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ORIGINAL PAPER

Redox regulation of cytokeratin 18 protein by NADPH oxidase 1 in preneoplastic human epithelial cells

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Abstract
Introduction A catalytic subunit of NADPH oxidase 1 (Nox1) is implicated to be involved in neoplastic progression in human epithelial cancers. We had previously demonstrated that Nox1 overexpression of immortalized epithelial cells was able to induce the generation of progenitor cells that expressed fetal-type cytokeratins 8 and 18.
Purpose We aimed to investigate the direct effects and underlying mechanisms of Nox1 on expression of cytokeratin 18 (CK18).
Methods Immortalized human epithelial GM16 cells with low CK18 were used in Nox1 overexpression experiments. NuB2 cells with high CK18 were used in Nox1 knockdown experiments. Protein expression of CK18, phosphorylated and ubiquitinated CK18 were analyzed by Western blot.
Results With no effects on the mRNA levels, CK18 protein was increased upon Nox1 overexpression and decreased upon Nox1 knockdown. Treatment with proteasome inhibitor MG132 prevented CK18 degradation and increased CK18 protein indicating translational regulation of CK18. Treatment for NuB2 cells with N-acetyl-L-cysteine, diphenyleneiodonium, or apocynin decreased CK18 protein levels indicating its regulation involving reactive oxygen species and flavoprotein Nox. It has been known that phosphorylation of CK18 regulates CK18 turnover by ubiquination. Consistently, Nox1 modulated CK18 phosphorylation at ser52. Nox1 knockdown and treatment with diphenyleneiodonium accumulated the levels of ubiquinated CK18 enhancing degradation causing decreased CK18 protein.
Conclusion We demonstrated that Nox1 was able to induce CK18 stabilization by inhibiting CK18 protein degradation in a phosphorylation-dependent manner. CK18 accumulation induced by Nox1 is consistent with the persistence of fetal-type CK18 protein in many epithelial carcinomas.

Keywords Cytokeratin 18 · Nox1 · Ubiquitin–proteasome pathway · Reactive oxygen species · Immortalized epithelial cells

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Abbreviations

CK	Cytokeratin
DPI	Diphenyleneiodonium
EGF	Epidermal growth factor
GM16	Human gingival mucosal epithelial cells immortalized by HPV16
HPV16	Human papilloma virus type 16
KGM	Keratinocyte growth medium
NAC	N-acetyl-L-cysteine
Nox1	NADPH oxidase 1
qPCR	Quantitative real-time PCR
ROS	Reactive oxygen species
siRNA	Small interfering RNA

Introduction

Strong evidence has emerged that many of non-phagocytic homologs of NADPH oxidase (Nox) gp91*phox* mediate neoplastic progression by generating low levels of reactive oxygen species (ROS) to promote tumor cell growth (Lambeth et al. 2007). Among seven Nox family members, Nox 1–5 and Duox 1 and 2, the most studied Nox1 is highly expressed in human epithelial such as colon (Perner et al. 2003; Fukuyama et al. 2005) and prostate (Lim et al. 2005) cancers. In rodent fibroblasts, Nox1 induces tumorigenic conversion (Suh et al. 1999), and that Nox1, which is induced by Ras (Mitsushita et al. 2004), is capable of down-regulating Rho resulting in disruption of stress fibers and focal adhesions (Shinohara et al. 2007). Nox1 in a H_2O_2-dependent manner mediates neoplastic progression by triggering an angiogenic switch (Arbiser et al. 2002), stimulating tumor cell growth (Park et al. 2004; Arnold et al. 2007), and inducing genomic instability (Chiera et al. 2008). In K-Ras-transformed normal rat kidney cells, Nox1 increases cell migration and invasion (Shinohara et al. 2010). Direct effects of Nox1 on tumorigenic conversion of human cells have not been demonstrated; plausibly because Nox1 may be functionally active in cancer with the requirement of an additional oncogene, such as, mutated K-Ras (Laurent et al. 2008). This view is supported by our data as we have shown that Nox1 was able to increase the transformation steps in human epithelial cells, which had been immortalized by E6/E7 oncogenes of human papilloma virus (HPV) type 16 (GM16) (Chamulitrat et al. 2003, 2007). Upon selection of GM16 cells by prolonged cultures after chronic ethanol treatment (Chamulitrat et al. 2003) or Nox1 overexpression (Chamulitrat et al. 2007), progenitor cells with epithelial-like and elongated fibroblast-like morphologies were subsequently generated. Cells with elongated phenotype exhibited increased expression of Nox1 as well as mesenchymal intermediate filament vimentin and fetal-type cytokeratins 8/18 (CK8/18). Nox1 is thus implicated for an induction of epithelial mesenchymal transition that is consistent with the known role of CK8/18 on invasive behaviors of cultured cells (Chu et al. 1993; Raul et al. 2004). It is yet to be demonstrated whether Nox1 could directly modulate expression of these keratins.

CK8 and CK18 are the most common and characteristic members of the large intermediate filament gene family expressed in single layer or simple epithelial tissues. CK8 and CK18 are persistently expressed in tumor cells derived from simple epithelia (Oshima et al. 1996) as well as in transitional cell carcinomas as well as in adenocarcinomas, basal cell epithelioma, and squamous cell carcinoma of skin, tongue, esophagus, and oral cavity (Schaafsma et al. 1990; Fillies et al. 2006). CK8/18 can be activated by various oncogenes, such as E6/E7 of HPV16 (Pei et al. 1992), Ha-Ras, Src, Lck, and Raf (Pankov et al. 1994). Much earlier work has shown that CK8/18 is constitutively degraded at post-translational level (Kulesh et al. 1989), and this keratin phosphorylation regulates its turnover via ubiquitination (Ku and Omary 2000). With the interplay between Nox1 and oncogenes Ras (Mitsushita et al. 2004; Laurent et al. 2008) and E6/E7 of HPV16 (Chamulitrat et al. 2003, 2007), we hypothesize that Nox1 and Nox1-mediated ROS may regulate CK18 protein at post-translational level via CK18 phosphorylation.

In our previous studies, all progenitor cells generated from the parental GM16 cells expressed elevated levels of CK8/18 proteins (Chamulitrat et al. 2003, 2007). We utilized these cells to investigate regulation of CK8/18 by Nox1 by using genetic overexpression and knockdown. Alterations by Nox1 were found for CK18 but not for CK8. We demonstrated that Nox1 regulated CK18 protein involving CK18 phosphorylation and ubiquitination. This regulation was redox dependent. Our results shed light on mechanisms for CK18 regulation by Nox1 and may explain the persistence of CK18 in many epithelial carcinomas.

Materials and methods

Reagents

N-acetyl-L-cysteine (NAC) was from AppliChem, Darmstadt, Germany. Apocynin and MG132 were obtained from Merck, Darmstadt, Germany. Diphenyleneiodonium (DPI), epidermal growth factor (EGF), phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Deisenhofen, Germany.

Cell culture

GM16 cells were grown in Clonetics keratinocyte growth medium (KGM) supplemented with singlequots from Cell Systems (Lonza, Verviers, Belgium). Cell lines produced from our previous studies (Chamulitrat et al. 2007) were cultured in DMEM containing 10% FCS (PAA, Cölbe, Germany).

Nox1 plasmids and transfection

Nox1 cDNAs were prepared from HT29 total RNA using Nox1-specific primers (Chamulitrat et al. 2003, 2007). DNA fragments of human Nox1 (between nucleotides 205 and 1838; GenBank accession# NM_007052) were amplified using primers indicated in Chamulitrat et al. (2007). This resulted in two cDNA fragments from Nox1 and Nox1-tv, which were gel-purified and cloned into pcDNA3.1/NT-GFP-TOPO (Invitrogen, Karlsruhe, Germany). These fragments were subcloned into pcDNA3.1. The correct molecular size

of pcDNA3.1/Nox1 and pcDNA3.1/Nox1-tv was confirmed by DNA sequencing. Overexpression with Nox1 or Nox1-tv plasmids was performed by transfection of GM16 cells (1–2 $\times$ 10^6 cells) in KGM with 5 μg supercoiled plasmids using the Nucleofector cell line V kit with T-20 program (Amaxa, Cologne, Germany). Transfected cells were harvested for analyses 6–48 h later.

siRNA studies

NuB2 cells at 0.5–2 $\times$ 10^5 cells per well in a 12-well plate were transfected with small interfering RNA (siRNA) against Nox1 or with scrambled control siRNA (Scr.) in serum-free DMEM. A scrambled and Nox1-specific siRNA (siNox1) was obtained from Eurogentec (Seraing, Belgium). The Nox1 siRNA sequences were (5′-CUG GAG UGA UCA UGA CAA-3′ and 5′-AUU GUC AUG AUC ACU CCA-3′). Transfection of NuB2 cells was performed with 100 nM siRNA using ICAFectin™ 442 (Eurogentec, Seraing, Belgium) for 24 h in Optimem (Invitrogen), and cells were harvested for RT-PCR. For Western blot analysis, transfected cells were cultured 24 h further in DMEM containing 10% FCS prior to addition of lysis buffer.

RNA isolation, RT-PCR, and qPCR

Total RNA isolation was performed using RNeasy Minikit (QIAGEN, Germany). Total RNA was reverse transcribed with First-Strand Beads (Amersham, Freiburg, Germany) or First-strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). For conventional RT-PCR, cDNA was amplified with specific primers using QIAGEN HotStarTaq Master Mix kit. Forward and reverse primers for Nox1 were 5′-GTT TTA CCG CTC CCA GCA GAA-3′ and 5′-GGA TGC CAT TCC AGG AGA GAG-3′ and for CK18 were 5′-TGG TCA CCA CAC AGT CTG CT-3′ and 5′-CCA AGG CAT CAC CAAGAT TA-3′. PCR conditions were 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min for CK18; 95°C for 30 s, 56°C 30 s, and 72°C for 30 s for Nox1; and 95°C for 30 s, 65°C 30 s, and 72°C for 45 s for GAPDH. Final extension was performed at 72°C for 7 min in all PCR reactions. Molecular sizes of PCR products were 156 bp for Nox1, 348 bp for CK18, and 460 bp for GAPDH. Quantitative real-time PCR (qPCR) analysis of Nox1 and CK 18 mRNA was performed on a 7500 Fast Real-Time PCR System using Nox1, CK18, and GAPDH and TaqMan® primers from Applied Biosystems (Darmstadt, Germany).

Luminescence assay

Redox activity in transfected cells was determined by ROS generation using a sensitive luminol probe L-012 (8-amino-5-chloro-7-phenylpyridol [3,4-d] pyridazine-1,4(2H,3H) dione) (Ii et al. 1993), which was obtained from Wako Chemicals, Neuss, Germany. After 48 h post-transfection of GM16 cells, cells were washed and treated with 50 μM L-012 for 30 min and luminescence was measured with a Fluostars OPTIMA (BMG LABTECH GmbH, Germany).

Western blot analysis

Cell lysis was performed using 20 mM Tris.HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2.5 mM β-glycerophosphate, 1 mM NaF, 1 mM DTT, 1 mM PMSF, 200 μM sodium orthovanadate, and protease inhibitor cocktails (Calbiochem, Darmstadt, Germany). Lysates were obtained after centrifugation at 16,000$\times g$, 4°C for 10 min. Protein concentrations were determined with a Bio-Rad DC Protein kit.

For immunoprecipitation, cell lysates (250 μg protein) were incubated with 2 μg anti-CK18 antibody (Santa Cruz, Heidelberg,Germany) at 4°C for 1 h. Protein G PLUS-agarose beads (Santa Cruz) were added and incubated overnight at 4°C with end-over-end rotation. After washing, Laemmeli buffer was added to beads and mixture was heated at 95°C for 3 min. Following centrifugation, ubiquinated CK18 in supernatants were analyzed by Western blot using anti-ubiquitin antibody.

Total cell lysates (30 μg protein) in Laemmeli buffer or 20 μl of immunoprecipitates were separated by SDS–PAGE electrophoresis and transferred onto a PVDF membrane. After blocking and washing, membranes were incubated with primary antibody 1:1,000 anti-Nox1 antibody (Chamulitrat et al. 2007); 1:3,000 anti-CK18 (DC-10, Dianova, Hamburg, Germany); 1:50,000 anti-GAPDH (Cell Signaling, Cologne, Germany) as well as 1:20,000 anti-ubiquitin (FL-76), 1:2,000 anti-ser52-phospho-CK18, and 1:1,500 anti-Mox1 (Santa Cruz, Heidelberg, Germany) at 4°C overnight. Membranes were incubated for 1 h at RT with anti-mouse or anti-rabbit secondary antibody. Proteins were visualized with an ECL system (Millipore, Schwalbach, Germany).

Statistics

Data are expressed as mean ± SEM. All experiments were reproducible from at least three independent experiments. Intensities of Western blot bands were determined using Image J. Statistical significance was evaluated by Student's t test. A value of $P < 0.05$ was considered significant.

Results

Increased expression of CK18 protein in Nox1-expressing cells

From our previous work, our so-called Nox1 cell lines (NuB1, FuB1, NuB2, FuB2, and NuB3) were generated following Nox1 transfection of GM16 cells and allowing preneoplastic progenitor cells to emerge and proliferate in standard DMEM (Chamulitrat et al. 2007). Similar to GM16 cells in KGM, NuB2 cells in DMEM exhibited typical epithelial-like cobblestone morphology (Fig. 1a). NuB1 cells with elongated phenotype were classified as more transformed exhibiting anchorage-independent growth. Compared with cells exhibiting cobblestone morphology, NuB1 cells expressed elevated levels of CK18 (Fig. 1b). Among cobblestone cells, the parental GM16 cells in KGM [labelled as GM16 (KGM)] and those adapted to proliferate in DMEM [labelled as GM16 (DMEM)] expressed lower CK18 protein than FuB1 and NuB2 cells (Fig. 1b). In multi-step carcinogenesis, NuB2 cells with higher CK18 expression were considered to be more transformed than the parental GM16 cells.

GM16 cells which expressed low CK18 in KGM were chosen for Nox1 overexpression. Transfection with Nox1 plasmid was found to increase CK18 protein, but not CK8 protein (Fig. 1c). NuB1 cells constitutively expressed elevated of CK8/CK18 proteins indicating association of these proteins with spindle elongated phenotype and de-differentiation state. Truncated Nox1 (Nox1-tv) has been characterized as Nox1 with the missing 49 amino acids (Bánfi et al. 2000), and it does not exhibit a functional oxidase property (Geiszt et al. 2004). Transfection of GM16 cells with pcDNA3.1/Nox1-tv did not increase CK18 protein (Fig. 1d). Thus, functional oxidase of Nox1 and its activity was required for increased CK18 protein expression. It is noted that Nox1 overexpression of FuB1 cells did not increase CK18 expression. Furthermore, Nox1 overexpression of GM16 cells did not increase intermediate filament vimentin.

Nox1 increases CK18 protein but not mRNA

To demonstrate involvement of Nox1 in regulation of CK18, we measured Nox1 and CK18 proteins, mRNAs in overexpressed GM16 cells, and in Nox1-silenced NuB2 cells. Upon Nox1 overexpression of GM16 cells in KGM, Nox1 protein was increased 48 h post-transfection (Fig. 2a). Nox1 mRNA expression was significantly increased starting at 6 h and further increased at 14, 24, and 48 h post-transfection as determined by real-time TaqMan® qPCR (Fig. 2c), and at 24 h with conventional RT-PCR (Inset of Fig. 2c). Nox1 transfection of GM16 cells caused

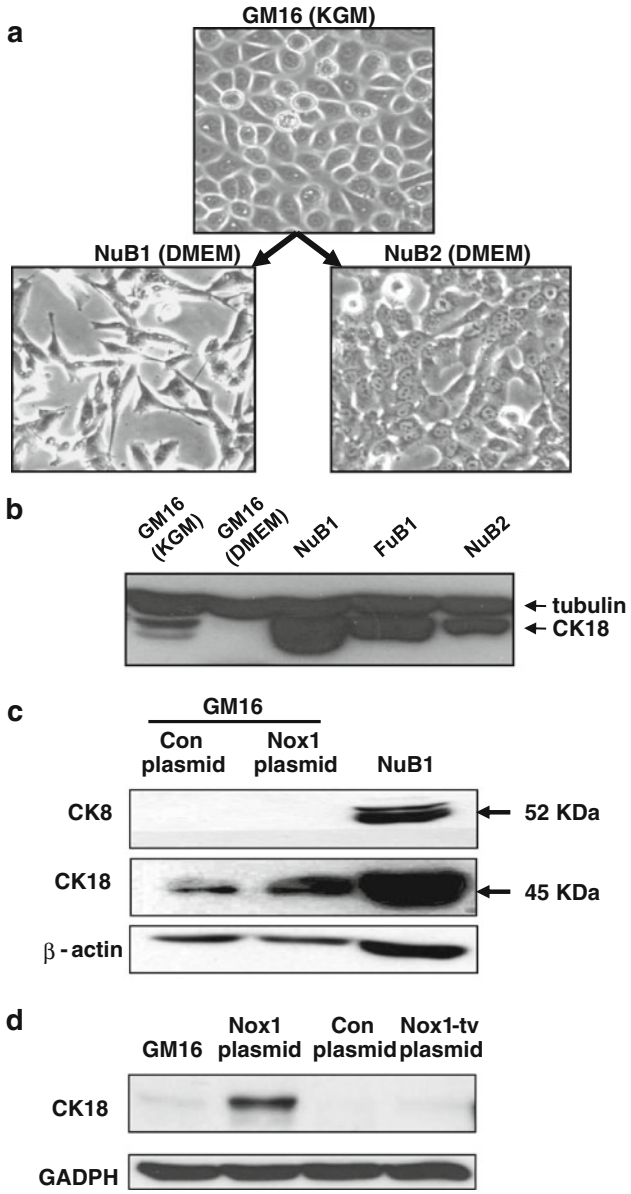


Fig. 1 Expression of cytokeratins in model human epithelial cell lines and effects of transfection with Nox1. **a** Phase contrast pictures showed morphology of the parental GM16 cells in keratinocyte growth medium (KGM) and progenitor NuB2 and NuB1 cells in DMEM. **b** Expression of CK18 was higher in spindle NuB1 cells compared with cobblestone cell lines including FuB1, NuB2 as well as GM16 cells, which were cultured in KGM [GM16 (KGM)] or adapted to grow in DMEM [GM16 (DMEM)]. **c** Transfection of GM16 cells with Nox1 plasmid increased CK18 but not CK8 protein in KGM. **d** Plasmid containing full-length Nox1, but not truncated version of Nox1 (Nox1-tv), increased CK18 protein expression in GM16 cells cultured in KGM

significant increases in CK18 protein (Fig. 2a); however, CK18 mRNA expression was not affected (Fig. 2e). These data indicate post-translational regulation of CK18 in GM16 cells, and this was supported by significant increases in CK18 protein upon treatment with proteasome inhibitor MG132 (Fig. 2g).

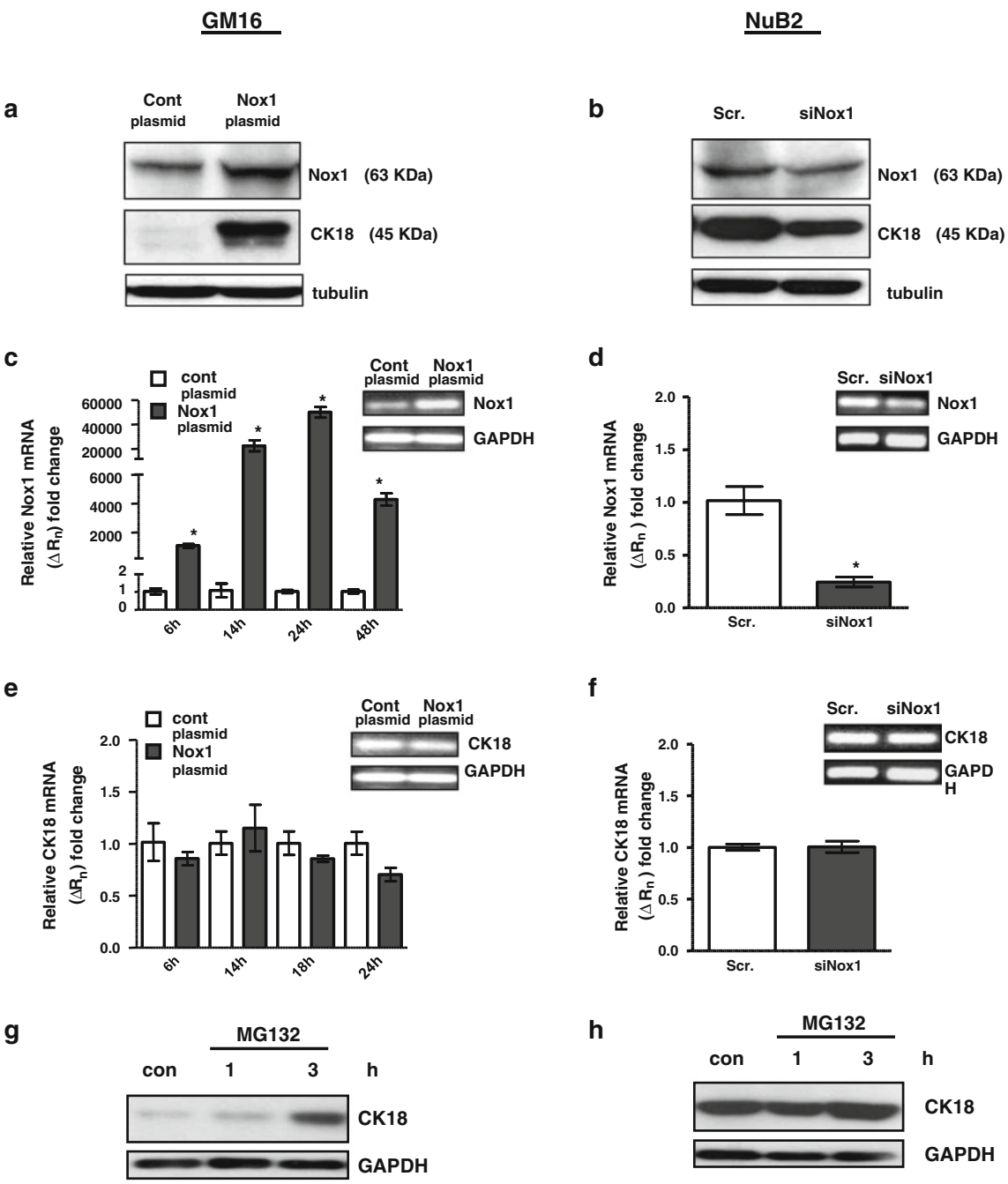


Fig. 2 Post-translational regulation of CK18 protein by Nox1 evidenced by Nox1 overexpression in GM16 cells and Nox1 knockdown in NuB2 cells. For Nox1 overexpression experiments, GM16 cells were nucleofected with 5 μg pcDNA3.1 or pcDNA3.1/Nox1 and harvested after 48 h post-transfection for protein and 6–48 h for mRNA analyses. Transfection of GM16 cells with pcDNA3.1/Nox1 increased Nox1 and CK18 proteins (**a**), and Nox1 mRNA (**c**). However, Nox1 overexpression did not alter CK18 mRNA levels (**e**). For Nox1 knockdown experiments, NuB2 cells were transfected with 100 nM scrambled (Scr.) or Nox1 siRNA (siNox1). Cells were harvested and subjected for protein and mRNA analyses at 48 h and 24 h post-transfection, respectively. Nox1 knockdown decreased Nox1 and CK18 proteins (**b**), and Nox1 mRNA (**d**). However, Nox1 knockdown did not alter CK18 mRNA levels (**f**). Treatment for GM16 cells (**g**) or NuB2 cells (**h**) with proteasome inhibitor MG132 at 20 μM for 1 and 3 h increased CK18 protein expression indicating post-translational stabilization of CK18. In (**c**), (**d**), (**e**), and (**f**), quantitative real-time PCR was performed by TaqMan® RT-PCR with relative expression (ΔRn) of the target gene versus GAPDH mRNA (data were mean ± SEM, $N = 3$, $^*P < 0.001$, vs. cont plasmid or Scr.), and the corresponding Insets by conventional RT-PCR using primers stated in Materials and Methods. Cell lysates were prepared and analyzed for Nox1 and CK18 expression. β-Tubulin or GADPH was used as loading control

For Nox1 knockdown experiments, NuB2 cells expressing high CK18 protein were used. NuB2 cells were transfected with Scrambled (Scr.) or Nox1 siRNA (siNox1). Cells were analyzed at 24 h and 48 h post-transfection for mRNA and protein analyses, respectively. Nox1 knockdown resulted in decreased expression of Nox1 on both protein (Fig. 2b) and mRNA levels (Fig. 2d). Upon Nox1 knockdown, CK18 protein expression was markedly decreased (Fig. 2b), while CK18 mRNA was not affected (Fig. 2f). Post-translational regulation of CK18 in NuB2 cells was again supported by increased CK18 protein upon treatment with proteasome inhibitor MG132 (Fig. 2h). It is noted that Nox1 knockdown of NuB1 cells did not alter CK18 expression.

Redox regulation of CK18 protein expression in Nox1-expressing cells

Nox1 overexpression of GM16 cells induced increased rates of ROS generation (Fig. 3a). Alternatively, Nox1 knockdown decreased ROS generation rates. To determine redox regulation of CK18, we treated NuB2 cells with a flavoprotein inhibitor DPI (Fig. 3b), antioxidant NAC (Fig. 3c), or a Nox inhibitor apocynin (Fig. 3d). As expected, all of these agents consistently decreased expression of CK18 protein indicating involvement of ROS from flavoprotein Nox enzymes, especially, Nox1 in regulation of CK18 protein (Fig. 2b).

Nox1 increases CK18 protein accumulation via ubiquination in a phosphorylation-dependent manner

It is known that CK18 turnover occurs via ubiquitination in a phosphorylation-dependent manner (Ku and Omary 2000). It is also known that ser52 is the major physiologic phosphorylation site of CK18 during increased DNA synthesis and mitotic cell division (Ku and Omary 1994; Liao et al. 1995). This phosphorylation is thus proposed to occur during Nox1-stimulated proliferation of preneoplastic cells. CK18 protein accumulation was found to be Nox1- and ROS-dependent (Figs. 2, 3). To elucidate ubiquitination of CK18 as a test assay system, we performed treatment for NuB2 cells with proteasome inhibitor MG132, which resulted in increased CK18 accumulation. Proteasome inhibition by MG132 should cause accumulated ubiquinated CK18 (Ub-CK18) (see scheme in Fig. 4d). By performing immunoprecipitation of CK18 and probing for ubiquitin, we expectedly found that MG132 caused an increase in Ub-CK18 detectable at ~53.5 kDa (Fig. 4a). NuB2 cells treated with DPI contained decreased amount of total CK18 and ser52-phosphorylated CK18 (pS52CK18) (Fig. 4b). This was concomitant with increased Ub-CK18 protein. In addition to DPI, we also found that NAC also increased Ub-CK18 protein (Data not shown). In a similar manner as DPI, Nox1 knockdown of NuB2 cells caused a decrease in CK18 and pS52CK18 expression and this was concomitant with an increase in Ub-CK18 levels (Fig. 4c). In immunoprecipitated CK18, we also found that the extent of CK18 phosphorylation at ser52 was decreased upon Nox1 knockdown of NuB2 cells (data not shown). Thus, down-regulation of Nox1 by knockdown was associated with increased CK18 ubiquination, which resulted in decreased CK18, and this process was associated with reduced CK18 phosphorylation. Alternatively, this demonstration could be confirmed by Nox1 overexpression of GM16 cells. Nox1-overexpressed GM16 cells showed increased CK18 and pS52CK18 (Fig. 4d). Due to lack of sufficient protein required for immunoprecipitation, we were not able to detect Ub-CK18 in transfected GM16 cells. The increased phosphorylation by Nox1-derived ROS (Fig. 3a) upon Nox1 overexpression would likely cause decreased ubiquitination, which resulted in increased CK18 accumulation (Fig. 4d, Scheme).

Discussion

Overexpression of Nox1–GM16 cells generated progenitor cells selectable by differentiation resistance after prolonged cultures (Chamulitrat et al. 2007). Increased expression of CK18 in the progenitor, i.e., NuB2 cells compared to GM16 cells provided a hint that Nox1 may directly regulate expression of this class of intermediate filaments. We herein demonstrated that with unchanged mRNA levels, CK 18 protein levels were significantly elevated upon Nox1 overexpression, and the opposite was found for Nox1 knockdown. We clearly demonstrated that Nox1 at post-translational level increased CK18 stabilization by reducing CK18 degradation. Proposed mechanisms for stabilization of CK18 by Nox1 are depicted in Fig. 4d. Nox1's ability to stabilize CK18 may be an early event in cell transformation rendering its persistence in many human epithelial carcinomas.

Our GM16 and NuB2 cell lines represent human immortalized epithelial cells that are considered preneoplastic, but not yet fully transformed. Nox1's ability to stabilize CK18 may be associated with physiological role of Nox1 in mitogenic signaling for proliferation of preneoplastic and cancer cells (Park et al. 2004; Arnold et al. 2007; Chamulitrat et al. 2004). During cell proliferation, ROS from Nox enzymes increase expression of cyclinD1 (Ranjan et al. 2006) and induce G2/M cell cycle progression (Yamaura et al. 2009). Indeed, phosphorylation of CK18 at ser52 is increased by three- to fourfold during S and G2/M phase of cell cycle (Ku and Omary 1994; Liao et al. 1995). Furthermore, Nox1 has been shown to mediate cell invasion and migration (Shinohara et al. 2010). Our data consistently showed that

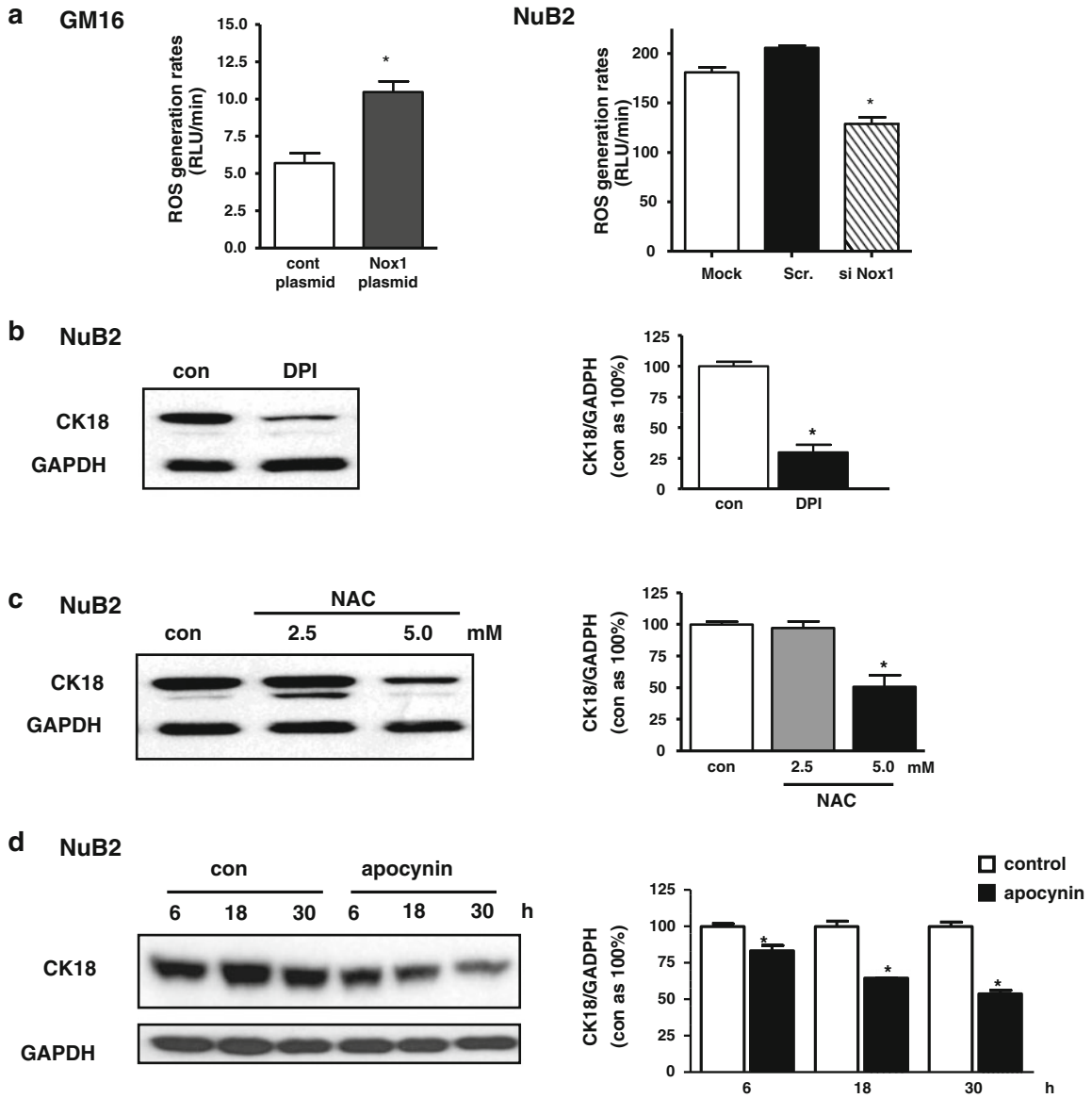
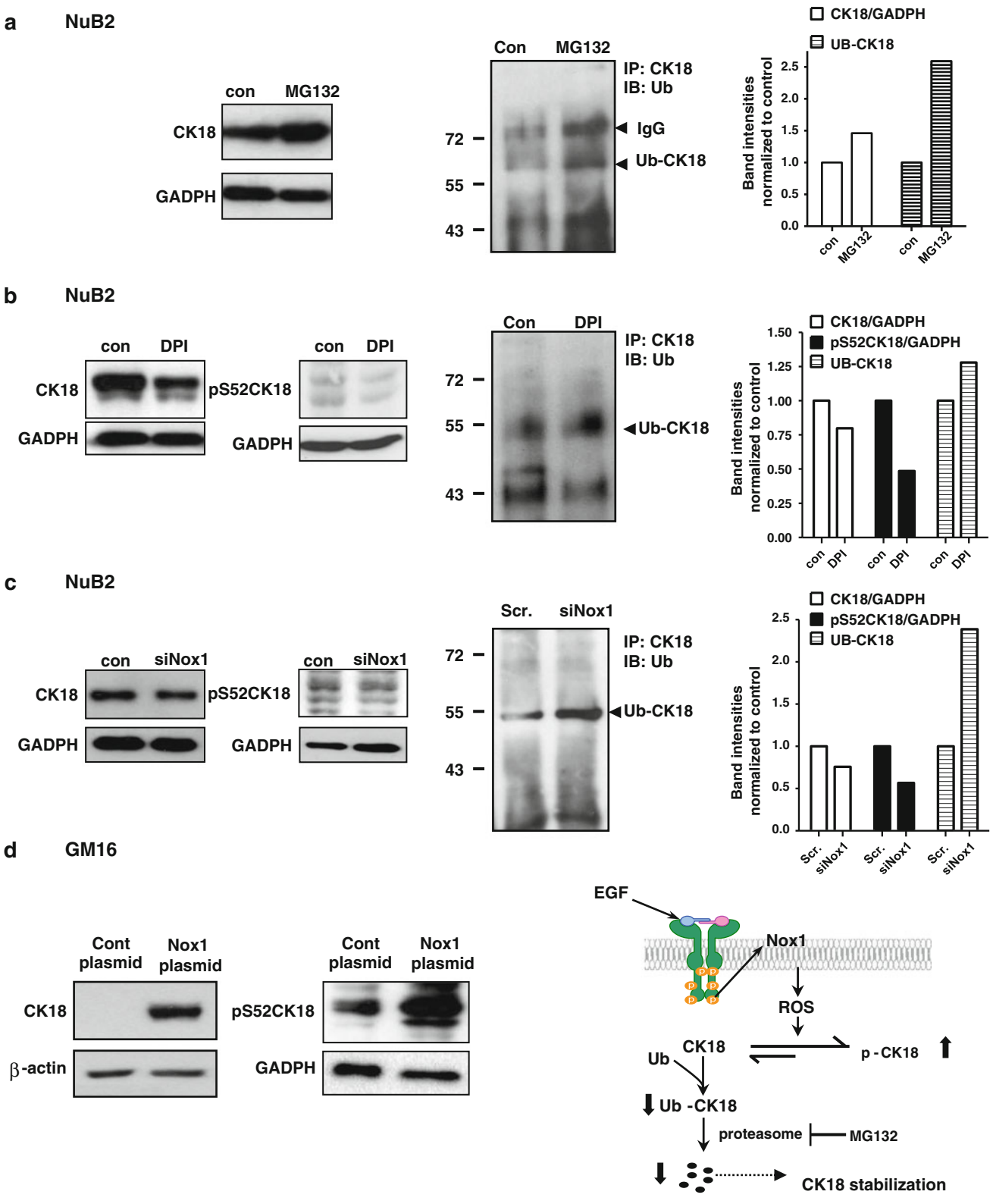


Fig. 3 Regulation of CK18 protein was ROS-dependent. (**a**, *left panel*) ROS generation was increased upon Nox1 overexpression in GM16 cells. GM16 cells (2×10^4) were nucleofected with pcDNA3.1 or pcDNA3.1/Nox1, and ROS generation was determined 48 h later. The generation of ROS was determined by chemiluminescence using L-012. Data were mean ± SEM, $N = 10$, $^*P < 0.0001$, versus cont plasmid. (**a**, *right panel*) ROS generation was inhibited in siNox1-transfected NuB2 cells. NuB2 cells (2×10^4) were transfected with 100 nM scrambled (Scr.) or Nox1 siRNA (siNox1). After 48 h incubation, transfected cells were treated with EGF (100 ng/ml) in serum-free medium for 24 h and ROS generation using L-012 was determined. Data were mean ± SEM, $N = 30$, $^*P < 0.0001$, versus Scr. Inhibitory effects of CK18 by flavoprotein inhibitor DPI, antioxidant NAC, and Nox inhibitor apocynin indicated redox regulation of CK18. NuB2 cells were treated with (**b**) 10 μM DPI for 10 h, (**c**) 2.5 and 5 mM NAC for 24 h, or (**d**) 1 mM apocynin for 6, 18, and 30 h. CK18 in cell lysates were determined by Western blot. GAPDH was used as a loading control. Normalized band intensities from Western blot data shown in the corresponding *right panels* were mean ± SE, $N = 3$ experiments

CK18 phosphorylation at ser52 was dependent on Nox1 and ROS. These events may lead to cytoskeletal dynamics associated with the reorganization of keratin filaments in cell division and migration. As depicted in Fig. 4d, ligation of EGF tyrosine kinase receptor during cell growth activates leading to Nox1 activation and oxidant generation (Park et al. 2004; Busso et al. 1994). Nox1-mediated oxidants may induce phosphorylation of CK18. The use of DPI, apocynin, and NAC was able to reverse the processes resulting in decreased CK18 protein levels.

Phosphorylation of CK18 stabilizes and protects its degradation by ubiquination (Ku and Omary 2000). Thus, phosphorylated CK18 at ser52 upon Nox1 activation may be as a physiological mechanism of preneoplastic cells to proliferate and resist apoptosis. During an early event in apoptosis, hyperphosphorylation of CK18 has been



observed and attributed for protection against caspase-mediated CK18 degradation (Ku and Omary 2001). Interestingly, CK18 and CK19 are the only keratins that undergo degradation during apoptosis, and the sparing of type II keratins, such as CK8, is related to lack of caspase recognition sequences (Ku and Omary 2001). In line with this, we did not observe Nox1 regulation of CK8 protein in our experiments. This implies that Nox1 may also mediate

◀ **Fig. 4** Nox1-regulated CK18 protein expression by CK18 ubiquitination in a phosphorylation-dependent manner. **a** Upon treatment of NuB2 cells with proteasome inhibitor MG132 at 20 μM for 3 h, CK18 expression was increased concomitant with ubiquintination of CK18 (Ub-CK18 detectable at ~53.5 kDa) complex. **b** Treatment with 10 μM DPI for 10 h decreased CK18 and its phosphorylated protein (pS52CK18). This was concomitant with increased Ub-CK18. **c** Nox1 knockdown of NuB2 cells increased the levels of CK18 and pS52CK18 concomitant with increased Ub-CK18. **d** Nox1 overexpression of GM16 cells caused increased CK18 and pS52CK18. In **d**, *right panel*, proposed mechanisms for stabilization of CK18 by Nox1 in an ROS-, and CK18 phosphorylation-dependent manner. In human epithelial cells, growth factor ligation leads to Nox1 and subsequent ROS activation. ROS induces increased phosphorylation of CK18 rendering a decrease level of total CK18 that is subjected for ubiquitination. Hence, CK18 degradation is inhibited resulting in CK18 stabilization via Nox1/ROS pathway

resistance against apoptosis by stabilizing CK18 in cancer cells. In support of this, Nox1 has been shown to prevent TNF-α-induced necrotic cell death (Byun et al. 2008) and suppress apoptosis by causing impaired p53 proapoptotic transcriptional activity (Puca et al. 2010). Conversely, inactivation of Nox1 as well as EGF receptor inhibition enhances apoptosis in hepatoma cells (Sancho et al. 2009). Consistent with this view, CK18 stabilization has been shown to elicit epithelial resistance to stress and apoptosis (Marceau et al. 2001).

We provided novel evidence for CK18 stabilization by Nox1 by reducing CK18 degradation in an ROS- and phosphorylation-dependent manner. This post-translational mechanism for CK18 accumulation is consistent with the persistent presence of CK18 in many epithelial carcinomas. Nox1 may thus be used as targets to inhibit progression of preneoplastic and cancer cells.

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Conflict of interest There is no conflict of interests to declare.

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