with His-NLK before performing the ubiquitination assay. Nevertheless, we did not observe any significant changes in FOXO4 ubiquitination in the situation where both Mdm2 and FOXO4 were prephosphorylated (lanes 9–12).

USP7 is a deubiquitinating enzyme (DUB) for FOXO4 (46), and therefore we considered the possibility that NLK enhanced deubiquitination of FOXO4 by promoting USP7 activity toward FOXO4. Using a coimmunoprecipitation assay, we did not observe any effect of NLK on the interaction between USP7 and FOXO4 (Fig. 7D). Consequently, NLK was still able to inhibit ubiquitination of FOXO4 after depletion of USP7 by siRNA, suggesting that NLK changes the ubiquitination status of FOXO4 regardless of USP7 (Fig. 7E and Supplementary Fig. S5B).

Finally, previously we have shown Pin1 to also act as a negative regulator of monoubiquitination of FOXO4 (3), suggesting the possibility that Pin1 and NLK act together in this process. Nevertheless, as shown in Figure 7F and Supplementary Fig. S5C, NLK was still sill able to completely abolish ubiquitination of FOXO4 in cells lacking Pin1, indicating that Pin1 is not necessary for NLK-mediated inhibition.

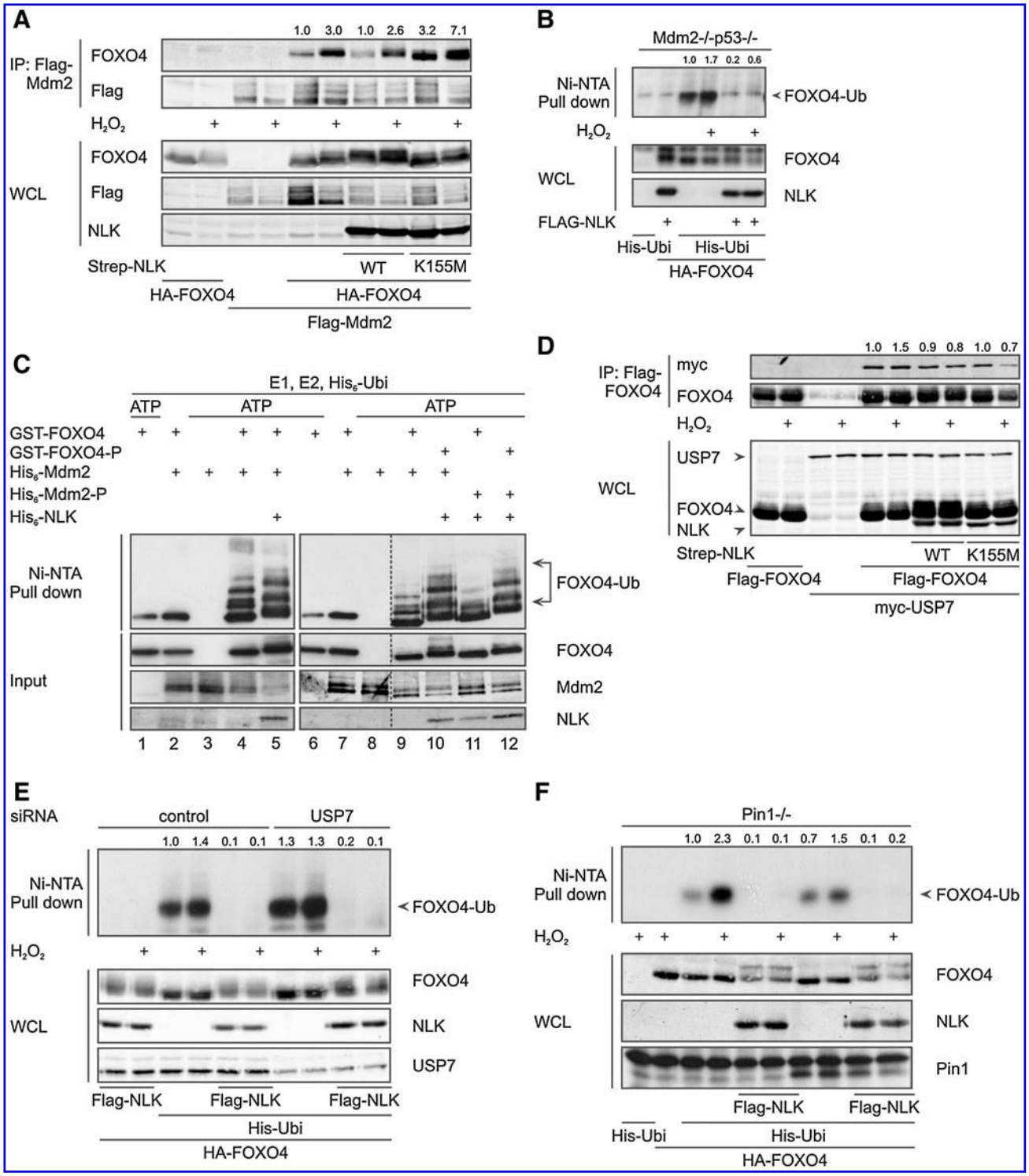
Thus, we conclude that negative regulation of monoubiquitination of FOXO4 by NLK occurs independently of Mdm2, USP7, and Pin1 activity.

Both NLK and p38 exhibit similar effect on monoubiquitination of FOXO4 as well as its transcriptional activity

MAP3K TAK1 can activate NLK in response to Wnt signaling (21, 22, 24, 30, 40). Therefore, we addressed whether TAK1 could also act as an upstream activator of NLK in case of FOXO4 repression. We performed an *in vivo* ubiquitination assay of FOXO4 and we observed that ectopic expression of TAK1 but not its kinase dead mutant ($TAK1^{D175A}$) led to the increase in ubiquitination of FOXO4 (Fig. 8A and Supplementary Fig. S6A). Hence, TAK1 is unlikely to be an upstream kinase of NLK in regard to the inhibition of FOXO4.

Given that it has been published recently that MAPK p38 serves as an activator of NLK, we investigated the effect of p38 on ubiquitination of FOXO4 (35). Similarly to NLK, p38 also exhibited a negative effect on ubiquitination of FOXO4 (Fig. 8B and Supplementary Fig. S6B). Moreover, ectopic expression of p38 suppressed the FOXO4-induced $p27^{kip}$ protein levels (Fig. 8C and Supplementary Fig. S6C). Taken together, these find-

FIG. 7. NLK-dependent inhibition of FOXO4 monoubiquitination does not require activity of Mdm2, USP7, and Pin1. **(A)** FOXO4 binds to Mdm2 in the presence of NLK. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-Mdm2 was immunoprecipitated and binding of FOXO4 to Mdm2 was analyzed by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(B)** FOXO4 ubiquitination assay in $Mdm2^{-/-}p53^{-/-}$ MEFs. MEFs were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(C)** *In vitro* FOXO4 ubiquitination assay. Recombinant GST-FOXO4, His_6-Mdm2, E1, E2 and His_6-ubiquitin were incubated for 2 h after adding ATP. To detect ubiquitinated GST-FOXO4, samples were subjected to Ni-NTA pull down followed by SDS-PAGE and immunoblotting using α-FOXO4 antibody. When indicated recombinant His_6-NLK was added to the reaction or GST-FOXO4 or His_6-Mdm2 were treated with His_6-NLK for 30 min before the addition of E1, E2 and ubiquitin. The levels of recombinant proteins were verified by SDS-PAGE and immunoblotting using the indicated antibodies. Separate lanes of SDS-PAGE are numbered 1–12 for reference purposes (see text results section). **(D)** FOXO4 binds to USP7 in the presence of NLK. Experiments were performed essentially as in **(A)** using the indicated constructs. **(E)** Effect of USP7 knockdown on NLK-mediated inhibition of ubiquitination of FOXO4. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(F)** FOXO4 ubiquitination assay in $Pin1^{-/-}$ MEFs. Experiments were performed essentially as in **(B)** using the indicated constructs but now using $Pin1^{-/-}$ MEFS. Additionally, in **(A, D)** relative amounts of USP7 or Mdm2 bound to FOXO4 for individual experiments are indicated and quantified as in Figure 1. The amounts of USP7 or Mdm2 bound to FOXO4 in the absence of NLK and H_2O_2 treatment were set to 1. **(B, E, F)** Relative levels of ubiquitinated FOXO4 for individual experiments are indicated and quantified as in Figure 5. The level of ubiquitinated FOXO4 in the absence of NLK and H_2O_2 treatment was set to 1. Mdm2, murine double minute 2; Pin1, peptidyl-prolyl isomerase 1; USP7, ubiquitin-specific peptidase 7.



ings point toward p38 as a likely upstream activator of NLK that could potentiate its negative activity against FOXO4.

However, siRNA-mediated knockdown of NLK did not significantly change the ability of p38 expression to reduce FOXO4 monoubiquitination (data not shown). In addition, we observed direct phosphorylation of FOXO4 by p38 at least *in vitro* (BMTB unpublished observations), and thus p38 may regulate FOXO4 through NLK-dependent and NLK-independent pathways (see summarizing Fig. 8D).

Discussion

Here we provide evidence for NLK as a novel regulator of FOXO4 that negatively influences its transcriptional activity.

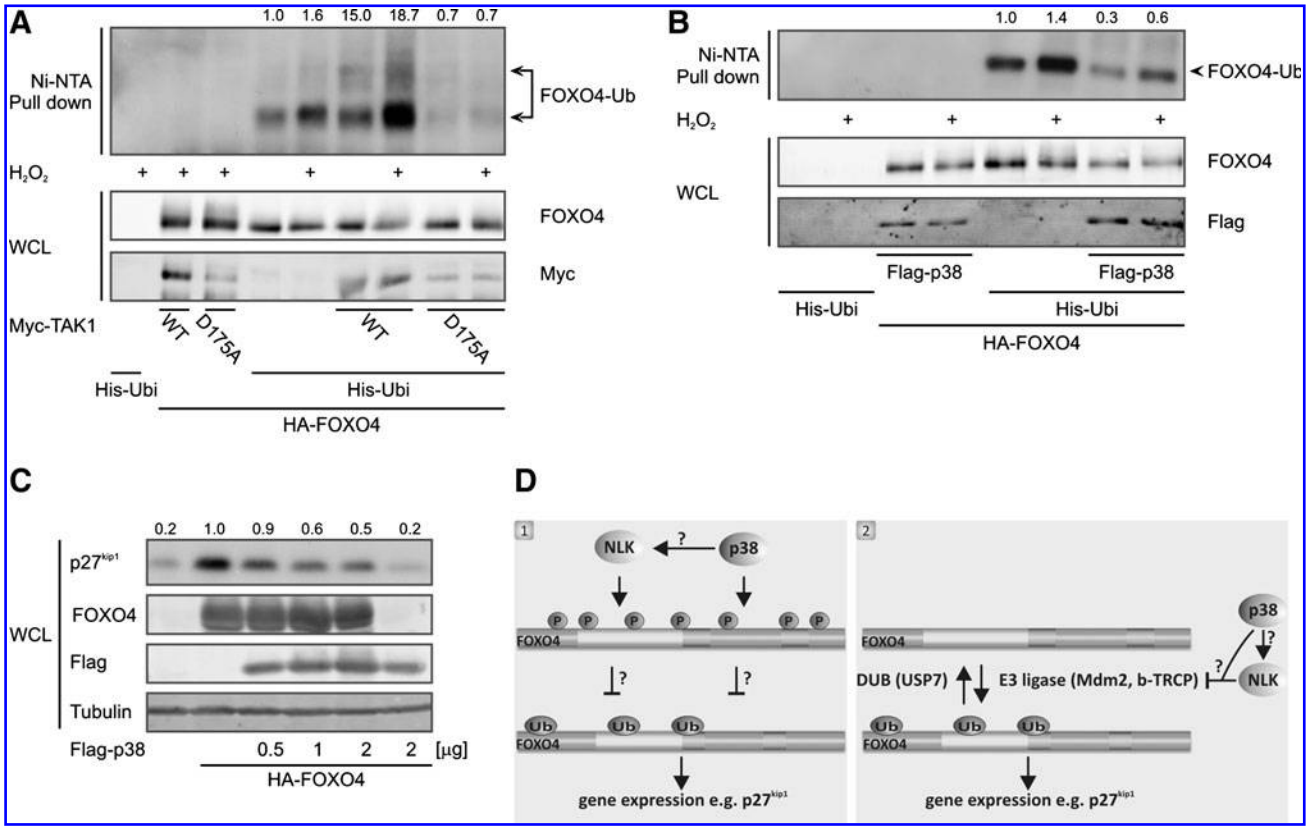


FIG. 8. TAK1 enhances ubiquitination of FOXO4, whereas p38 MAPK decreases ubiquitination of FOXO4 and FOXO4-induced $p27^{kip1}$ protein levels. (A) TAK1 enhances ubiquitination of FOXO4. HEK293T cells were transfected with HA-FOXO4 and wild-type TAK1 or kinase dead TAK1 ($TAK1^{D175A}$). Cells were treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(B)** p38 MAPK decreases ubiquitination of FOXO4. Experiments were performed essentially as in **(A)** using the indicated constructs. **(A, B)** Relative amounts of ubiquitinated FOXO4 for individual experiments are indicated and quantified as in Figure 5. The levels of ubiquitinated FOXO4 in the absence of TAK1 or p38 MAPK and H_2O_2 treatment were set to 1. **(C)** Exogenous expression of p38 MAPK reduces $p27^{kip1}$ protein levels. Western blot analysis of $p27^{kip1}$ expression in puromycin-selected HEK293T cells expressing HA-FOXO4 and Flag-p38 as indicated. Relative levels of $p27^{kip1}$ are indicated and quantified as in Figure 3. The $p27^{kip1}$ level in the presence of WT-FOXO4 was set to 1. **(D)** Here we show that NLK could interact with FOXO4 in a ROS-dependent manner. In addition, NLK can phosphorylate FOXO4 on multiple residues. NLK inhibits transcriptional activity and monoubiquitination of FOXO4; however, this appears not to be linked to its ability to directly phosphorylate FOXO4 (panel 1). Nevertheless, since NLK-mediated inhibition of FOXO4 ubiquitination and its activity depends on NLK kinase activity, we hypothesize that NLK could act on the ubiquitination machinery (*e.g.*, E3 ligase) (panel 2). This is indicated by our result that NLK inhibits residual monoubiquitination in $Mdm2^{-/-}$ cells and Mdm2-reconstituted cells. The stress kinase p38, a potential upstream regulator of NLK, also inhibits FOXO4 monoubiquitination and directly phosphorylates FOXO4 *in vitro* (not shown). P38 MAPK may therefore regulate monoubiquitination in an NLK-dependent and NLK-independent manner (panels 1 and 2). MAPK, mitogen-activated protein kinase; TAK1, transforming growth factor β-activated kinase 1.

In response to increased cellular oxidative stress, complex formation between NLK and FOXO4 is strongly enhanced and this results *in vivo* in phosphorylation of FOXO4 on at least one of the eight S/T-P motifs, that is, Ser^{268}. Although the interaction between NLK and FOXO4 is enhanced after increased ROS, we were unable to show that an increment in the ROS level directly increases the activity of NLK as measured by an *in vitro* kinase assay. In addition to the above, we were unable to show significant changes in the *in vitro* kinase activity of NLK after treatment of NLK-expressing HEK293T cells with Wnt5a or coexpression of TAK1. In agreement, whereas NLK inhibits FOXO4 monoubiquitination, we observe TAK1 expression to enhance FOXO4 monoubiquitination in concordance with TAK1 signaling toward JNK (33) and JNK-regulating FOXO4 monoubiquitination (manuscript in preparation). Taken together, this suggests the possibility that under the experimental conditions employed here NLK is actually a constitutively active kinase, and its potential to specifically regulate FOXO4 activity therefore depends on the ability of NLK to colocalize with its substrate, which is in this case FOXO4. A similar mechanism has been described for some other kinases. For example, the activation of PKB by constitutively active 3-phosphoinositide-dependent protein kinase-1 (PDK1) is regulated through the growth factor-induced phosphatidylinositol 3,4,5-trisphosphate [PtdIns $(3,4,5)P_3$] production resulting in simultaneous recruitment of PKB and PDK1 to the plasma membrane. This induced colocalization of PKB and PDK1, and then enforces a PtdIns

(3,4,5)P_3-dependent conformational change of PKB to allow PDK1 to phosphorylate the critical threonine 308 within the T-loop of PKB (36).

Taking into the consideration that NLK is mainly localized in the nucleus (4), it is likely that in this case FOXO4 translocates upon peroxide treatment to colocalize with NLK. This is consistent with previous reports that elevated ROS leads to nuclear translocation of FOXO4 (13). Moreover, the observation that in an *in vitro* kinase assay the presence of FOXO4 actually appears to increase NLK activity (Fig. 2C), suggesting the possibility that the actual increase in NLK activity is prompted by its direct binding to the substrate.

Oxidative stress regulates the transcriptional activity of FOXOs in a positive manner (15). This leads to expression of genes that contribute to detoxification of cellular ROS and repair of DNA damage (18, 28, 42, 43). Furthermore, transactivation of FOXOs induces cell cycle arrest, which provides time for scavenging of ROS and repairing of DNA damage to take place (10, 14, 32, 39). In apparent contradiction, elevation in cellular ROS level simultaneously leads to inhibition of FOXO4 by NLK. However, the cellular response to ROS is tightly linked to the cellular concentration of ROS. Thus, whereas ROS is often studied in the context of a damaging signal eventually leading to cell death, ROS also performs a role in normal nonpathological signaling. Hence, it is conceivable that when the level of oxidative stress within the cell exceeds a certain threshold, it becomes important to downregulate in this case the FOXO4-dependent antioxidant defense program to allow apoptosis. NLK induces apoptosis in DLD1 cells (49). In addition, we observed that ectopic expression of NLK in U2OS cells suppressed the growth of these cells and induced apoptosis in a kinase-dependent manner (data not shown). Hence, the NLK-mediated transrepression of FOXO4 activity in response to elevated ROS might be important for overcoming the FOXO4-induced cell cycle arrest to trigger apoptosis. In agreement with this hypothesis, ectopic expression of NLK causes downregulation of FOXO4-induced $p27^{kip1}$ levels. This inhibition partially depends on kinase activity, as much weaker inhibition is observed after overexpression of kinase-dead form of NLK.

In contrast, downregulation of NLK protein levels increases $p27^{kip1}$ expression. Our previous study has shown that monoubiquitination of FOXO4 increases its nuclear localization and consequently enhances its transcriptional activity (46). Here we observe that ubiquitination of FOXO4 is abolished by NLK, which might provide a mechanism for the inhibition of FOXO4 by NLK. Considering that inhibition of both FOXO4 monoubiquitination and FOXO4-triggered expression of $p27^{kip1}$ requires kinase activity of NLK, it became interesting to determine the involvement of NLK-dependent phosphorylation of FOXO4 in the inhibition of its activity. NLK belongs to the MAPK family and has been reported previously to phosphorylate a broad spectrum of transcription factors on multiple Ser or Thr residues lying within the S/T-P motif. For example, NLK-mediated repression of Notch1 requires phosphorylation of at least 7 residues in Notch1. Similarly, in case of c-Myb, NLK has been reported to phosphorylate 15 different residues and substitutions of all of them with Ala residues were necessary to reverse NLK-mediated degradation of c-Myb.

Despite the fact that FOXO4 contains only eight S/T-P motifs, we were unable to unequivocally define which residue is targeted by NLK phosphorylation. Even mutation of all S/T-P sites failed to abrogate NLK-dependent repression of FOXO4 transcriptional activity, which may indicate that phosphorylation of FOXO4 is not essential for its inhibition. However, using an *in vitro* kinase assay followed by LC-MS/MS analysis, we identified 28 residues in FOXO4 being phosphorylated by NLK. This, together with previous studies, suggests that the extent of NLK-mediated phosphorylation rather than site-specific phosphorylation might be crucial for NLK to exert its negative effects on substrate activity or stability.

It has been shown for other transcription factors, including Ets-1, that decrease in its activity strongly correlates with the number of sites phosphorylated within its serine-rich region (37). Therefore, it is possible that to efficiently repress FOXO4 activity NLK needs to target other residues in addition to the S/T-P motifs to achieve the required degree of phosphorylation. However, additional studies are needed to determine the relevance of this possibility with respect to FOXO4 regulation. Also, it can be questioned how relevant the analysis of a mutant of any protein harboring such a number of mutations is in comparison to the function of wild-type protein.

To further clarify the molecular mechanism leading to NLK-mediated inhibition of monoubiquitination of FOXO4, we hypothesized that NLK could influence the activity of enzymes being directly involved in ubiquitination/deubiquitination of FOXO4. Previously, we have been able to provide evidence that Mdm2 acts as an E3 ligase for FOXO4 (2), which is counteracted by USP7-mediated deubiquitination (46). However, based on our results obtained in $Mdm2^{-/-}p53^{-/-}$ cells as well as in an *in vitro* ubiquitination assay, it seems unlikely that NLK decreases FOXO4 ubiquitination by repression of Mdm2 activity. In addition, also USP7 appears not required for NLK-mediated regulation of ubiquitination of FOXO4. Finally, even though the peptidyl isomerase Pin1 attenuates ubiquitination of FOXO4, loss of Pin1 does not eliminate the inhibitory effect of NLK.

Despite these essentially negative results with respect to how NLK may regulate ubiquitination of FOXO4, we cannot exclude the involvement of other E3 ligases or DUBs necessary for NLK to negatively modulate ubiquitination of FOXO4. For instance, like FOXO4, p53 is also a substrate for USP7, yet other DUBs, for example, USP10 have been identified to also deubiquitinate p53 and contribute to its activation (50). Therefore, it would be interesting to address whether USP10 can also target ubiquitinated FOXO4.

In mammals the FOXO family consists of four members: FOXO1, FOXO3a, FOXO4, and FOXO6 (7, 25). We show here that similarly to FOXO4, NLK can also interact with FOXO1 and negatively modulate its transcriptional activity, suggesting that NLK acts as general regulator of all FOXOs. Moreover, while this article was under preparation, it was reported that the TAK1-NLK pathway inhibits activity of FOXO1 *via* triggering FOXO1 translocation out of the nucleus (27). Although the mechanism for this was not determined, it is clear that these observations are complementary to what would be expected for inhibition of monoubiquitination. However, in contrast to FOXO4, mutation of eight S/T-P sites present in FOXO1 alleviated the effect of NLK. A rationale for this different behavior of mutant FOXO4 *versus* mutant FOXO1 is unclear at present. Nevertheless, it is apparent that NLK antagonizes FOXOs transcriptional activity. Therefore,

it would be interesting to determine whether NLK-mediated regulation of FOXOs activity is conserved throughout evolution in a manner similar to the regulation by PKB and JNK (26, 34).

Acknowledgments

The authors are grateful to Dr. P. Cohen and Dr. L. Smit for TAK1 and NLK expression plasmids, respectively. The authors also thank Dr. P. van der Sluijs and Dr. A.G. Jochemsen for $Pin^{-/-}$ and $Mdm2^{-/-}p53^{-/-}$ MEFs, correspondingly. The authors also appreciate helpful discussion with comembers of the Bos-Burgering lab. This work was supported by grants from the Center for Biomedical Genetics (CBG), the Cancer Genomics Center (CGC), and the Netherlands Proteomics Center (NPC).

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, April 9, 2010; date of final revised submission, September 28, 2010; date of acceptance, September 28, 2010.

Abbreviations Used

CBP = CREB-binding protein
CHX = cycloheximide
DUBs = deubiquitinating enzymes
FOXO = Forkhead box O
GST = glutathione *S*-transferase
H_2O_2 = hydrogen peroxide
JNK = c-Jun N-terminal Kinase
LEF/TCF = lymphoid enhancer factor/T-cell factor
MDM2 = murine double minute 2
MEF = mouse embryonic fibroblast
NAC = N-acetyl cysteine
NARF = NLK-associated ring finger protein
Ni-NTA = nickel-nitrilotriacetic acid
NLK = nemo-like kinase
p38 MAPK = p38 mitogen-activated protein kinases
p53 = protein 53
PAGE = polyacrylamide gel electrophoresis
PDK1 = 3-phosphoinositide-dependent protein kinase-1
PI3K = phosphoinositide 3-kinase
Pin1 = peptidyl-prolyl isomerase 1
PKB = protein kinase B
PPAR = peroxisome proliferator-activated receptor
PtdIns(3,4,5)P_3 = phosphatidylinositol 3,4,5-trisphosphate
ROS = reactive oxygen species
SD = standard deviation
SDS = sodium dodecyl sulfate
SETDB1 = SET domain, bifurcated 1
TAB1 = TAK1-binding protein
TAK1 = transforming growth factor β-activated kinase 1
USP7 = ubiquitin-specific peptidase 7
USP10 = ubiquitin-specific peptidase 10

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