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# NADPH oxidase overexpression in human colon cancers and rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

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**NADPH oxidase/dual-oxidase (Nox/Duox) family members have been implicated in nuclear factor kappa-B (NFκB)-mediated inflammation and inflammation-associated pathologies. We sought to examine, for the first time, the role of Nox/Duox and NFκB in rats treated with the cooked meat heterocyclic amine carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). In the PhIP-induced colon tumors obtained after 1 year, Nox1, Nox4, NFκB-p50 and NFκB-p65 were all highly overexpressed compared with their levels in adjacent normal-looking colonic mucosa. Nox1 and Nox4 mRNA and protein levels also were markedly elevated in a panel of primary human colon cancers, compared with their matched controls. In HT29 human colon cancer cells, Nox1 knockdown induced G1 cell cycle arrest, whereas in Caco-2 cells there was a strong apoptotic response, with increased levels of cleaved caspase-3, -6, -7 and poly(ADP-ribose)polymerase. Nox1 knockdown blocked lipopolysaccharide-induced phosphorylation of IκB kinase, inhibited the nuclear translocation of NFκB (p50 and p65) proteins, and attenuated NFκB DNA binding activity. There was a corresponding reduction in the expression of downstream NFκB targets, such as *MYC*, *CCND1* and *IL1β*. The results provide the first evidence for a role of Nox1, Nox4 and NFκB in PhIP-induced colon carcinogenesis, including during the early stages before tumor onset. Collectively, the findings from this investigation and others suggest that further work is warranted on the role of Nox/Duox family members and NFκB in colon cancer development.**

**Key words:** colon carcinogenesis, heterocyclic amines, NFκB, reactive oxygen species, apoptosis

**Abbreviations:** GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IKK: IκB kinase; IL-1β: interleukin-1β; IL6: interleukin-6; NFκB: nuclear factor kappa-B; Nox/Duox: NADPH oxidase/dual-oxidases; LPS: lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP: poly(ADP-ribose)polymerase; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; TMAs: tissue microarrays; TNF-α: tumor necrosis factor-α; TNFR: TNF receptor

Additional supporting information may be found in the online version of this article.

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Nuclear factor kappa-B (NFκB) is a key transcription factor regulating the expression of genes involved in inflammation, immune modulation and apoptosis, as well as in various stages of cancer development.<sup>1-3</sup> In mammals, five members of the NFκB family have been identified, p50, p65 (RelA), RelB, c-Rel and p52.<sup>4</sup> These subunits exist as homodimers or heterodimers, under the control of inhibitors of NFκB (IκB). The IκB family comprises IκBα, IκBβ, IκBγ, IκBε, IκBζ, BCL3, p100 and p105. Activation of NFκB involves IκB kinase (IKK)-dependent phosphorylation and degradation of IκB proteins.<sup>5</sup> IKK contains the catalytic subunits IKKα and IKKβ, and a regulatory component, IKKγ/NEMO. Nuclear trafficking of NFκB results in the activation of genes encoding cytokines, chemokines, growth factors and antiapoptotic factors.<sup>1</sup>

A plethora of physiological stimuli activate NFκB. These include lipopolysaccharide (LPS) and proinflammatory cytokines, such as interleukin-6 (IL6) and interleukin-1β (IL-1β), as well as tumor necrosis factor-α (TNF-α), acting *via* the TNF receptor (TNFR). Activation of NFκB is associated with the production of reactive oxygen species (ROS), and the best-studied ROS-producing enzyme is the phagocyte-derived NADPH oxidase (Nox), which plays a pivotal role during

bacterial infection and inflammation.<sup>6,7</sup> Non-phagocyte-derived Nox homologues also have been identified, designated collectively as the Nox/dual oxidase (Duox) family, which comprises Nox1, Nox2, Nox3, Nox4 and Nox5, plus Duox1 and Duox2.<sup>8</sup> Each Nox/Duox isoform exhibits a distinct cellular and tissue distribution pattern.<sup>7</sup> In lung, aberrant expression of Nox2, Duox1 and Duox2 contributes to chronic obstructive pulmonary disease, asthma and cystic fibrosis,<sup>9</sup> whereas in forebrain abnormal levels of Nox2 and Nox4 have been implicated in the pathogenesis of schizophrenia.<sup>10</sup>

Accumulating evidence supports a role for Nox/Duox members in other pathologies, including cancer. Nox1 stimulates mitogenesis, cell transformation and tumorigenesis when ectopically expressed in NIH3T3 fibroblasts and DU-145 prostate epithelial cells, with a corresponding increase in angiogenesis.<sup>11,12</sup> Overexpression of Nox1 was observed in prostate, breast and ovarian cancers,<sup>13,14</sup> and Nox4 was detected at high levels in glioblastoma cells.<sup>15</sup> Information on Nox/Duox involvement in colon cancer is somewhat inconsistent. Geiszt *et al.*<sup>16</sup> first reported that Nox 1 was expressed mainly in differentiated colonic epithelial cells, and Szanto *et al.*<sup>17</sup> detected no statistical difference for *NOX1* mRNA expression between adenomas and poorly- or well-differentiated colon adenocarcinomas. Szanto *et al.*<sup>17</sup> concluded that Nox1 is an enzyme that is constitutively expressed in colonic epithelium and is not associated with tumorigenesis. However, using immunohistochemical analyses, Fukuyama *et al.*<sup>18</sup> observed Nox1 overexpression in human colon adenomas and well-differentiated adenocarcinomas, and Laurent *et al.*<sup>19</sup> reported that Nox1 was overexpressed in human colon cancers and was correlated with activating mutations in K-ras.

To clarify the role of Nox/Duox family members during colon carcinogenesis, rats were treated with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), as reported before,<sup>20,21</sup> and the expression levels of Nox/Duox and NFκB were examined before and after the appearance of frank tumors. A panel of primary human colon cancers and human colon cancer cell lines also was studied for expression of NFκB and Nox/Duox isoforms.

## Material and Methods

### Rat colon tumors

Colon tumors and adjacent normal-looking mucosa were from a study in which male F344 rats received intermittent exposure to PhIP alternating with a high-fat diet, as reported elsewhere.<sup>20</sup> An interim sacrifice was included to assess early molecular changes, before the onset of tumors. Specifically, 24 h after the last dose of PhIP, rats in each group ( $n = 5$ ) were euthanized, the colon was removed and opened longitudinally, and the mucosa was scraped and frozen in liquid nitrogen before storing at  $-80^{\circ}\text{C}$ . The remaining animals in each group ( $n = 36$ ) were euthanized at 52 weeks (Supporting Information Fig. 1). A complete necropsy examination

was performed on each animal.<sup>20</sup> This work received prior approval from the Institutional Animal Care and Use Committee.

### Human primary colon cancers

Ten pairs of primary human colon cancers and their matched adjacent normal-looking tissues were kindly provided by Steven F. Moss, M.D. and Lelia Simao (Rhode Island Hospital, Providence, RI). The patients (6 female; 4 male, 53-93 years of age) had been diagnosed with adenocarcinoma of the colon.

### Quantitative real-time RT-PCR (qPCR)

Frozen colon tumor samples and their matched controls were thawed, and mRNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RNA (2  $\mu\text{g}$ ) was reverse-transcribed in 20  $\mu\text{l}$  of 1X RT buffer, containing 10 U RNase inhibitor (Invitrogen, Carlsbad, CA), 0.5 mM each dNTP, 4 U Omniscript Reverse Transcriptase (Qiagen) and 50 ng random hexamers (Invitrogen). Primers were as listed in Supporting Information Table I. Forty cycles of PCR were run on an Opticon Monitor 2 system (Finnzymes, Finland), in 20  $\mu\text{l}$  total reaction volume containing cDNAs, SYBR Green I dye (DyNAmo master solution, Finnzymes) and primer set. The PCR conditions were  $95^{\circ}\text{C}/10\text{s}$ ,  $58^{\circ}\text{C}/20\text{s}$  and  $72^{\circ}\text{C}/20\text{s}$ , except for rat *Nox1* and rat *Nox4*, in which the annealing temperature was  $60^{\circ}\text{C}$ . The amount of specific mRNA was quantified by determining the point at which the fluorescence accumulation entered the exponential phase ( $C_t$ ), and the  $C_t$  ratio of the target gene to *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* was calculated for each sample. At least two separate experiments were performed for each sample. In some experiments, malignant regions were microdissected from PhIP-induced colon tumors using a Zeiss PALM MicroBeam IV Laser Capture System (Carl Zeiss, Thornwood, NY), and compared with microdissected normal colon. Briefly, mRNA from captured cells was extracted using an RNeasy Micro Kit (Qiagen) and cDNA was synthesized using the Superscript III kit (Invitrogen). Forty-five cycles of qPCR ( $95^{\circ}\text{C}/10\text{s}$ ,  $60^{\circ}\text{C}/10\text{s}$ ,  $72^{\circ}\text{C}/10\text{s}$ ) were run on a Roche LightCycler 480 II (Roche, Indianapolis, IN), in a 10  $\mu\text{l}$  reaction containing primer set, cDNA and SYBR Green I dye (Roche).

### Immunoblotting

ProteoExtract Native membrane protein extraction kit (EMD Biosciences, San Diego, CA) was used for enrichment of membrane proteins, whereas NE-PER reagents (Pierce Biotechnology, Rockford, IL) were used to separate cytoplasmic and nuclear fractions. Membrane-enriched fractions were subjected to SDS-PAGE and immunoblotted using anti-Nox1 (H-75, sc-25545, 1:400 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Nox4 (rat, 1:100 dilution, abcam, Cambridge, MA, ab41886; human, 1:500 dilution, Novus, Biologicals, NB110-58851, Littleton, CA). The correct molecular weights were confirmed by reference to the marker

ladder on each blot (Nox1 65-kD, Nox4 67-kD), and antibody specificity was corroborated *via* the use of blocking peptides (sc-5821p against sc-5821 Nox1) (Santa Cruz), 1:200 dilution; Nox4 peptide (ND110-58851 PEP Novus Biologicals), 1:250 dilution). Whole cell lysates were immunoblotted with rabbit polyclonal antibody to IKK $\alpha$  and IKK $\beta$  (1:1000 dilution, Cell Signaling, Nos. 2682 and 2684) or phospho-IKK $\alpha$ / $\beta$  (1:500 dilution, Cell Signaling, no. 2681). Cytoplasmic extracts were probed with antibodies to cleaved caspase 3 (1:1000 dilution, Cell Signaling, no. 9661), cleaved caspase 6 (1:1000 dilution, Cell Signaling, no. 9761), and cleaved caspase 7 (1:1000 dilution, Cell Signaling, no. 9492). Nuclear fractions were immunoblotted with polyclonal rabbit antibody to NF $\kappa$ B p50 (1:600 dilution, Santa Cruz, sc-7178), NF $\kappa$ B p65 (1:600 dilution, Santa Cruz, sc-109), and poly-(ADP-ribose)polymerase (PARP, 1:1000 dilution, Cell Signaling, no. 9532). Amido black staining was used to ensure equal protein loading, followed by  $\beta$ -actin. For nuclear extracts, histone H1 also was used as a loading control (mouse monoclonal antibody sc-8030, Santa Cruz, 1:500 dilution). Proteins were visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston MA), with quantification *via* an AlphaInnotech photodocumentation system and associated software (AlphaInnotech, San Leandro, CA).

#### Immunohistochemistry

Rat colon tumors were processed to paraffin, sectioned at 4  $\mu$ m, and placed on charged slides. Sections were rehydrated through xylene, 100% ethanol, 95% ethanol, 80% ethanol and water. Antigen retrieval was carried out in a microwave pressure cooker for 10 min, followed by 20 min at room temperature. Antigen retrieval solution was Dako Target Retrieval Solution pH 6.0 (Dako, Carpinteria, CA). Slides were washed in water and loaded into a Dako autoimmunostainer. Endogenous peroxides were blocked with 3% H<sub>2</sub>O<sub>2</sub> in TBST (Dako Tris-buffered saline with Tween 20) for 10 min, and slides were then washed in TBST. Dako serum-free protein block was applied for 10 min, followed by a burst of air to blot the slides. Incubation with the primary antibody was for 30 min (Nox4, 1:250 dilution, Novus Biologicals, NB110-58851, Littleton, CA). As immunohistochemical controls, the corresponding blocking peptide (see above) was used to confirm antibody specificity, and Dako Universal Negative Rabbit control was used in place of the primary antibody. After washing in TBST, Dako Envision+ anti-rabbit HRP was applied for 30 min, followed by Nova Red (Vector Laboratories, Burlingame, CA) and hematoxylin (Dako) counter staining.

The same protocol was used to immunostain Nox4 in human tissues. Tissue microarrays (TMAs) were constructed using a Beecher Instruments MTA-1 tissue arrayer (Beecher Instruments, Sun Prairie, WI). At least duplicate tumor samples were taken from donor tissue blocks, and a retrospective analysis for outcome assessment was based on detailed clinical

copathological information linked to the TMA specimens. For further details, see Ashktorab *et al.*<sup>22</sup>

#### Cell culture, siRNA transfection and LPS treatment

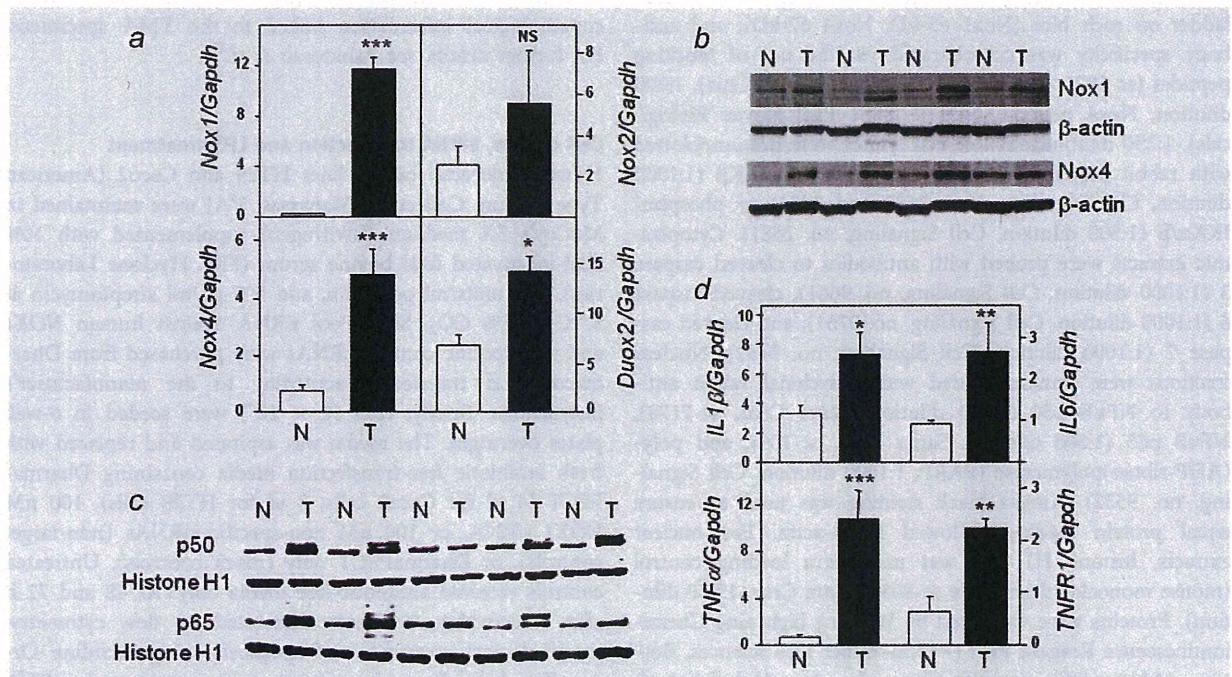
Human colorectal cancer lines HT29 and Caco2 (American Type Culture Collection, Manassas, VA) were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in 5% CO<sub>2</sub>. SmartPool siRNA against human NOX1 and non-specific control siRNAs were purchased from Dharmacon and transfected according to the manufacturer's instructions. Briefly, cells ( $1 \times 10^5$ ) were seeded in 6-well plates overnight. The media was aspirated and replaced with fresh antibiotic free-transfection media containing DharmaFECT (4  $\mu$ l for Caco2 cells, 8  $\mu$ l for HT29 cells), 100 nM NOX1 siRNA, or 100 nM non-specific siRNAs (non-target controls), or DharmaFECT only (mock controls). Untreated controls received antibiotic-free media only. At 48 and 72 h after transfection, cells were harvested for flow cytometry, morphological assessment of apoptosis using Acridine Orange/Ethidium Bromide staining, and protein and mRNA analyses. In additional experiments, 72 h after transfection of NOX1 siRNAs the media was aspirated and replaced with fresh media containing 1:2  $\mu$ g/ $\mu$ l of LPS (Sigma), or media alone. Thirty minutes later the cells were washed twice with PBS and lysed in IP buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail I and II (Sigma). Insoluble debris was removed by centrifugation at 10,000g for 5 min at 4°C. Cell lysates were subjected to SDS-PAGE, as described above, and immunoblotted for phospho-IKK $\alpha$ / $\beta$  (p-IKK, Ser176/180, 1:500, Cell Signaling, no. 2697).

#### MTT assay

Cells ( $5 \times 10^3$ ) in 100  $\mu$ l media were seeded in 96-well plates overnight and transfected with siRNA, as described above. At 24, 48, 72 and 96 h after transfection 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 3 h, followed by 100  $\mu$ l of 10% SDS in 0.01 N HCl. Formation of colored formazan dye was assessed colorimetrically at 550 nm.

#### Cell cycle distribution

Cells treated with NOX1 siRNA for 72 h (see above) were harvested in cold PBS, fixed in 70% ethanol, and stored at -20°C for 48 h. Fixed cells were washed with PBS and resuspended in propidium iodide/Triton X-100 staining solution containing RNase A. Samples were incubated in the dark for 30 min before determining the DNA content on an EPICS XL Beckman Coulter flow cytometer. Cell cycle distribution was assessed using Multicycle Software (Phoenix Flow Systems, San Diego, CA).



**Figure 1.** Overexpression of Nox isoforms and NFκB in PhIP-induced rat colon tumors. (a) *Nox1*, *Nox2*, *Nox4* and *Duox2* mRNA levels determined by qPCR and normalized to *Gapdh*; solid bars, tumors (T); open bars, adjacent normal-looking tissue (N). (b) Immunoblotting of Nox1 and Nox4 proteins in membrane-enriched fractions isolated from rat colon tumors and adjacent normal-looking tissue; loading control, β-actin. (c) Immunoblotting of NFκB-p50 and NFκB-p65 proteins in nuclear extracts obtained from rat colon tumors and adjacent normal-looking tissue; loading control, histone H1. (d) Expression of NFκB targets *IL1β*, *IL6*, *TNFα* and *TNFR1* in colon tumors and matched normal-looking tissues determined by qPCR. Data = mean ± SE,  $n = 9-12$ ; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### Cell morphology

Cell suspensions (25 μl) were incubated with 1 μl acridine orange/ethidium bromide solution (50 μg/ml of each reagent in PBS), mixed gently, and placed onto a microscope slide. Cell morphology was examined under a fluorescence microscope for signs of chromatin condensation, fragmented nuclei and/or membrane blebbing, indicative of apoptosis. At least 500 cells were counted per treatment, and three independent experiments were performed.

### DNA binding activity of NFκB-p50

Nuclear extracts were examined via enzyme-linked immunosorbent assay (NFκB-p50 transcription factor assay kit, Cat no. 10006912, Cayman Chemical, Michigan), according to the manufacturer's instructions.

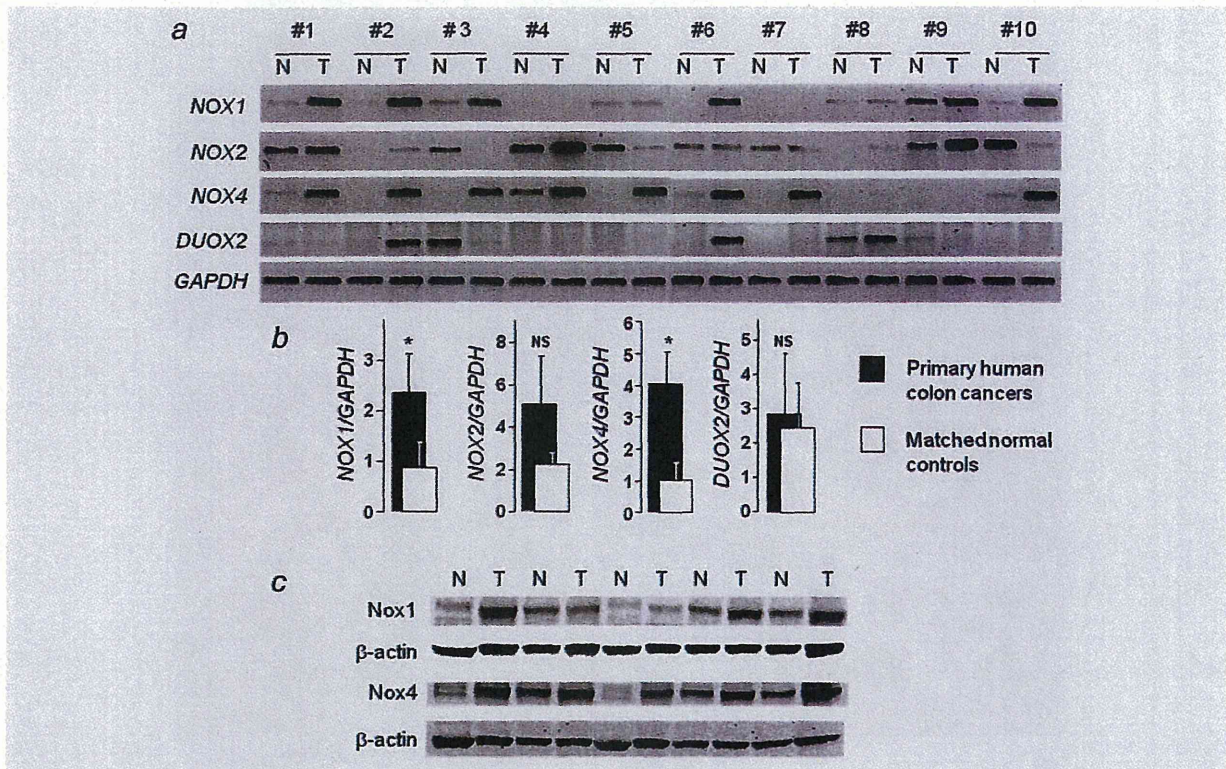
### Statistics

Unless stated otherwise, results shown in each figure were from a single experiment, and are representative of the findings from three or more independent experiments. Data were expressed as mean ± standard error (mean ± SE), and comparisons between control and treatment groups were made using the paired *t*-test (SigmaPlot 8.0). In the figures, significant results were indicated as follows: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

### Results

#### Nox is overexpressed in PhIP-induced colon tumors

In the colon tumors obtained from rats after 1 year, Nox/Duox mRNA levels were highly overexpressed compared with adjacent normal looking tissue (T vs. N in Fig. 1a, solid vs. open bars). The increase was highly significant for *Nox1* (133-fold,  $p < 0.001$ ) and *Nox4* (6.5-fold,  $p < 0.001$ ), and significant for *Duox2* (2.1-fold,  $p < 0.05$ ), but not significant for *Nox2* (2.0-fold,  $p > 0.05$ , NS). Adenocarcinoma cells also were captured by laser microdissection from PhIP-induced colon tumors, and these areas expressed significantly increased levels of *Nox1* mRNA (Supporting Information Fig. 2). Because *Nox1* and *Nox4* were most highly overexpressed at the mRNA level, the corresponding proteins were examined by immunoblotting (Fig. 1b). After normalizing to β-actin, densitometric analyses confirmed that Nox1 and Nox4 proteins were elevated 2- to 3-fold in tumors versus adjacent normal-looking tissue ( $p < 0.01$ ,  $n = 12$ ). A doublet was detected for Nox1, with the upper band stronger in some tumors and the lower band dominating in others (Fig. 1b, upper panel). This Nox1 doublet has been observed previously, for example in immortalized human keratinocytes,<sup>23</sup> Ras(+) NIH3T3 cells,<sup>24</sup> rat smooth muscle cells,<sup>25</sup> and human T84 large intestinal cells,<sup>26</sup> but the significance remains unclear. Subsequent studies examined nuclear NFκB



**Figure 2.** Overexpression of Nox isoforms in human primary colon cancers. (a) conventional RT-PCR data for 10 human colon adenocarcinomas (T, tumor) and their matched controls (N, normal-looking tissue from the same patient). (b) *NOX/DUOX* mRNA expression in human primary colon tumors determined by qPCR. Data = mean  $\pm$  SE,  $n = 10$ ; \* $p < 0.05$ ; NS, not significant. (c) Immunoblotting of Nox1 and Nox4 proteins in human primary colon tumors and their matched controls.

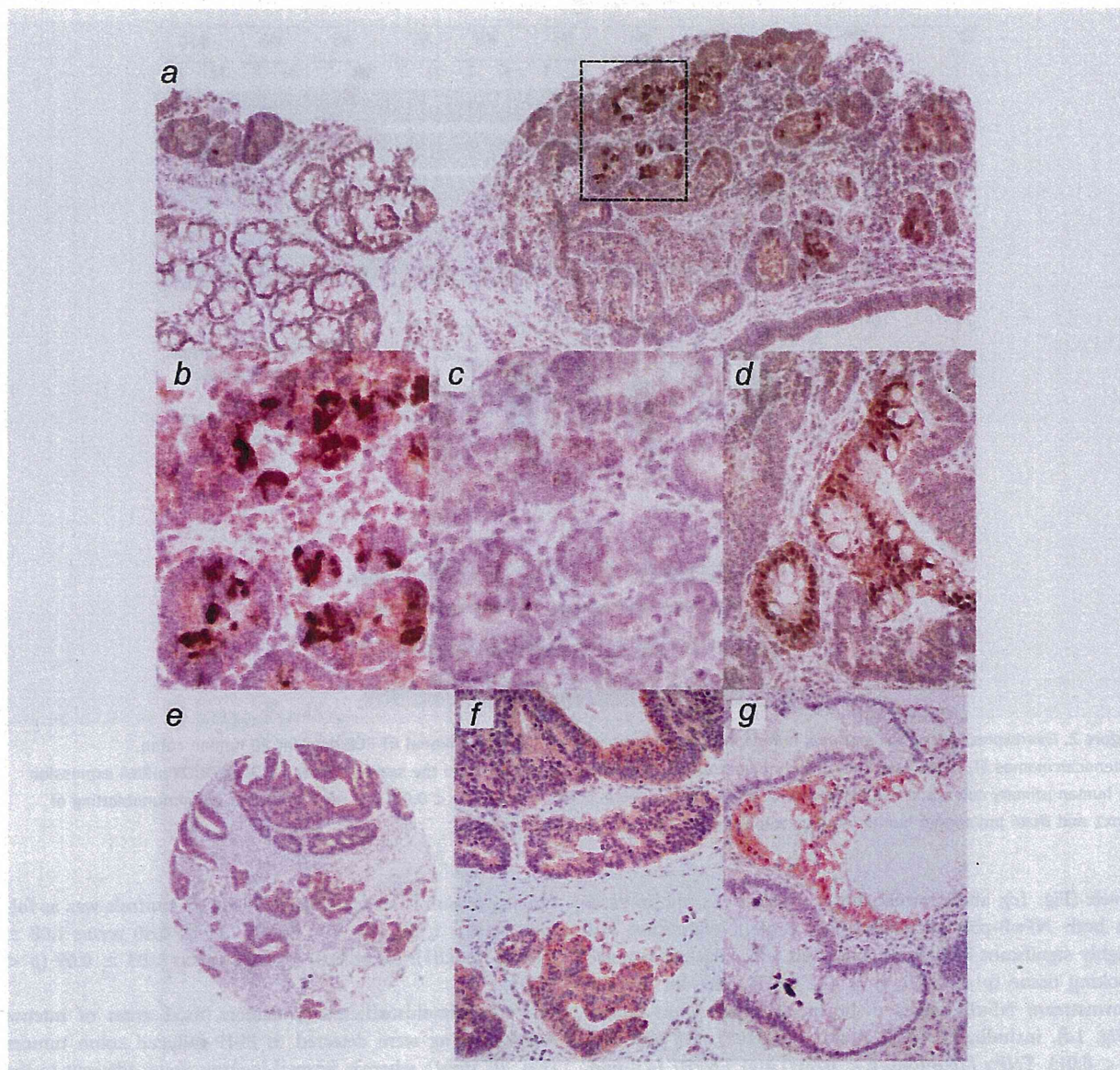
levels (Fig. 1c); after normalizing to histone H1, the increase in both NF $\kappa$ B-p50 and NF $\kappa$ B-p65 protein expression was highly significant in tumors compared with adjacent normal-looking tissue ( $p < 0.001$ ,  $n = 12$ ). qPCR analyses revealed downstream NF $\kappa$ B targets to be increased in colon tumors (Fig. 1d), including *IL1 $\beta$*  (2.2-fold,  $p < 0.05$ ), *IL6* (2.9-fold,  $p < 0.01$ ), *TNF $\alpha$*  (16.6-fold,  $p < 0.001$ ) and *TNFR1* (4.0-fold,  $p < 0.01$ ).

#### Nox is overexpressed in primary human colon cancers

We next examined the expression of Nox/Duox family members in a panel of primary human colon cancers. By conventional RT-PCR (Fig. 2a), cancer specimens nos. 1, 2, 3, 6 and 10 had high levels of both *NOX1* and *NOX4* mRNA, whereas *NOX2* and *DUOX2* mRNA levels were more inconsistent, being higher in some cancers and lower or undetectable in others. These findings were confirmed by qPCR, which revealed a 2.5- to 3.0-fold increase in *NOX1* and *NOX4* mRNA expression in primary colon cancers versus matched controls ( $p < 0.05$ , Fig. 2b). No significant difference was seen for *NOX2* and *DUOX2* in the qPCR analyses. Thus, Nox1 and Nox4 were selected for subsequent immunoblotting studies (Fig. 2c). After normalizing to  $\beta$ -actin, the rela-

tive expression in cancers versus matched controls was as follows (mean  $\pm$  SE,  $n = 10$ ): Nox1,  $2.36 \pm 0.40$  versus  $1.08 \pm 0.09$  ( $p < 0.01$ ); Nox4,  $2.87 \pm 0.22$  versus  $1.03 \pm 0.09$  ( $p < 0.001$ ).

In immunohistochemistry studies, focal areas of intense Nox4 staining were detected in PhIP-induced colon tumors (Fig. 3a, right), whereas normal-looking tissue adjacent to the tumor had no such focal Nox4 expression (Fig. 3a, left). Staining of serial sections in the absence (Fig. 3b) and presence of a Nox4 blocking peptide (Fig. 3c) confirmed the specificity of the primary antibody. Cells containing high levels of Nox4 protein typically were interspersed with other cells having little or no such staining. In some of the rat colon tumors, however, islands of intense Nox4 staining were observed, surrounded by areas that were predominantly negative (Fig. 3d). These "islands" corresponded to the more differentiated regions within a tumor. In human colon cancer tissue microarrays, a core occasionally stained entirely negative for Nox4 (not shown), but most cores had areas that were strongly Nox4 positive (Fig. 3e). Nox4 was detected in the cancer epithelial cells, and was absent from the surrounding stroma (Fig. 3f). In some cores, islands of Nox4 staining were associated with differentiated regions within the human



**Figure 3.** Immunodetection of Nox4 in rat (a)–(d) and human (e)–(g) colon tumors. (a) Expression of Nox4 in a PhIP-induced colon tumor (right) compared with adjacent normal-looking tissue (left). (b,c) Enlarged view of the dotted region from (a) showing serial sections stained with Nox4 primary antibody in the (b) absence and (c) presence of a Nox4 blocking peptide. (d) Differentiated regions in a rat colon tumor staining positive for Nox4. (e) View of an entire core from a human colon cancer tissue microarray (TMA) immunostained for Nox4, and (f) the same core at higher magnification. (g) Differentiated regions in a TMA core staining positive for Nox4. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

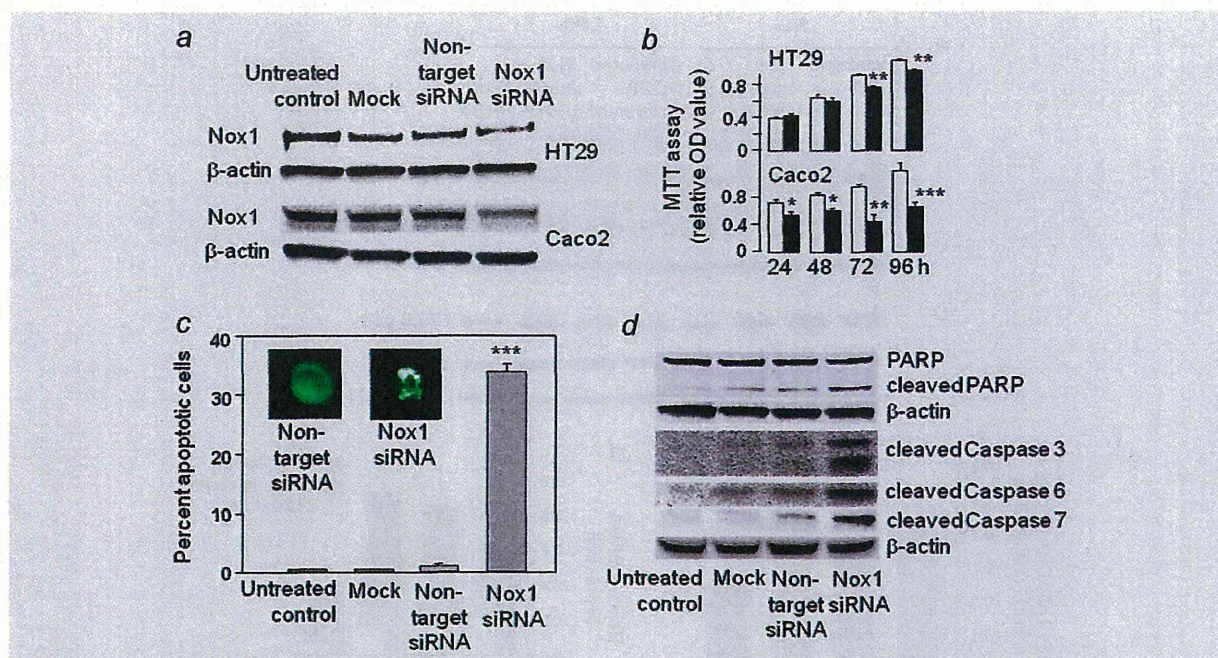
colon cancer (Fig. 3g), as seen in the rat (Fig. 3d). Our interpretation is that Nox4 staining patterns were similar in rat and human colon tumors, and that the PhIP model might provide useful insights into the role of Nox4 in colon cancer development.

Attempts to immunolocalize Nox1 protein in rat and human colon tissues were unsuccessful. In our hands, antibodies from commercial sources (cs-25545 from Santa Cruz, ab78016 from abcam, and LS-B1832 from Lifespan Biosciences,

Seattle, WA) gave high background expression with non-specific labeling, despite exhaustive protocol modifications and antibody dilution experiments (data not shown).

#### Nox1 knockdown induces cell cycle arrest and apoptosis

HT29 and Caco2 cells were selected from among a panel of human colon cancer cell lines due to their high *NOX1* mRNA content (data not shown), and *NOX1* siRNA was used as a knockdown strategy. Based on the results of three



**Figure 4.** Nox1 knockdown in human colon cancer cells. (a) Immunoblot analyses of Nox1 expression in HT29 and Caco2 cells treated with *NOX1* siRNA or nontarget siRNA. Untreated and mock controls (transfection agent alone) were included in the assays. (b) MTT assay data following *NOX1* siRNA (solid bars) or nontarget siRNA (open bars); mean  $\pm$  SE, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001,  $n$  = 3. (c) Percent apoptosis in Caco2 cells, 72 h after treatment with Nox1 siRNA (mean  $\pm$  SE,  $n$  = 3; \*\*\* $p$  < 0.001 versus nontarget siRNA); inset: acridine orange/ethidium bromide dual staining showing nuclear condensation and other hallmarks of apoptotic morphology. (d) Cleavage of PARP and caspases 3, 6, and 7, 72 h after *NOX1* knockdown in Caco2 cells. Caspase-3 cleaved products, 17-kD and 19-kD. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

replicate qPCR experiments, *NOX1* mRNA levels in HT29 cells typically were diminished by 48% at 48 h and 92% at 72 h, whereas in CaCo2 cells the reduction was 60% at 48 h and 94% at 72 h. Despite the similar knockdown efficiency for *NOX1* mRNA at 72 h, Nox1 protein expression was reduced more dramatically in Caco2 cells than in HT29 cells (Fig. 4a). Densitometry measurements were performed on Nox1 normalized to  $\beta$ -actin. Compared with the non-target siRNA control at 72 h, the relative expression of Nox1 protein was reduced by 48% in HT29 cells ( $1.03 \pm 0.12$  vs.  $0.54 \pm 0.07$ ,  $p$  < 0.05) and by 82% in Caco2 cells ( $0.96 \pm 0.07$  vs.  $0.18 \pm 0.05$ ,  $p$  < 0.001).

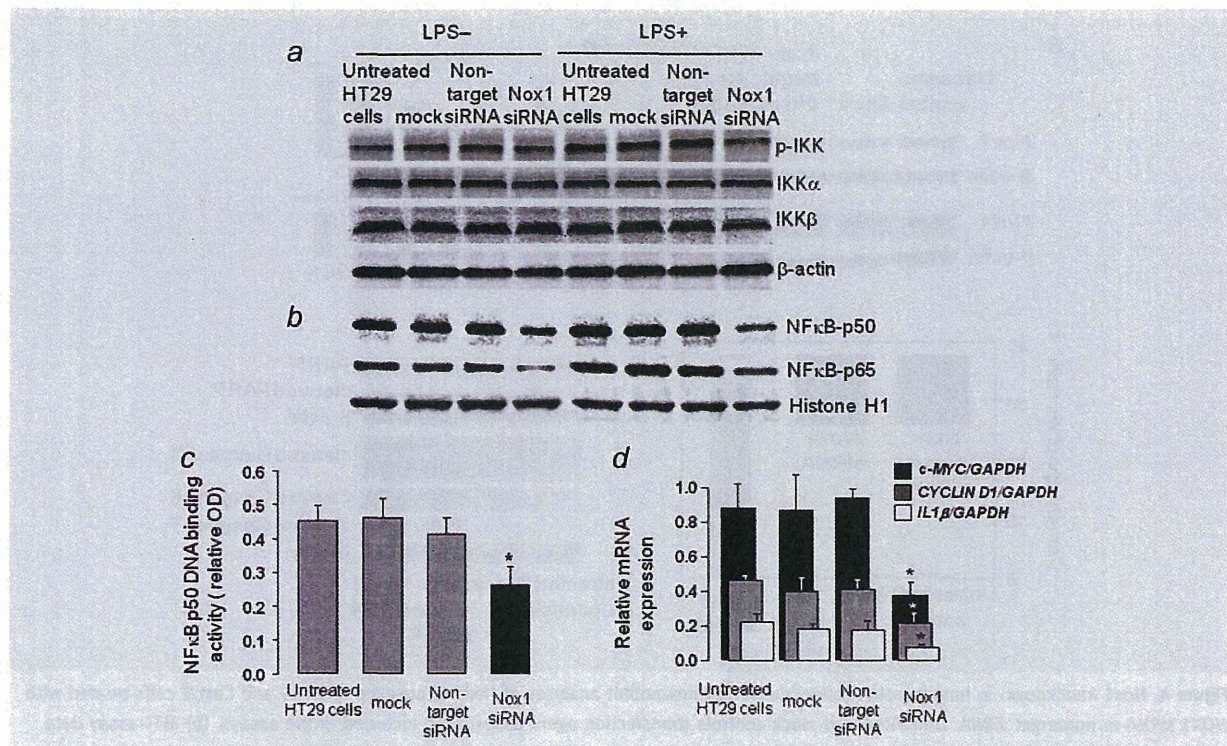
Inhibitory responses in the MTT assay following Nox1 knockdown were significant at all time-points after 24 h in Caco2 cells, and after 72–96 h in HT29 cells (Fig. 4b). To clarify whether the MTT assay data might be indicative of changes in cell cycle kinetics or apoptosis, further experiments were performed. In HT29 cells, changes in the cell cycle were detected 72 h post-transfection, as follows:  $77.4 \pm 2.5\%$  versus  $52.5 \pm 2.9\%$  in G1,  $14.6 \pm 2.2\%$  versus  $30.5 \pm 1.6\%$  in S, and  $8\%$  versus  $16.6\%$  in G2/M (*NOX1* siRNA vs. nontarget siRNA, mean  $\pm$  SE,  $n$  = 3 separate experiments). No significant changes were detected in the proportion of HT29 cells undergoing apoptosis (2–3% in all groups, data not shown). In contrast, Caco2 cells exhibited clear hallmarks

of apoptosis (Fig. 4c, inset), and the percentage of apoptotic cells at 72 h increased from 1–2% after exposure to non-target siRNA to >30% following treatment with *NOX1* siRNA ( $p$  < 0.001). In Caco2 cells treated with *NOX1* siRNA, there was an increase in the cleaved (active) forms of Caspase-3, -6 and -7, as well as elevated PARP cleavage (Fig. 4d). Taken together, these findings suggested that human colon cancer cells undergo cell cycle arrest or apoptosis, depending on the cell line and extent of Nox1 knockdown.

#### Nox1 knockdown blocks LPS-induced IKK phosphorylation and NF $\kappa$ B activity

In HT29 cells, low constitutive levels of phospho-IKK were detected in the absence of LPS treatment, whereas an increase in phospho-IKK was evident within 30 min of LPS exposure (Fig. 5a, LPS– vs. LPS+, respectively). Interestingly, LPS-induced phospho-IKK expression was blocked in cells that had been transfected with *NOX1* siRNA (Fig. 5a, top right). When the nuclear extracts were examined 6 h after treatment with LPS or media alone, NF $\kappa$ B-p50 and -p65 proteins were reduced markedly by Nox1 knockdown (Fig. 5b). Knockdown of Nox1 reduced significantly the DNA binding activity of NF $\kappa$ B in nuclear extracts ( $p$  < 0.05, Fig. 5c), and lowered the mRNA expression levels of downstream NF $\kappa$ B targets, such as *MYC*, *CCND1* and *IL1 $\beta$*  ( $p$  < 0.05, Fig. 5d).





**Figure 5.** Nuclear NFκB expression and DNA binding activity following Nox1 knockdown in human colon cancer cells. (a) Seventy-two hours after transfection with *NOX1* siRNA, HT29 cells were treated with LPS (1.2 μg/ml) or media alone for 30 min, and whole cell lysates were immunoblotted for phospho-IKKα/β (p-IKK), IKKα, or IKKβ, with β-actin as loading control. (b) Nuclear extracts were immunoblotted for NFκB-p50 and NFκB-p65, 72 h after HT29 cells were treated with *NOX1* siRNA, and 6 h following treatment with LPS or media alone. (c) NFκB (p50) DNA binding activity in nuclear extracts from HT29 cells, 72 h after Nox1 knockdown. Data = mean ± SE,  $n = 3$ ; \* $p < 0.05$  versus the corresponding nontarget siRNA control. (d) *c-MYC*, *CYCLIN D1* and *IL1β* mRNA expression following Nox1 knockdown; qPCR data normalized to *GAPDH*; mean ± SE,  $n = 3$ ; \* $p < 0.05$  versus the corresponding nontarget siRNA control.

## Discussion

Oxidative stress in the colon originally was thought to involve Nox2, and was ascribed to resident and recruited phagocytic cells with important roles in host-defense mechanisms.<sup>7,27</sup> However, this idea has evolved with the discovery of non-phagocyte derived Nox/Duox homologues. Nox1 is expressed in gastric pits and colonic epithelial cells and is required for normal gut physiology. For example, Nox1-induced oxidative stress, involving intermediates such as  $O_2^{\bullet-}$  and  $H_2O_2$ , regulates mucosal 5-hydroxytryptamine levels, which affects normal secretion and motility in the colon.<sup>28</sup> Transcriptional activation of *NOX1* by IFN-γ produces  $O_2^{\bullet-}$  in colonic epithelial cells, contributing to mucosal host defense mechanisms.<sup>26</sup> Duox2 is expressed in normal human colorectal barrier epithelial cells,<sup>16</sup> and its loss following *duox2* silencing in flies markedly enhances mortality following infection.<sup>29</sup> Nox family members also have been implicated in Crohn's disease and other inflammatory bowel disorders.<sup>7</sup>

Although the cancer-associated expression of Nox1 has been reported in human stomach,<sup>30</sup> there is some debate

over the precise role of Nox1 in colon cancer, as noted in the introduction. The most recent study found that Nox1 was overexpressed in human colon cancers and was correlated with activating mutations in K-ras.<sup>19</sup> Colon tumors induced by PhIP and other heterocyclic amines lack mutations in K-ras, but harbor genetic changes in β-catenin or Apc.<sup>20,21,31</sup> We performed mutation screening for the corresponding genes in the PhIP-induced colon tumors reported here, but observed no correlations with the expression of Nox1 or other Nox/Duox members (data not presented). Nonetheless, results from the present investigation support a role of Nox1 in human colon cancer and expand this observation to Nox4, including the first such evidence using a rat colon carcinogenesis model.

Interestingly, increased Nox1, NFκB-p50, and NFκB-p65 protein levels were detected in colonic mucosa obtained from rats immediately after completing PhIP treatment, several weeks before the appearance of frank tumors (Supporting Information Fig. 3). Thus, Nox1, NFκB-p50, and NFκB-p65 might play a role during the early stages of PhIP-induced colon carcinogenesis. Charalambous *et al.*<sup>32</sup> recently postulated

that NF $\kappa$ B-p65 and IKK $\alpha$  are early post-initiation events in human colorectal tissues, perhaps involved in tumor progression. Heterocyclic amine mutagens have been studied extensively,<sup>31–36</sup> but this is the first report to show that PhIP-induced colon tumors have increased nuclear NF $\kappa$ B-p50 and NF $\kappa$ B-p65 expression, and higher levels of IL1 $\beta$ , IL6, TNF- $\alpha$  and TNFR1.

In HT29 cells, LPS-induced phosphorylation of IKK was blocked following Nox1 knockdown. This suggests that NF $\kappa$ B activation induced by cytokines, growth factors, and other external stimuli might be mediated, at least in part, by Nox1. Even in the absence of LPS treatment, however, Nox1 knockdown lowered nuclear NF $\kappa$ B-p50 and NF $\kappa$ B-p65 protein expression, attenuated NF $\kappa$ B-p50 DNA binding activity, and reduced transcription of NF $\kappa$ B downstream targets. We were also interested in the reverse scenario, namely that loss of NF $\kappa$ B might attenuate Nox1 levels. However, NF $\kappa$ B knockdown did not alter Nox1 mRNA or protein expression in human colon cancer cells (data not presented), suggesting that alternative mechanisms regulate Nox1.

Finally, we observed that Nox1 knockdown in HT29 cells resulted in the accumulation of cells in G1, whereas the same knockdown strategy in Caco2 cells strongly induced apoptosis. These cell-specific effects are intriguing and may suggest a causal role in tumorigenesis. However, the findings should be interpreted cautiously, since it is still not entirely clear whether Nox/Duox overexpression is a cause of tumorigenesis, or whether it results from tumor formation. The data showing increased Nox1 expression in colonic mucosa several weeks before colon tumor formation (Supporting Information Fig. 3) suggest a causal role, but additional work is needed to

confirm this possibility. Moreover, immunohistochemical analyses of rat colon tumors revealed increased NF $\kappa$ B expression in areas that were either positive or negative for Nox4 (data not presented). This is perhaps not surprising, given that multiple pathways activate NF $\kappa$ B,<sup>2</sup> and further studies appear to be warranted on other Nox/Duox family members in the context of NF $\kappa$ B signaling and colon cancer development. The present report is the first to show that PhIP-induced colon tumors have increased Nox/Duox expression and NF $\kappa$ B activation, but these events are probably not specific to heterocyclic amines, and other colon carcinogens may act in a similar fashion.

In summary, we provide here the first evidence for the involvement of Nox1, Nox4 and NF $\kappa$ B during PhIP-induced colon carcinogenesis, and provide further support for a role of Nox/Duox isoforms in primary human colon cancers and colon cancer cell lines. In cultured human colon cancer cells, Nox1 knockdown blocked LPS-induced phosphorylation of IKK, reduced nuclear NF $\kappa$ B levels and DNA binding activity, and attenuated the transcription of downstream NF $\kappa$ B targets. We conclude that the interplay between Nox/Duox family members and NF $\kappa$ B signaling during colon cancer development is worthy of further investigation.

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