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NADPH Oxidases: A Perspective on Reactive Oxygen Species Production in Tumor Biology

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Abstract

Significance: Reactive oxygen species (ROS) promote genomic instability, altered signal transduction, and an environment that can sustain tumor formation and growth. The NOX family of NADPH oxidases, membrane-bound epithelial superoxide and hydrogen peroxide producers, plays a critical role in the maintenance of immune function, cell growth, and apoptosis. The impact of NOX enzymes in carcinogenesis is currently being defined and may directly link chronic inflammation and NOX ROS-mediated tumor formation. ***Recent Advances:*** Increased interest in the function of NOX enzymes in tumor biology has spurred a surge of investigative effort to understand the variability of NOX expression levels in tumors and the effect of NOX activity on tumor cell proliferation. These initial efforts have demonstrated a wide variance in NOX distribution and expression levels across numerous cancers as well as in common tumor cell lines, suggesting that much remains to be discovered about the unique role of NOX-related ROS production within each system. Progression from *in vitro* cell line studies toward *in vivo* tumor tissue screening and xenograft models has begun to provide evidence supporting the importance of NOX expression in carcinogenesis. ***Critical Issues:*** A lack of universally available, isoform-specific antibodies and animal tumor models of inducible knockout or over-expression of NOX isoforms has hindered progress toward the completion of *in vivo* studies. ***Future Directions:*** *In vivo* validation experiments and the use of large, existing gene expression data sets should help define the best model systems for studying the NOX homologues in the context of cancer. *Antioxid. Redox Signal.* 20, 2873–2889.

Introduction

Angiogenesis, proliferation, and metastasis are hallmarks of cellular transformation and tumor growth that have been associated with oxidative stress and genomic instability (14, 18, 27, 105, 121, 126, 130). Redox imbalance in tumors may originate from a variety of sources, including mitochondrial or NADPH oxidase-derived reactive oxygen species (ROS). Although a role for ROS in tumor biology was postulated well before the discovery of the NOX enzymatic family, the functions of these oxidases are redefining the role of superoxide and hydrogen peroxide (H_2O_2) in tumor cell homeostasis (100).

The NOX family, comprising seven enzymatic isoforms, produces ROS by the NADPH-dependent one-electron reduction of oxygen to superoxide. Isoform-specific production of H_2O_2 is unique to NOX4 and the dual oxidase (DUOX) enzymes (31, 87) (Fig. 1); controversy surrounds this ROS categorization with some researchers noting superoxide production by the NOX4 isoform. Variability in the specific ROS reported to be produced by NOX4 has been attributed to the interpretation of fluorescent microscopy studies and/or diverse cellular localization (112, 122). Structurally, each NOX/DUOX protein is anchored to the plasma membrane through six transmembrane helices, which bind two heme cofactors and allow for NADPH oxidation through a C-terminal FAD/NADPH binding domain (Fig. 2); other features vary between isoforms, such as cytosolic amino-terminal EF-calcium binding regions present in NOX5 and DUOX1/2, and a unique DUOX structural extension

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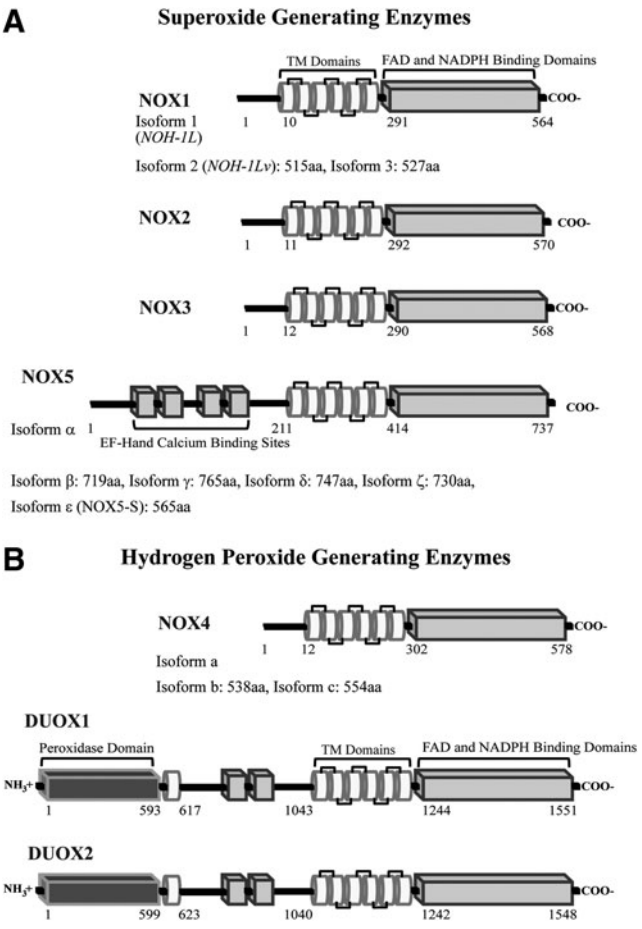


FIG. 1. Classification of the NADPH oxidase family members by ROS generation. Illustration of each NOX/DUOX protein depicts important structural motifs and glycosylation sites; each isoform is categorized by function: **(A)** superoxide generating enzymes and **(B)** hydrogen peroxide generating enzymes. The known number of variants and the amino acids predicted to comprise each structural domain are listed (amino acid assignments were generated by Jpred3 secondary structure and TMHMM 2.0 servers). Putative TM domains (white tubes), which bind two heme molecules, and cytosolic EF-hand and FAD/NADPH binding domains (light gray rectangles) are displayed; each DUOX protein contains an N-terminal extracellular peroxidase-like domain (dark gray rectangles). DUOX, dual oxidase; ROS, reactive oxygen species.

composed of a transmembrane helix and extracellular peroxidase-like domain, hence the DUOX nomenclature. Studies of the DUOX peroxidase-like domain suggest that it neither functions as a peroxidase, nor a superoxide dismutase (SOD), to account for the production of H_2O_2; as such, the role of the peroxidase-like domain and the complete mechanism of DUOX ROS production remains elusive (91, 92). The first NADPH oxidase discovered, the phagosomal membrane-associated NOX2 (originally gp91^{phox}), shares sequence identity with the other family members as follows: 56% (NOX1), 58% (NOX3), 39% (NOX4), and 27% (NOX5). DUOX isoforms, DUOX1 and 2 are 83% homologous; they share 57% and 43% sequence similarity with NOX2, respectively (9, 23, 42, 127). The structural homologies among the NOX family have hindered both targeted therapeutic intervention and antibody development (3, 32, 72).

ROS production by NOX members is regulated in response to interactions with a series of cytoplasmic and membrane-associated proteins (Table 1), as well as chemical stimuli such as calcium or phorbol 12-myristate 13-acetate. Phosphorylation events trigger the formation of an activated NOX enzymatic complex for isoforms 1–3 (19, 98). With the exception of NOX5, which to date has no known interaction partner essential for activity, all remaining NOX homologues (NOX1–4) form a heterodimer with p22^{phox}, a small membrane-bound association factor required for complex formation and stability (78, 151). NOX1, NOX2, and NOX3 organize through recruitment of further cytosolic regulatory subunits p47^{phox} (or the related NOX organizer 1 or NOXO1), p67^{phox} (or the related NOX activator 1 or NOXA1), and the GTPase Rac isoforms (predominantly Rac1 associates with NOX1 and NOX3, Rac2 with the NOX2 enzyme) (1, 7, 65, 131, 133). NOX4 apparently has no other activation requirements, aside from interaction with p22^{phox}, and is therefore constitutively active. The NOX proteins may further enhance their structural stability and/or activity through colocalization with other proteins such as p40^{phox}, tyrosine kinase substrate 4 or 5 (Tks4/5), protein disulfide isomerases (ERp72), Hsp90, c-Abl, or Poldip2 (21, 22, 29, 33, 44, 57, 84, 143). DUOX proteins have a unique protein association with a maturation factor (DUOXA) required to afford trafficking from the endoplasmic reticulum (ER) to the plasma membrane, where a functional membrane complex is formed (46). This association is known to be preferential for DUOX1–DUOXA1 and DUOX2–DUOXA2; DUOXA can cross-function in supporting DUOX membrane transport, however, mismatched associations promote cell line-dependent variability in the level and specificity of membrane targeting and ROS production (superoxide *vs.* H_2O_2) (83, 95). Complex formation, organization, and characterization of individual association partners, briefly outlined above, have previously been extensively reviewed (13, 77).

The epithelial NOX/DUOX family members are recognized to be widely distributed from the kidney, colon, and thyroid tissues to the brain and inner ear (68) (Table 1). Many cell lines have been found to express more than a single NOX/DUOX protein, complicating research efforts. In vascular smooth muscle cells, NOX1 and NOX4 are the predominant members expressed (73). NOX2 and NOX4 play a role in regulating kidney function; both DUOX isoforms are highly expressed in the thyroid (28, 31, 118).

Whereas tissue distribution varies greatly, cellular localization is somewhat less diverse. All NOX proteins exist as transmembrane species and have been found, to varying degrees, at the plasma membrane surface. Intracellular localization has been characterized for NOX1, NOX4, and NOX5 in such structures as trafficking vesicles, the ER, mitochondria, or nuclear membranes (40, 45, 51, 63). The effect of the specific ROS, superoxide (NOX1/5), or H_2O_2 (NOX4/DUOX), on the intracellular environment, including the potential for genomic instability, will be discussed in the framework of each enzyme and its relevant tumor biology.

To date, the role of the NOX/DUOX family in the context of cancer biology has primarily been examined using human tumor cell lines. These cell lines, however, are known to differ significantly in gene expression levels from patient tumors, because they rapidly adapt to cell culture *in vitro* by

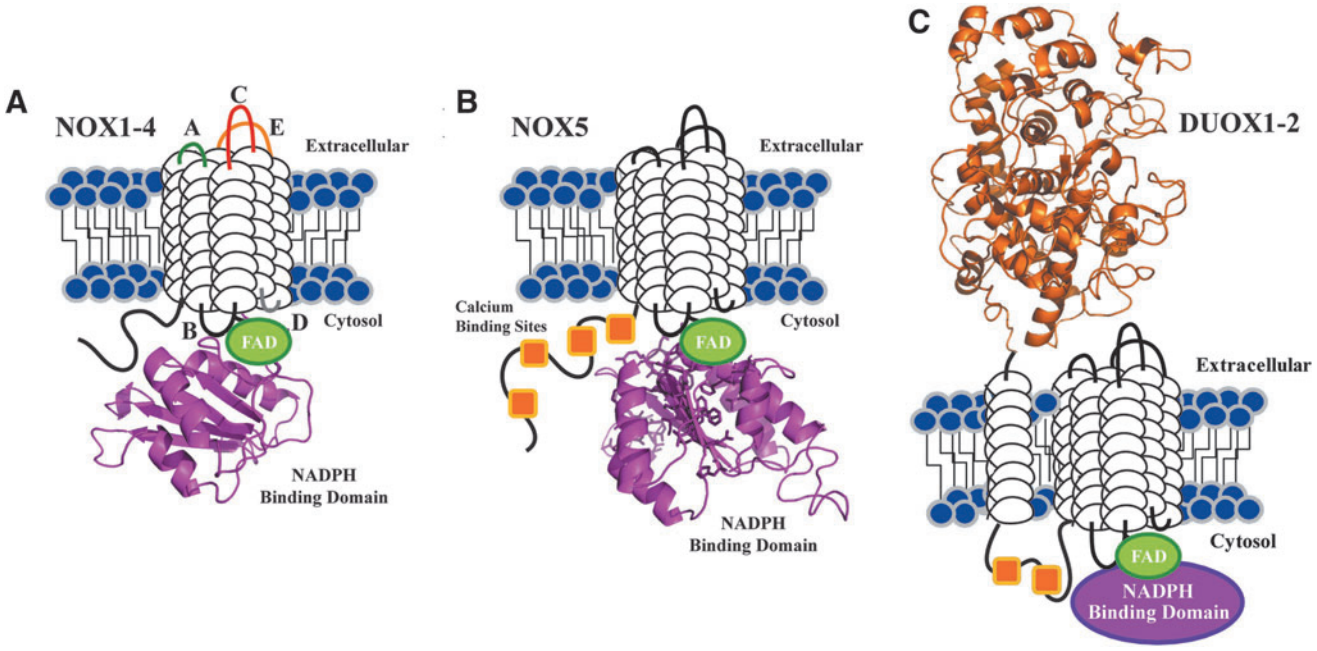


FIG. 2. Schematic view of the conserved structural features of the NADPH oxidase family. Each NOX/DUOX isoform contains six putative TM domains (white cylindrical loops), with C-terminal FAD (green) and NADPH binding domains (purple). The NADPH binding domain structural models were created by the SWISS-MODEL program server with hNOX2 (PDB: 3A1F) as the template. A model of the peroxidase-like domain of DUOX2 is also highlighted and was created as previously reported (92); all models were visualized by Pymol software. **(A)** NOX1-4 isoforms are depicted with loop regions labeled based on established designations: extracellular loops A (green), C (red), E (yellow) and intracellular loops B (black) and D (gray). **(B)** Each NOX5 isoform contains four N-terminal calcium binding sites (orange squares); the hNOX2 and hNOX5 NADPH binding domains share 36% amino acid sequence identity. Amino acids displayed as sticks represent the predicted NADPH binding site residues. **(C)** DUOX1-2 are unique to the NADPH oxidase family, as both isoforms contain an extracellular N-terminal peroxidase homology domain (orange) tethered to a TM domain, and two cytosolic calcium binding sites.

upregulating genes involved in cellular proliferation (107, 115). Decades of usage, as well as contamination, have promoted loss of many of their initial gene expression characteristics (41, 67, 89, 117, 153). Sandberg and Ernberg have created a tissue similarity index to help guide investigators toward the best cell lines for experimentation within 10 important cancer cell types, by direct comparison of gene expression levels in existing cancer cell lines to those in both normal and tumor tissues (116). This study demonstrated what many had already reported, that while cell lines are useful cell biology tools, they have many limitations. Their application should be accompanied by the perspective that substantial differences exist between cell lines and tumors originating from the same tissues. Progress in understanding

Table 1. Characteristics of the NADPH Oxidase Family

Enzyme	*Interaction partner(s)*[a]	*Cellular localization*	*Major tissue distribution*[b]
NOX1	$p22^{phox}$, NOXA1/$p67^{phox}$ NOXO1/p47phox, Rac, *Tks4/5*, *PDI (ERp72)*, *Hsp90*	Plasma membrane caveoli, lipid rafts	Colon epithelium
NOX2	$p22^{phox}$, $p67^{phox}$, $p47^{phox}$, Rac, *p40phox*, *Hsp90*	Plasma membrane	Phagocytes
NOX3	$p22^{phox}$, NOXA1/$p67^{phox}$, NOXO1/p47phox, Rac	Plasma membrane	Inner ear
NOX4	$p22^{phox}$, *Poldip2*, *Tks4/5*, *PDI*	Plasma membrane, ER, mitochondrial and nuclear membranes	Kidney, ovary, brain
NOX5	*Hsp90*, *c-Abl*	Plasma membrane, ER, nuclear membrane	Spleen, testis, lymph node
DUOX1	DUOXA1, *TPO*, *EFP1*, *NOXA1*	Plasma membrane	Thyroid, lung, prostate, testis
DUOX2	DUOXA2, *TPO*, *EFP1*, *NOXA1*	Plasma membrane, ER, vesicles	Thyroid, salivary gland, colon, pancreas

[a]Interaction partners nonessential for activity are in italics.
[b]Literature references for tissue distribution assignments: (5, 8, 9, 20, 28, 68, 127).
DUOX, dual oxidase; Tks, tyrosine kinase substrate.

the role of the NOX family in tumor biology awaits movement toward *in vivo* tissue/tumor studies and animal models. However, difficulties in developing well-characterized, NOX-specific antibodies have also hindered the conduct of *in vivo* investigations. The recent development of NOX5 and DUOX monoclonal antibodies should broaden the scope of NOX/DUOX research (3, 145).

This review will focus on the evolution of investigative efforts in the field to study the role of the NOXs and DUOXs in cancer, profiling progress from cell line experiments to *in vivo* tumor studies. This direction is important to define the relevance of these enzymes and ROS in cancer biology and to evaluate how the development of novel therapeutic agents targeting this enzyme family could benefit cancer patients. Of the known epithelial proteins to be addressed, discussion will focus on the NOX1, NOX4, NOX5, and DUOX1/2 isoforms. The utility of bioinformatics approaches to direct investigative efforts with existing platforms of tumor tissue gene expression and cell line data in the context of the NOX proteins will also be reviewed.

NADPH oxidases: from cell lines to tumors

NOX1 (colon cancer). Originally discovered during investigation of the human colon cancer cell line Caco2, NOX1 is expressed throughout the colonic epithelium (127). NOX1 protein levels vary according to anatomic localization along the gastrointestinal tract, with a general increase in enzymatic expression from small intestine to transverse, descending, and sigmoid colon (74). The functional role of NOX1-related superoxide production at the epithelial surface of the colon may extend beyond acknowledged host defense activities (43, 62, 76), through development of a mitogenic environment from intracellular ROS exposure, especially in cases of chronic inflammation.

Intracellular ROS production is known to promote base oxidation, cross-linking between DNA and proteins, and induction of DNA strand breaks. Oxidation of guanine (G) is commonly observed, due to its low nucleotide redox potential. These alterations are among the most prominent somatic mutations in lung, breast, ovarian, gastric, and colorectal cancers (135). The potential of human NOX1 to contribute to genomic instability was first characterized using active overexpression model systems in both HeLa cells and mouse embryo fibroblasts. Chronic oxidative stress by NOX1-induced ROS was shown to promote significant DNA damage (formation of 8-oxoGua); interestingly, no changes in the steady-state levels of DNA single-strand breaks were detected. The mechanism(s) underlying base damage remain to be clarified (24).

Beyond genomic instability, a novel connection to the proapoptotic tumor suppressor p53 has been described for the NOX1 family member. HIPK2, a transcriptional corepressor, which controls p53 function through phosphorylation and acetylation events, was shown to upregulate NOX1. In turn, NOX1 inhibited p53 Lys^{382} acetylation, a target of the deacetylase sirtuin 1 (SIRT1), responsible for deacetylation and inactivation of p53. This consequently impaired transcriptional induction of proapoptotic genes (110, 136). SIRT1 deacetylates a wide range of protein substrates, and has been implicated in chemotherapeutic resistance; therefore, the link to NOX1 could be important to many cancer-related biological outcomes (102).

Despite these investigations, debate continues regarding the role of NOX1 in colon carcinogenesis. One comparative study of NOX1 expression in adenomas and colonic adenocarcinomas demonstrated no statistical difference in expression levels between normal and malignant tissues (129). Subsequent studies, however, have detected significant NOX1 overexpression in precancerous tubular and villous adenomas as well as in moderate and well-differentiated adenocarcinomas (39, 58). In support of a role for NOX1 in colon carcinogenesis, activating mutations in the proto-oncogene K-Ras (codons 12 and 13) were discovered in concert with NOX1 overexpression in human colon cancers. Eighty percent of tumors with these mutations demonstrated a twofold or greater elevation in NOX1 mRNA *versus* normal tissues (74). Still, it remains unclear whether NOX1 is required for carcinogenesis, and whether NOX1 expression levels change during the transition from premalignant to advanced stage cancer (129).

Understanding the importance of NOX1 overexpression during tumorigenesis continues to be an area of active investigation. It has been suggested that NOX1-related ROS contribute to the development of inflammation-associated colonic malignancies. Nuclear factor-κB (NF-κB), a critical regulator of inflammation and cancer development, has been proposed as the link between NOX1 and the development of colon cancer (34, 85). Increased NOX1, and p50 and p65 (members of the NF-κB family) expression in colonic mucosa from rats exposed to a heterocyclic amine carcinogen, before formation of frank tumors, suggests a role for NOX1 in the initiation phase of PhIp-induced cancer (6, 39, 139). These experiments support the hypothesis that enhanced production of ROS, through increased NOX1 expression, activates NF-κB signaling, which could contribute to tumor initiation.

Beyond cancer initiation, the presence of an active NOX1 complex in advanced cancers suggests that NOX1-dependent ROS could support signaling pathways that promote colon cancer proliferation. Consistent with a role in growth, ROS production in colon tumor-derived cells expressing NOX1 is higher in subconfluent than in confluent (arrested) cells. ROS generation in this situation was suggested to be NOX derived by the use of a flavoprotein inhibitor, diphenylene iodonium (DPI), which blocked the NOX activity (106). NOX1-mediated ROS production has also proven essential for activation of the Wnt-β-catenin pathway involved in cell proliferation through nucleoredoxin oxidation and subsequent disruption of the nucleoredoxin dishevelled complex (60).

Further support for a functional NOX1 role in colon carcinogenesis was found by stable shRNA knockdown of NOX1 in HT-29 human colon cancer cells (59). Diminished constitutive ROS production was achieved as a result of significantly decreased NOX1 expression (80–90%). An accompanying G_1/S block responsible for a two- to threefold increase in tumor cell doubling time, without increased apoptosis and changes in tumor cell morphology, was also observed. This block in cell cycle transition was related to a steady-state decrease in the phosphorylation of other mitogen-activated protein kinase (MAPK) signaling pathway enzymes (Rac1, ERK1/2, CREB), and concomitant downregulation of cyclin D1. With the activities of protein tyrosine and serine/threonine phosphatases enhanced by a decrease of intracellular oxidant, a known phenotype (66, 103), the observed MAP kinase pathway changes may be the downstream result of c-Raf inactivation

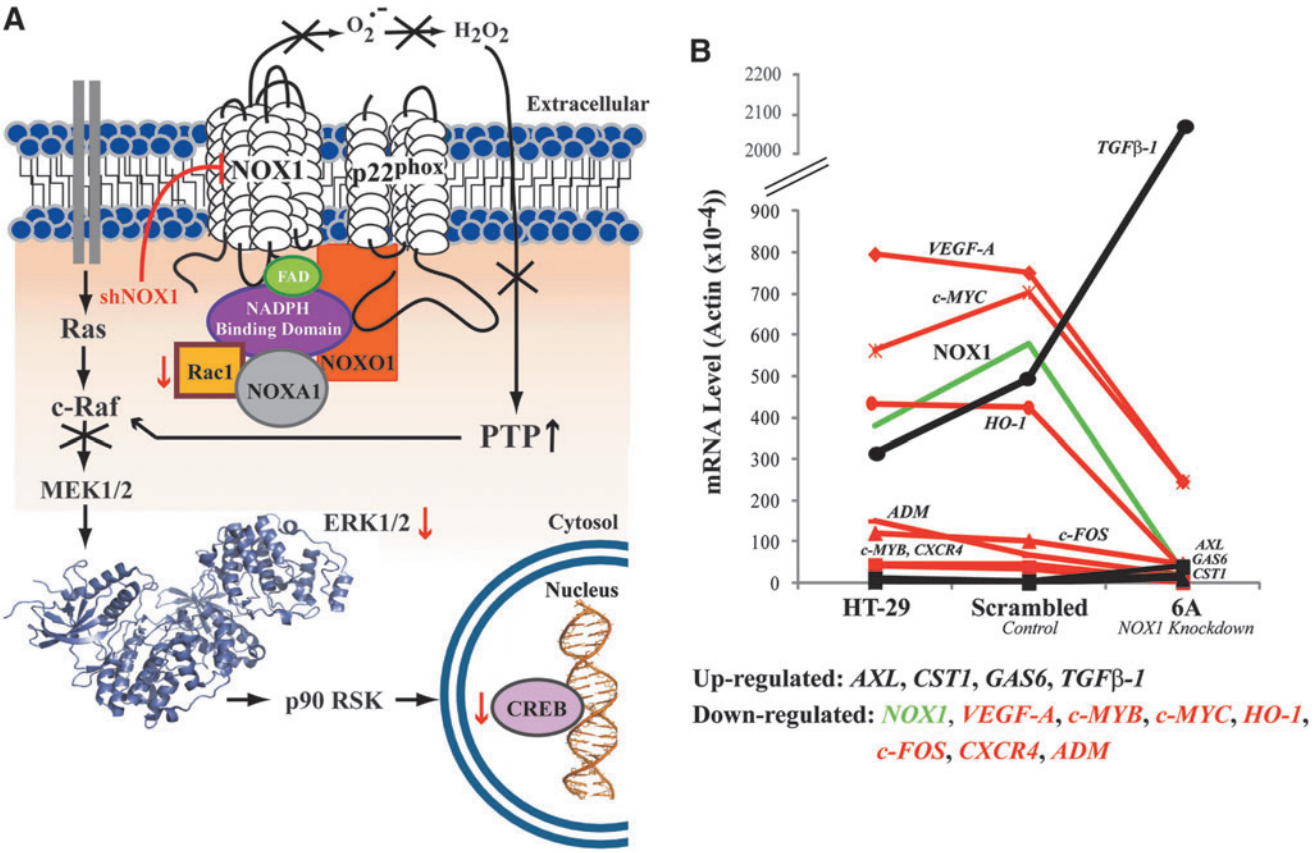


FIG. 3. Insights from *in vitro* and *in vivo* studies of NOX1 in HT-29 cells. (A) Schematic representation of the active NOX1 enzymatic complex and MAP kinase components affected by NOX1 expression levels. Stable shRNA knockdown of NOX1 in HT-29 cells results in a block in G_1 phase of the cell cycle related to a decrease in the phosphorylation of Rac 1, ERK 1/2 (PDB: 2ZOQ, ERK 1), and CREB (red *arrows*). A subsequent decrease in ROS production and increase in the level of PTP was also observed; immunoprecipitation demonstrated phosphatase binding to c-Raf, providing a potential mechanism of MAP kinase inhibition. **(B)** mRNA expression levels of genes related to cell proliferation, angiogenesis, and invasion identified in tumor xenografts from HT-29 cells, cells stably transfected with scrambled shRNA, and NOX1 knockdown cells. Genes identified as either upregulated (black) or downregulated (red) in response to the expression of NOX1, as measured by real-time reverse transcriptase-polymerase chain reaction, are plotted [accession number GSE4561 (59)]. PTP, protein tyrosine and serine/threonine phosphatases; TGFβ-1, transforming growth factor β-1.

through phosphatase binding (unpublished results, Fig. 3). No changes in the AKT or p38MAPK pathways were noted; microarray analysis found that NOX1 silencing was also associated with downregulation of several oncogenes, chemokines, and angiogenic factors. Corroborative *in vivo* studies provided direct comparison of gene expression levels, tumor growth, and angiogenesis for HT-29 xenografts from parental HT-29 cells *versus* knockdown cell xenografts. A significant decrease in tumor growth and angiogenesis was observed when NOX1 expression was attenuated; concomitant alterations in the expression of genes essential for proliferation were also observed *in vivo* following NOX1 knockdown (Fig. 3) (59). Interestingly, decreased NOX1 expression, and accordingly intracellular ROS, enhanced the expression of the growth inhibitor transforming growth factor β-1 (TGFβ-1) fourfold above scrambled control, consistent with a previous investigation (144).

Whereas studies of NOX1 have mainly addressed its role in colon cancer, a role for NOX1 has also been suggested in gastric, prostate, and breast carcinomas, as well as melanoma (25, 79, 81, 110, 132). The significance of NOX1 expression in these tumors is speculative; further development of specific NOX1 antibodies may clarify the prevalence of NOX1 in these diseases.

NOX4 (glioblastoma, melanoma, renal, and ovarian cancer). As the only H_2O_2-liberating NOX enzyme, NOX4 is distinguished not only by its ROS generation, but also by its lack of known protein partners beyond $p22^{phox}$ or required calcium stimulus for functionality. Constitutive activity, coupled with a pattern of both intracellular and plasma membrane associations, has been used to support the potential for NOX4 to produce genomic instability (45, 108). There has been considerable interest in the relationship of NOX4 to carcinogenesis over the past 5 years (142). Increased NOX4 expression has been found prominently in tumors of the brain, breast, ovary, pancreas, and kidney, as well as melanoma. However, in depth investigations have been hindered by the lack of a widely available, specific NOX4 monoclonal antibody and transgenic animal models for investigations of the role of NOX4 in tumor biology (45, 86, 94, 124, 141, 148, 149). Three different transgenic NOX4 overexpression mouse models have been published; one endothelial-specific system

and two cardiomyocyte-specific systems (2, 112, 152). Interestingly, the endothelial tgNOX4 mouse had a lower systemic blood pressure compared to wild-type litter mates. This may be due to a bystander effect on $p22^{phox}$, or perhaps, non-physiologically overexpressed NOX4 localizes differently than endogenous proteins. Whereas overexpression of NOX4 in this mouse model was not noted to promote carcinogenesis, the development of more tumor-specific animal models is required to afford a proper insight into the role of NOX4 in cancer biology.

The initial suggestion that NOX4-derived ROS had an impact on the development of the most aggressive form of brain tumor, glioblastoma multiforme (GBM), originated from the demonstration of increased NOX4 expression levels in cultured glioma cells. NOX4-mediated ROS inhibition has also been shown to decrease the formation of invadopodia (phosphotyrosine-rich structures involved in glioma motility) (29). The *in vivo* relevance of NOX4 in GBM was validated by detection of high levels of NOX4 expression in both GBM cell lines (U87, KNS81, and KNS42), and in surgically resected human gliomas (124). Targeted knockdown of NOX4 by dsRNA in KNS81 and KNS42 cells promoted morphological changes within 48 h of transfection, including changes that were not similarly observed after DPI treatment. Retardation of cell growth and increased vulnerability to apoptosis from the chemotherapeutic agent cisplatin were also observed following NOX4 downregulation, through an unknown pathway. Immunohistochemical analyses, performed with a polyclonal antibody raised against the C-terminus of NOX4 (amino acids 559–578), suggest that NOX4 expression is increased in high-grade tumors. Reverse transcriptase-polymerase chain reaction (RT-PCR) evaluation demonstrated an increase in the expression of NOX4 with increasing tumor stage; grade IV GBM tumors demonstrated significantly higher NOX4 levels than either grade III (anaplastic astrocytoma) or grade II (diffuse astrocytoma) cancers. NOX4, therefore, may serve as a marker of adverse prognosis for patients with brain tumors.

Regulation of growth in melanoma cells by NOX4 has been suggested since 2002 (17). Yamaura *et al.* found by RT-PCR that 13 melanoma cell lines demonstrated significant NOX4 gene expression. Of these, MM-BP cells were chosen for focused experiments because no significant expression of other NOX isoforms could be demonstrated (149). DPI exposure and siRNA knockdown produced growth inhibition. This was accompanied by a decrease in intracellular ROS and anchorage-independent growth, indicating that NOX4-generated ROS were required to sustain melanoma development. MM-BP cells transfected with scrambled or NOX4-specific siRNAs produced tumors within 2 weeks of subcutaneous injection in athymic mice; however, significantly decreased tumor volumes were observed in the xenografts produced from NOX4 knockdown cells. Flow cytometry of melanoma cells depleted of ROS by DPI exposure, catalase treatment, or NOX4 siRNA knockdown resulted in a G2/M cell cycle arrest, suggesting that NOX4-derived ROS promote progression across the G2/M boundary. Dephosphorylation of CDK1 (cdc2) kinase is required for the transition from G2 to M phase. NOX4-mediated ROS production appears to increase the phosphorylation of CDK1, contributing to the G2 block. Further investigation is needed to determine the molecular signals connecting NOX4-induced ROS production and CDK1 phosphorylation.

NOX4 was first isolated from human fetal kidney cDNA; immunostaining of the human kidney cortex has established that abundant NOX4 expression occurs in distal renal tubular cells (23, 123). NOX1 and NOX4 are both expressed in renal cell carcinomas (RCCs); however, NOX4, rather than NOX1, appears to be upregulated in RCC lines when compared to normal cells of the kidney (15). RCC has been associated with inflammation, through observed upregulation of hypoxia inducible factor 1-alpha (HIF-1α), a component of the heterodimeric HIF-1 complex (HIF-1α and HIF-1β). Biallelic inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene is also linked to RCC formation through a hypoxic response. Mutations in VHL occur in $\sim$80% of RCCs; VHL-mediated degradation of HIF is significantly inhibited in the case of VHL mutation, producing increased levels of HIF proteins and their downstream targets (69). A link between NADPH oxidases and HIF proteins in the context of RCC was noted when VHL-deficient cells (786-O cell line) were found to downregulate HIF-2α expression after DPI treatment or stable NOX4 knockdown when compared to a normal kidney (15). Furthermore, NOX4 expression, under control of glycolytic intermediates, may be related to the production of the proinflammatory cytokines interleukin (IL)-6 and IL-8 in kidney cancer cells during hypoxia (30, 36).

Similar to renal cancer, the NOX4 isoform is the predominant NOX found to have increased expression in ovarian cancer cell lines; markedly higher NOX4 mRNA levels have been found in both OVCAR-3 and A2780 cells relative to immortalized normal ovarian cells (148). To determine whether NOX4-mediated ROS production plays a role in ovarian cancer-related angiogenesis through HIF-1 expression, OVCAR-3 cells were subjected to hypoxia, with a resultant upregulation of HIF-1α; DPI inhibited the upregulation of HIF-1α expression, without changing HIF-1β levels. Identification of the specific ROS, which affect HIF-1α expression, was determined using specific ROS scavengers (SOD, catalase, and sodium formate). Only exposure to catalase decreased HIF-1α expression, suggesting that H_2O_2 was responsible for enhancing HIF-1α levels. *In vivo*, DPI treatment decreased the growth of OVCAR-3 cell xenografts; adenoviral transduction of OVCAR-3 cells with catalase abolished H_2O_2 production and reduced tumor angiogenesis by 80%. Importantly, evaluation of ovarian tumor tissues has provided support for the relevance of NOX4 expression. Immunohistochemical staining of human ovarian tumors found that 63% expressed NOX4; however, no correlation could be demonstrated between NOX4 expression and tumor grade (45).

Recently, a role has been suggested for NOX4-related ROS in ovarian cancer metastasis and invasion. BLT-2, a low-affinity leukotriene B_4 receptor, plays a critical role in the metastasis of ovarian cancer cells through STAT3 activation and related upregulation of matrix metalloproteinase 2 (MMP2). BLT-2 is highly expressed in the ovarian cancer cell lines OVCAR-3 and SKOV-3; when NOX4 siRNAs were transfected into these cells, STAT3 activation and MMP2 expression were diminished, decreasing the invasive properties of the cells. In SKOV-3 xenografts, treatment with a BLT-2 inhibitor significantly reduced the number of metastatic nodules by 60% (120).

NOX5 (prostate cancer, hairy cell leukemia, and esophageal cancer). Discovery of a unique, calcium-regulated

NOX protein was first reported in 2001 from mRNA transcripts in the human spleen, testis, and kidney (9, 23). Currently, there are six identified human splice variants of the NOX5 enzyme (α, β, γ, δ, ζ, and a truncated variant NOX5-S or ε); isoforms α and β are the most abundantly expressed in cells, and have been established as functional superoxide generating enzymes. Interestingly, in esophageal cancer cells, NOX5-S is the dominant isoform. Reports of the activity level of this N-terminal truncation, devoid of calcium stimulatory ability, have varied between nonfunctionality and basal level ROS production (9, 38, 104, 125). For the remaining NOX isoforms, γ, δ, and ζ activity has not yet been reported (104).

Studies on the relevance of NOX5 in tumor biology have been limited, in part, because of the absence of NOX5 in the genome of rodents and a lack of reliable immunological tools. The first report of the possible involvement of NOX5 in cancer was in 2003, where Brar *et al.* implicated NOX5 in the regulation of the growth and apoptosis of DU145 prostate cancer cells (16). However, three independent studies report the absence of detectable NOX5 mRNA in DU145 cells; thus, questions arise regarding the relevance of NOX5 in the proliferation of this cell line (10, 56, 58). In contrast, significant levels of NOX5 have been reported in PC-3 human prostate cancer cells. Detection of NOX5 in PC-3 cells coupled with the observation that NOX5 expression is upregulated by sterol regulatory element-binding protein-1 both *in vitro* and *in vivo* in the context of prostate cancer progression, does implicate a proliferative role for NOX5 in prostate cancer (54). There have been two other detailed studies of NOX5 in the context of tumor biology; in 2005, the expression of NOX5 in hairy cell leukemia was reported to regulate constitutive phosphorylation signals mediating proliferation, and in 2006, acid-induced NOX5-S expression was reported to contribute to increased cell proliferation and decreased apoptosis in Barrett's esophageal adenocarcinoma cells (38, 61).

Although there is a growing base of information regarding NOX5 regulation, signaling, and various biological functions, the role of NOX5-generated ROS in tumor biology is still largely unexplored (12). Recently, advances have been made in the development of a validated mouse monoclonal antibody against NOX5 and the characterization of NOX5 expression in human tumors and tumor cell lines (4). Raised against a truncated recombinant NOX5 protein (amino acids 600–746), sequence conservation over the antigenic C-terminal region provided a novel antibody that detects all isoforms of the NOX5 protein. Analysis of human tumor microarrays by immunohistochemistry with this tool has revealed high levels of NOX5 expression, with a frequency of intermediate to strong expression of 57–70% for several human tumors evaluated, including prostate and ovarian cancers. Although the relevance of NOX5 in tumors still remains unclear, the detection of elevated expression of NOX5 in tumor specimens provides an impetus for further exploration of the functional significance of NOX5 in the context of cancer development and progression.

DUOX1/2 (thyroid, lung, and pancreatic cancer). H_2O_2-generating enzymes, DUOX1 and DUOX2, coexist in thyroid tissue, where they were first discovered to provide ROS for oxidation of iodine (by way of thyroid peroxidase [TPO]). Oxidized iodine is, in turn, covalently linked to available tyrosine residues in thyroglobulin, subsequently crosslinking tyrosines to form the thyroid hormone (both T_3 and T_4) (99). Analysis of DUOX expression in the thyroid has demonstrated that localization occurs at the apical membrane, with positive staining for DUOX in 40–60% of thyrocytes (20).

Studies of the role of DUOXs in carcinogenesis began when isolated thyroid carcinomas were first analyzed by RT-PCR for variation in DUOX gene expression by Lacroix *et al.* in 2001 (71). Analysis of both DUOX isoforms demonstrated no distinct trend toward gene upregulation or downregulation *versus* normal tissue. TPO, on the other hand, was observed to be downregulated in nine of ten cancers compared to matched normal tissues. Analysis at the protein level was more compelling; immunohistochemical staining of 86 primary carcinomas revealed positive DUOX expression in 67 (78%). In 29 of the samples with a high number of positive follicular cells, DUOX immunostaining was found at both the apical membrane and in the cytoplasm. This shift in protein distribution implies improper targeting or destabilization of the DUOX enzyme. The DUOX protein was found in 43 of 50 primary tumors with metastases (86%), and radioiodine uptake was observed for 37 of these 43 DUOX-positive tumors. H_2O_2 exposure from DUOX expression in thyroid cancer cells may enhance DNA damage, contributing to genetic instability in thyroid cancer (26).

The two DUOX isoforms demonstrate 83% homology in amino acid sequence; this has contributed to the inability to generate a monoclonal isoform-specific antibody. DUOX polyclonal isoform-specific antibodies were first reported in 2009 (83). Thus, the aforementioned immunohistochemical studies measured both DUOX1 and DUOX2, considering that both isoforms are expressed at significant levels in the normal thyroid. Evidence suggests that DUOX2 may provide most thyrocyte oxidase activity, because *DUOX2* mono- or biallelic inactivating mutation(s) are linked to congenital hypothyroidism, a phenotype not rescued by DUOX1 (96, 101, 109, 138). However, DUOX1 is the main H_2O_2 source in the rat thyroid line PCCl3, suggesting that factors such as differential cellular localization or alternate activation mechanisms may prohibit DUOX1 substituting for DUOX2 in the human thyroid (113). Indeed, *in vitro* studies have shown that DUOX isoforms are differentially regulated by separate phosphorylation pathways; DUOX1 is activated by protein kinase A (G_s-PKA pathway), whereas DUOX2 is stimulated by protein kinase C (G_q-phospholipase C pathway) (114). Recent insights into DUOX transcriptional regulation have also favored DUOX2 through upregulation under inflammatory conditions. Raad *et al.* demonstrated that both *DUOX2* and *DUOXA2* genes were upregulated upon thyrocyte exposure to T helper (Th) 2 cytokines IL-4/IL-13, an effect that correlated with an increase in protein and extracellular H_2O_2 production (111). No effect on *DUOX1/DUOXA1* was found. Therefore, while the Lacroix study was limited to demonstrating a relationship between DUOX expression and thyroid cancer, it seems likely that human cancer-related effects from ROS production may relate predominantly to DUOX2, although a contribution from DUOX1 cannot be ruled out.

As the dominant isoform expressed in the airway epithelium, DUOX1 has been linked to lung development, differentiation, mucus production, and host defense. DUOX2 is also present in lung epithelial cells, but at a significantly lower level of mRNA expression than DUOX1 (37, 134). These expression levels were perturbed upon treatment with Th1

(interferon-gamma [IFN-γ]) and Th2 (IL-4/IL-13) cytokines, with modest DUOX1 (Th2 related) and significant DUOX2 (Th1 related) mRNA increases, validated at the protein level by H_2O_2 production assay (50). DUOXs localize to the leading edge of migrating cells as functional calcium-activated complexes with a distinct distribution on the apical side of a differentiated lung epithelium. Overexpression of DUOX1-DUOXA1 in DUOX-deficient NCI-H661 lung cancer cells revealed that DUOX1 is present at the edge of plasma membrane protrusions (colocalized with DUOXA1). Expression of DUOX2 and its partner maturation factor DUOXA2 in the same cells led to enzymatic localization at the ER and intracellular structures (trafficking vesicles), with infrequent presentation at the plasma membrane (83).

Investigation of lung cancer cell lines and primary tumors demonstrated a consistent loss of DUOX gene expression. Whereas abundant in normal or immortalized lung epithelial cells, DUOX1 expression was lost in 75% of lung cancer cell lines screened, including A549, NCI-H661, NCI-H157, NCI-H441, and SHP-77 cells (82). DUOX2, DUOXA1, and DUOXA2 expression levels were also decreased from those in normal cells; DUOX2 and DUOXA2 are, however, routinely expressed at significantly lower levels in the lung than DUOX1. Recovery of DUOX1 expression was increased by treatment with the DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine in several lung cancer cell lines, suggesting that *DUOX* gene silencing is regulated through aberrant promoter hypermethylation. This observation is consistent with the presence of CpG islands, sites for potential methylation, in the promoters of both the *DUOX1* and *DUOX2* genes. Methylation was confirmed through bisulfate sequencing, at levels ranging from dense *DUOX1* promoter hypermethylation in A549 and NCI-H661 cells, to 30–35% coverage of CpG sites in NCI-H157 cells; normal primary lung epithelial cells demonstrated insignificant methylation. Modest methylation was also noted for the *DUOX2* promoter and was highest in A549 cells. These results were translated to primary tumors derived from non-small-cell lung cancer patients. Real-time PCR with DUOX-specific primers revealed a statistically significant reduction of *DUOX1* expression in tumor tissue *versus* normal lung in 9 of 11 patients. Of these tumors, five that demonstrated significant *DUOX1* downregulation were chosen for further study; promoter hypermethylation was detected in all five cases. Stable expression of the DUOX1 and DUOXA1 mRNAs in A549 and NCI-H157 lung cancer cells permitted calcium-dependent H_2O_2 production, which promoted greater cell migration, but showed no effect on proliferation. Because DUOX1 has been linked to airway host defense and wound healing, silencing of this gene could increase inflammation-related pre-neoplastic tissue injury (often triggered by inhalation of foreign substances) (140).

Recent studies have suggested that DUOX enzymes may be involved in the etiology of pancreatic cancer. Pancreatic inflammation plays a critical role in the development and progression of pancreatic tumors; experimentally, pancreatic cancer cells generate higher concentrations of ROS than respective normal cells (35, 48, 75, 88, 150). IFN-γ, a soluble proinflammatory cytokine, stimulates ROS production in human neutrophils and macrophages by upregulation of $gp91^{phox}$ and $p67^{phox}$ NADPH oxidase components (70). IFN-γ dramatically increased both DUOX2 and DUOXA2 mRNA expression in BxPC-3 and AsPC-1 pancreatic cancer cell lines without altering the expression of other NADPH oxidase family members. A newly characterized DUOX monoclonal antibody, developed against a truncated peroxidase-like domain antigen, confirmed that this increase in gene expression translated to the protein level (145). Both intra- and extracellular calcium-dependent ROS production followed IFN-γ-related increases in DUOX2-DUOXA2 expression, suggesting that a fully functional DUOX2-DUOXA2 enzymatic complex was formed following IFN-γ exposure. Enhanced DUOX2 expression was demonstrated to be the result of Stat-1 binding to a putative Stat recognition site in the DUOX2 promoter.

The role of Toll-like receptor 4 (TLR4), a known target of bacterial lipopolysaccharide (LPS), in the upregulation of DUOX2 in pancreatic cancer cells was evaluated in a subsequent study (147). Exposure to both IFN-γ and LPS synergistically upregulated the expression of DUOX2 and DUOXA2 in BxPC-3 cells, and increased intra- and extracellular H_2O_2 production by way of TLR4. DUOX2 expression was also markedly upregulated in BxPC-3 cell xenografts in the absence of cytokine treatment, presumably as a result of the proinflammatory milieu of the tumor microenvironment. Patients with chronic pancreatitis were also found to have increased DUOX expression adjacent to areas of inflammatory cell infiltrates by immunohistochemistry, further supporting a role for inflammatory stimuli in the regulation of DUOX2 expression (147).

Immunohistochemical examination of a human tumor panel in related studies has found expression of DUOX to be highest in prostate adenocarcinomas; greater than 60% of breast and colon cancers also demonstrated high level DUOX expression (strong + intermediate staining, Fig. 4) (145). Expression of DUOX2 was observed to be significantly increased in human colon biopsies and isolated intestinal epithelial cells from patients with chronic inflammatory conditions that are predisposed to cancer, Crohn's disease, and ulcerative colitis, compared to adjacent normal colon mucosa (80). DUOX2 is also upregulated 10-fold in premalignant adenomatous polyps *versus* adjacent mucosa by RNA microarray analysis as well as in some surgically resected colon cancers (58, 64). These recent observations in human tumors suggest that further study of DUOX2 in colon adenocarcinoma is warranted.

Bioinformatics tools for the study of NOX family members in cancer. In the context of cancer biology, where prohibitive cost may preclude many laboratories from pursuing *in vivo* studies, bioinformatics tools provide access to gene expression profiles and patient-specific tumor data that would otherwise be unavailable (49, 93, 119, 137). Large-scale clinical data sets, coupled with high-throughput analytical techniques, can also afford a perspective focusing on gene networks or pathways rather than individual genes (97). Evaluation of difficult biological systems, where lack of specific antibodies, subtle tumor *versus* normal tissue gene expression variations, and broad tissue distribution are hindrances, may greatly benefit from large-scale screening and predictive tools to define *in vivo* relevance. Hence, current bioinformatics platforms may advance NOX/DUOX research through insights drawn regarding gene expression in both cancer cell lines and human tumors. Outlined below are databases that have provided some initial insights regarding NOX/DUOX enzymes in oncology.

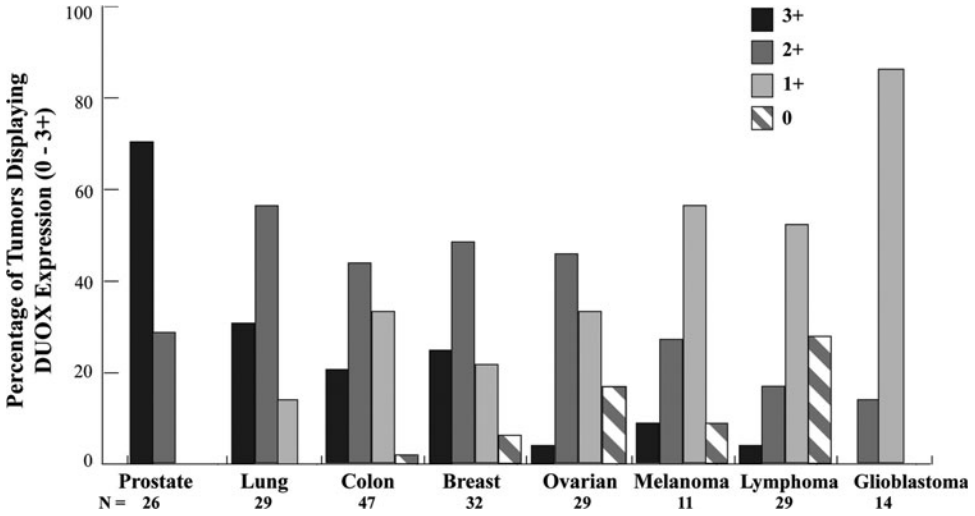


FIG. 4. Distribution of DUOX expression levels in tumors from a multitumor tissue microarray (TARP MTA-3). DUOX expression was studied by immunohistochemistry in 217 tumor samples as well as negative control tissues. Staining of DUOX protein occurred in a cytoplasmic pattern and was scored on a scale from 0 to 3 as follows: negative stain (0, stripes), weak staining (1+, light gray), intermediate staining (2+, dark gray), and strong staining (3+, black). Bar graphs depict the staining percentage achieved for each tumor type at each expression level, with the total number of samples (*N*) evaluated for each cancer listed. DUOX expression was weakly positive in normal bone marrow, pancreas, and stomach tissues, while negative in other normal tissues evaluated, including the brain, liver, lung, small bowel, and testis. Distribution of positive staining was statistically different between tumor types, $p < 0.01$.

The Cancer Cell Line Encyclopedia (www.broadinstitute.org/ccle)

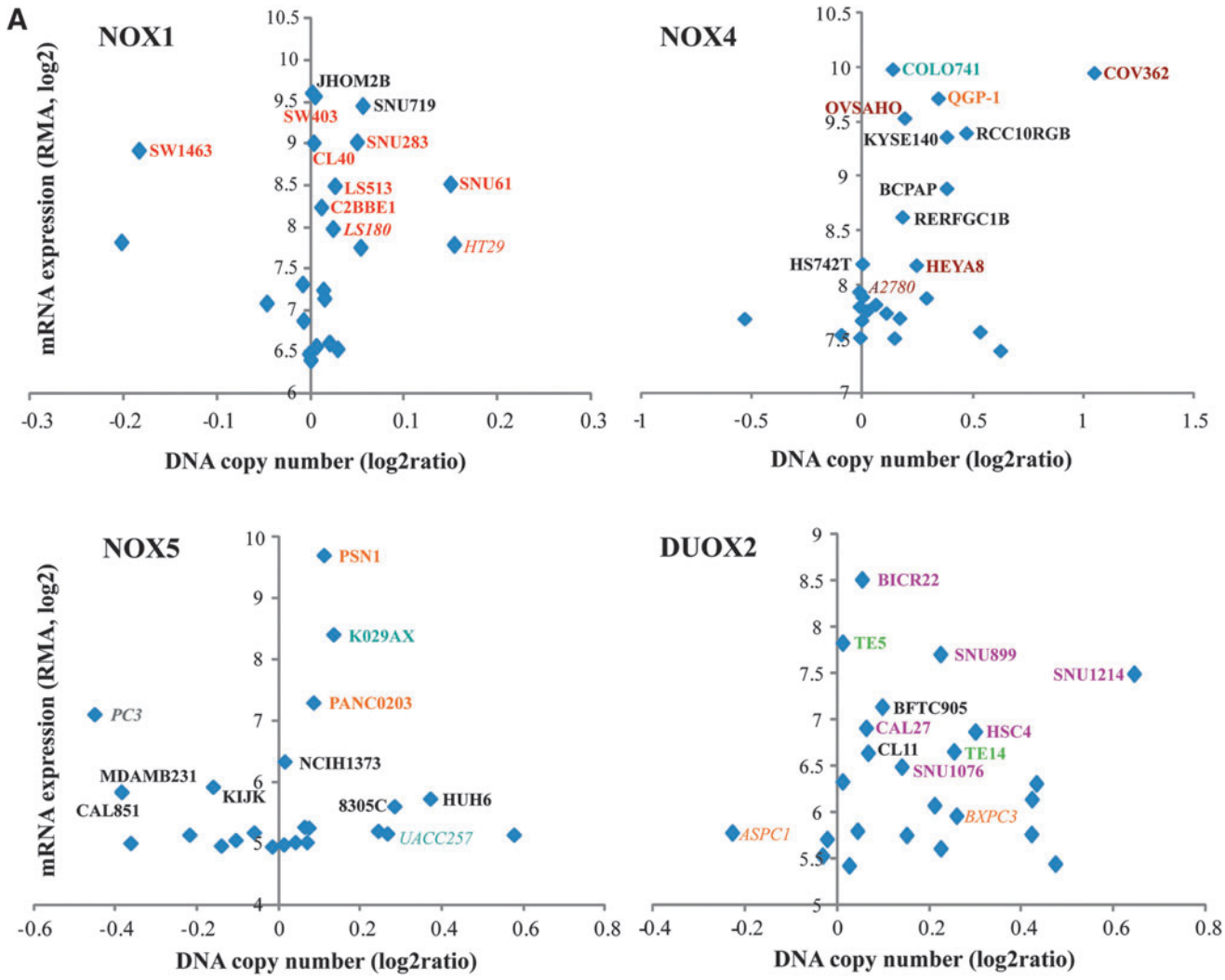
A large-scale genomic dataset for 1047 human cancer cell lines from 36 tumor types, together with pharmacologic profiling of tumor cell line sensitivity for 24 anticancer compounds, was produced by the Broad Institute in collaboration with the Novartis Institutes for Biomedical Research (10). As an ongoing project, the collection of gene expression, chromosomal copy number, and parallel sequencing data is constantly growing.

Analysis of this database for the NOX enzymes resulted in profiles for NOX1, NOX4, NOX5, and DUOX2, relating mRNA expression levels to specific cancer cell lines (Fig. 5). The top 25 cell lines with the highest gene expression for each protein were graphed; the names for the top 10 cell lines, plus literature relevant examples, are displayed. Color association by tumor type helps to illustrate correlations to specific NOX family members; for NOX1, 8 of 10 cancer cell lines with the highest expression levels are from the colon, while 6 of the top DUOX2 cell lines are head and neck cancers. Correlation of several cell lines to published expression values is also demonstrated; HT-29 cells have significant NOX1 mRNA, and have been studied extensively at the protein level and in xenograft models, while A2780 cells possess high NOX4 mRNA expression levels relative to normal ovarian cells and demonstrate increased ROS production (59, 148). These investigative observations aid in validating the information available from this database; however, no direct estimate of the translation of mRNA to protein can be drawn. This must be evaluated on a case by case basis. Interestingly, two cell lines of questionable oxidase-specific cancer relevance, DU145 (prostate cancer and NOX5) and U251 (GBM and NOX4) were not found by the Cancer Cell Line Encyclopedia (CCLE) screen to have significant levels of either NOX protein (Fig. 5) (16, 53).

Oncomine (www.oncomine.org)

Originally conceived by scientists at the University of Michigan, Oncomine was first released in 2003, with 40 microarray data sets. As a cancer microarray database, the latest version, Oncomine 4.4.3, provides registered users from the academic and nonprofit cancer research communities free access to data from over 600 gene expression datasets (99, 100). This resource acts as a hub for cell line data from the CCLE (917 cell lines), and the Sanger Institute (www.sanger.ac.uk/, 732 cell lines), and provides the ability to survey over 10,000 gene expression and DNA copy number sets from The Cancer Genome Atlas project. Differential expression analyses comparing many major cancer types to respective normal tissues, as well as clinical outcomes, pathology subtype (cancer stage), and drug sensitivity data are available for exploration. Data can be queried and visualized for a specific gene or multiple genes across a variety of selected analyses. This bioinformatics tool has been successfully used to produce data on numerous cancers, including colon, breast, and prostate adenocarcinomas (11, 47, 90, 128).

To illustrate the value of the Oncomine database for the study of NADPH oxidases in cancer, differential expression analyses for the H_2O_2 producing members of the NOX/DUOX family, NOX4, DUOX1 and DUOX2, were performed for cancer *versus* normal tissues from microarray datasets of primary tumors (Fig. 6). NOX4 was found to be upregulated in nine of the ten cancer types profiled. Interestingly, NOX4 was significantly upregulated in three of the five gastric cancer analyses, a tumor type that has not been previously associated with this NOX isoform; NOX4 was downregulated in 7 of 16 renal adenocarcinoma analyses, whereas RCC cell line-based studies have demonstrated upregulation (15). This suggests that critical differences may exist between the *in vitro* cell line and *in vivo* tissue studies in the context of RCC. A



B

Cell Line	NOX1	NOX2	NOX3	NOX4	NOX5	DUOX1	DUOX2
A2780	12*	6	13	**6505**	0	0	2
ASPC1	4	81	0	0	0	39	**1159**
BXPC3	65	16	0	0	1	532	**2271**
HT29	**15429**	1167	8	9	1	15	1
LS180	**6050**	107	8	0	0	1	0
PC3	8	11	8	0	**18873**	0	0
DU145	22	477	10	5	93	496	137
U251	8	116	0	0	43	6	5

*Table values normalized to 18S rRNA expression ($\times 10^{-8}$)

FIG. 5. Comparison of mRNA expression levels for NOX/DUOX enzymes in cell lines acquired and analyzed by the Broad Institute CCLE. (A) The top 25 cell lines showing the highest expression levels of NOX1, NOX2, NOX5, and DUOX2 were graphically presented based on mRNA values, with the names of the top 10 cell lines for each enzyme displayed (bold). The cell names are further highlighted by a color code to portray the pattern of cancer types associated with each enzyme. Cancer cell lines listed in italics were evaluated by quantitative polymerase chain reaction for the mRNA levels of NOX and DUOX proteins **(B)** and/or have been listed as a recommended cell line based on precedent literature (Table 2), demonstrating consistency with the CCLE obtained data [(58) and unpublished results]. U251 and DU145 human tumor lines are highlighted in **(B)** (underline), demonstrating a correlation with the CCLE results and disparity with the literature, as negligible levels of NOX4 and NOX5 RNA were measured. CCLE, Cancer Cell Line Encyclopedia.

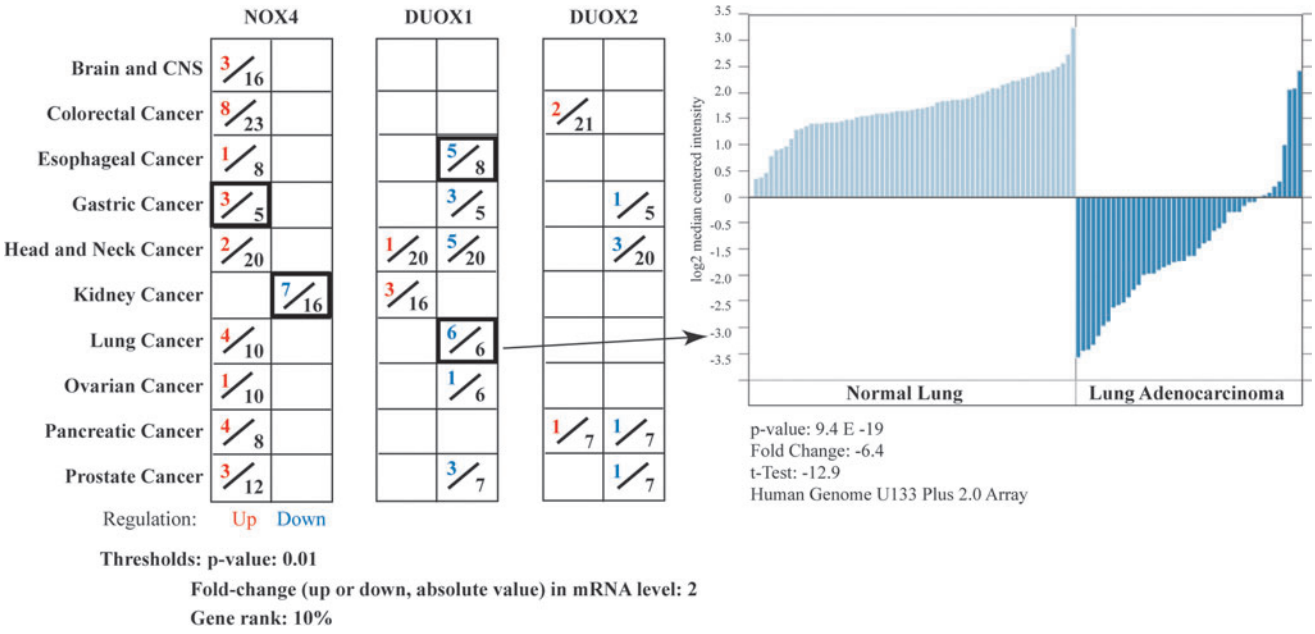


FIG. 6. Comparison of analyses of mRNA expression values for cancer *versus* normal samples of NOX4, DUOX1 and DUOX2 obtained from the Oncomine database. The hydrogen peroxide converting members of the NOX/DUOX family are profiled, with the number of analyses of cancer *versus* normal tissue demonstrating significant gene upregulation (red) or downregulation (blue) out of total analyses (black) that met the threshold criteria shown across 10 cancer types. One analysis set for DUOX1 expression in lung adenocarcinoma tissue is highlighted [215800_at, (52)], which demonstrates the downregulation of DUOX1 in lung adenocarcinoma.

trend for DUOX1 downregulation across analyzed tumor types is supportive of the initial epigenetic study of DUOX in lung cancer (82); significant downregulation in both esophageal and lung cancers was noted. One of the six sets of analyses for lung cancer is highlighted in Figure 6, representative of the data available from individual analyses of each tumor type (52). No significant datasets or distinct trend in regulation was found relating DUOX2 expression to cancer under the chosen thresholds.

Conclusions

Research into the biochemical role of NADPH oxidases in the initiation and progression of human cancers is in its

Table 2. Suggested *In Vitro* and *In Vivo* Models for Study of hNOX Isoforms in Tumor Biology

Tumor	*Cell line(s)*	*Xenograft(s)*	*NOX/DUOX proteins*	*References*
Glioblastoma multiforme	U87 KNS81 KNS42		NOX4	(124)
Colon cancer	HT-29[a] LS174T[a] Caco-2[a]	HT-29 LS-174T	NOX1, DUOX2	(145–147)
Lung cancer	NCI-H661 NCI-H157		DUOX1, DUOX2	(82)
Melanoma	UACC257[a] MM-BP	MM-BP A375	NOX4, NOX5	(4, 55, 149)
Ovarian cancer	OVCAR-3[b] A2780[b] SKOV-3	OVCAR-3	NOX2, NOX4	(45, 120, 148)
Pancreatic cancer	BxPC-3[a] AsPC-1[a] CFPAC-1[a]	BxPC-3 AsPC-1	DUOX2	(146, 147)
Renal cell carcinoma	786-O RCC4 KPK13	786-O	NOX2, NOX4	(15, 36, 123)

[a]Validated by our laboratory at the RNA and protein levels.
[b]Validated by our laboratory at the RNA level; validated at the protein level in the literature.
PDI, protein disulfide isomerase; RCC, renal cell carcinoma.

earliest stages. Hindered by a lack of available, well-qualified antibodies and *in vivo* models, as well as crystal structures for targeted inhibitor development, *in vitro* studies have dominated the field. Definition of *in vivo* relevance should be the focus of future research efforts, coupled with the refinement of reliable *in vitro* systems (Table 2). Recent studies of the tumor microenvironment have implicated oxidative stress-related chronic inflammation as a mediator of tumorgenesis, linking cancer to the upregulation of several NADPH oxidases. Further validation of the NOX/inflammation/cancer connection through investigation of DNA damage and studies of gene networks should be pursued, aided by bioinformatics strategies.

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Abbreviations Used

CCLE = Cancer Cell Line Encyclopedia
DPI = diphenylene iodonium
DUOX = dual oxidase
H_2O_2 = hydrogen peroxide
HIF-1α = hypoxia inducible factor 1-alpha
IFN-gamma = interferon-γ
IL = interleukin
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
MMP2 = matrix metalloproteinase 2
NF-κB = nuclear factor-κB
PDI = protein disulfide isomerase
RCC = renal cell carcinoma
ROS = reactive oxygen species
SIRT1 = sirtuin 1
SOD = superoxide dismutase
TGFβ-1 = transforming growth factor β-1
Th = T-helper
Tks = tyrosine kinase substrate
TLR4 = Toll-like receptor 4
TPO = thyroid peroxidase
VHL = von Hippel-Lindau