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PARADOXICAL ROLES OF DUAL OXIDASES IN CANCER BIOLOGY

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Abstract

Dysregulated oxidative metabolism is a well-recognized aspect of cancer biology, and many therapeutic strategies are based on targeting cancers by altering cellular redox pathways. The NADPH oxidases (NOXes) present an important enzymatic source of biological oxidants, and the expression and activation of several NOX isoforms are frequently dysregulated in many cancers. Cell-based studies have demonstrated a role for several NOX isozymes in controlling cell proliferation and/or cell migration, further supporting a potential contributing role for NOX in promoting cancer. While various NOX isoforms are often upregulated in cancers, paradoxical recent findings indicate that dual oxidases (DUOXes), normally prominently expressed in epithelial lineages, are frequently suppressed in epithelial-derived cancers by epigenetic mechanisms, although the functional relevance of such DUOX silencing has remained unclear. This review will briefly summarize our current understanding regarding the importance of reactive oxygen species (ROS) and NOXes in cancer biology, and focus on recent observations indicating the unique and seemingly opposing roles of DUOX enzymes in cancer biology. We will discuss current knowledge regarding the functional properties of DUOX, and recent studies highlighting mechanistic consequences of DUOX1 loss in lung cancer, and its consequences for tumor invasiveness and current anticancer therapy. Finally, we will also discuss potentially unique roles for the DUOX maturation factors. Overall, a better understanding of mechanisms that regulate DUOX and the functional consequences of DUOX silencing in cancer may offer valuable new diagnostic insights and novel therapeutic opportunities.

Graphical abstract

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Keywords

NOX; DUOX; NADPH oxidase; reactive oxygen species; epithelial; EGFR; epigenetics

Introduction

Oxidative stress is typically defined as an imbalance between reactive oxygen species (ROS) production and the cellular response(s) to counteract their actions (Fig. 1). Originally considered a simple by-product of mitochondrial respiration and other metabolic pathways, it is now well understood that ROS can be generated by various endogenous cellular sources in a regulated fashion. Chief among these are the NADPH oxidases (NOXes), a family of enzyme complexes (NOX1–5 and DUOX1/2) that generate ROS (O_2^- and/or H_2O_2) as their primary product of enzymatic activity[1, 2], implicating the biological importance of ROS. During the past decade, extensive research has implicated ROS in a variety of cellular signaling pathways involved in cell proliferation, migration, and differentiation[3–5], and altered ROS production or metabolism has been demonstrated in a variety of human pathologies such as aging[6], diabetes[7], atherosclerosis[8], and cancer[9–11]. Indeed, cancer cells are typically characterized by elevated amounts of ROS, and various lines of evidence indicate their ability to contribute to cancer progression[9, 10, 12]. The contribution of ROS in cancer is clearly very complex, and likely occurs at several levels. For instance, ROS can contribute to genomic instability by inducing direct oxidative damage to DNA[10] or by promoting lipid peroxidation to generate mutagenic byproducts such as malondialdehyde or 4-hydroxynonenal, which can adduct DNA[10, 13] (Fig. 2A,B). Furthermore, ROS are capable of activating or inactivating cell signaling pathways that control proliferation, migration, differentiation, or survival, by inducing specific redox-mediated post-translational modifications in redox-sensitive targets such as protein kinases or phosphatases[13, 14] (Fig. 2C).

Because of the presence of elevated ROS in cancer cells, which likely contributes to imbalanced redox-dependent cellular signaling, antioxidant approaches to minimize cellular ROS have been proposed as supplementation to chemotherapy regimens, such as vitamin C and E[15, 16], *N*-acetyl cysteine (NAC)[15, 17], and glutathione[18–22]. However, clinical trials using antioxidant-based approaches have shown varying degrees of success, and in some cases have contributed to worse outcomes[9]. Indeed, recent studies in animal models of e.g. lung cancer or melanoma demonstrated that antioxidant supplementation (NAC, Trolox) can actually promote tumor progression and metastasis[23, 24]. Opposite strategies that are also being explored are approaches to either inhibit antioxidant defense or enhance ROS generation within cancer cells, to levels that exceed their adaptive capacity and thus induce cancer cell death[25, 26]. In fact, the antioxidant vitamin C may paradoxically act as

a pro-oxidant, likely due to extracellular conversion to dehydroascorbic acid (DHA), thereby inducing the generation of H_2O_2 [27]. High doses of intravenous vitamin C were found to significantly reduced levels of prostate-specific antigen (PSA), an appreciated serum biomarker for prostate cancer[28]. Pro-oxidant effects of DHA have also been explored in non-small cell lung cancer (NSCLC) and glioblastoma (GBM), where H_2O_2 induced by the extracellular conversion of ascorbic acid to DHA, selectively disrupted iron metabolism in cancer cells, leading to cytotoxic effects, and its efficacy has been demonstrated in preclinical and clinical trials when paired with standard radiation and chemotherapy regimens[29].

Beyond directly targeting ROS with antioxidants as a therapeutic strategy, alternative approaches involve strategies to inhibit major cellular sources of ROS production. The largest cellular source of ROS in cancer cells may be mitochondria[30], due to glycolytic reprogramming and upregulation of glucose metabolism leading to loss of regulation between glycolysis and aerobic respiration[31–34]. Such altered mitochondrial metabolism in cancer cells, known as the Warburg effect, is thought to lead to enhanced steady-state levels of intracellular ROS which in turn may drive enhanced addiction to glucose to increase glycolysis and pentose phosphate cycle activity to provide reducing equivalents for hydroperoxide metabolism[31]. Indeed, enhanced mitochondrial-derived ROS has been implicated in pathways promoting oncogenic cell behavior[33, 35], and several drugs that suppress mitochondrial electron transport and ROS production (e.g. complex 1 inhibitors (metformin)[36], complex III inhibitors[37], others) have been utilized recently to treat cancer. Hence, alterations in mitochondrial dynamics, function, and regulation in cancer cells may position them as attractive targets for therapeutic approaches, although they may also have untoward consequences for normal cell biology.

Although mitochondrial ROS can contribute to redox-dependent cell signaling[38, 39], the main sources of ROS involved in redox regulation of cell signaling pathways are thought to be the NADPH oxidases (NOXes). Based on growing numbers of studies indicating altered expression or activation of NOX family members in various cancers[13], they are increasingly considered as additional attractive targets for chemotherapeutic development in cancer[40, 41]. For example, NOX1 and 4 have been implicated for their contributions to cancer, based on their roles in invadopodia formation[42] and tumorigenesis/angiogenesis[43], through redox-dependent activation of signaling pathways such as Akt and NF- κ B [10, 13, 40, 41]. It is also worth noting that emerging evidence for the existence of cross-talk mechanisms between NOX enzymes and mitochondria[44, 45] may imply that altered expression or activation of NOX enzymes may contribute to alterations in mitochondrial metabolism or, conversely, that altered mitochondrial metabolism in cancer cells may contribute to dysregulated NOX expression or activation. However, to our knowledge, the significance of such NOX-mitochondrial interactions for cancer biology has not been explicitly explored. Importantly, there is a wide variance of NOX expression and distribution across different cancers, which suggests that the contributions of NOX-derived ROS may be highly variable[46]. Moreover, as will be discussed in more detail in this review, some NOX homologs also appear to be downregulated in cancer cells, primarily due to epigenetic mechanisms [47]. This applies in particular to the dual oxidases DUOX1 and DUOX2, which are normally expressed in the thyroid and in epithelia of mucosal

tissues[48–51], and are frequently suppressed in many epithelia-derived cancers[52–54], primarily through hypermethylation of its promoter[13, 52]. This review will first briefly discuss the known contributions of NOX enzymes to cancer biology and then focus specifically on the biological functions of the DUOX proteins in epithelial biology, and the functional implications of altered expression of DUOX1 and DUOX2 in various cancers.

NOX enzymes and Cancer: A Brief Overview

The first NADPH oxidase to be described is the phagocyte oxidase flavocytochrome b_{558} of which the key transmembrane heme-containing protein, gp91^{phox}, was renamed NOX2 after discovery of six other mammalian homologues, NOX1, NOX3, NOX4 and NOX5, and the dual oxidases DUOX1 and DUOX2[10]. All NOX enzymes are transmembrane proteins that contain a C-terminus consisting of FAD and NADPH binding domains, and 6 transmembrane-spanning helical sequences containing two bis-his heme ligated by histidines located in helices III and V, which facilitate the intramolecular electron transfer from NADPH to O₂ to generate superoxide (O₂^{•-})[1, 55]. Cellular distribution of the NOXes varies widely, and include localization at the plasma membrane but also in other intracellular membranes in ER, mitochondria, nuclei, etc., largely dependent on cell type[13, 56]. NOX1–3 each require assembly with various cytosolic cofactors for activation, while NOX4 only requires p22phox and is thought to be constitutively active [57]. In contrast, NOX5 and DUOX1 and DUOX2 are activated by Ca²⁺ and do not require other cofactors[57]. NOX proteins were initially appreciated for their roles in host defense[1, 58] but, since their discovery in non-phagocytic cell types, have gained recognition for their participation in other cellular processes, such as cell survival[59, 60], migration[61], proliferation[59, 62], and differentiation[63]. Dysregulated expression and/or activation of NOX enzymes may therefore play a pivotal role in cancer cell behavior.

Table 1 provides a brief summary of presently established involvement of NOX enzymes in various cancers. The first NOX isoform that was linked to cancer biology is NOX1, which was originally identified as Mox1 and found to be capable of inducing cellular transformation and tumor growth [64]. NOX1 is primarily expressed in the colon, and was found to be overexpressed in colon cancers[65, 66], and also in radiation induced skin cancer[67], breast cancer [68], and oral squamous cell carcinoma[69]. Additionally, a growing number of studies have focused on a role for NOX4 in cancer biology. A recent meta-analysis and systematic review of the association between NOXes and lung cancer defined that overall NOX expression and activity significantly attributed to the tumorigenesis of lung cancer, and more specifically, NOX4, suggesting strategies to suppress it could have potential therapeutic benefits[70]. NOX1 and 4 have been shown to contribute to angiogenesis, an important feature of tumor growth by promoting generation of new blood vessels and enhancing oxygen and nutrient supply, which may in part be associated with oxidation-dependent upregulation of vascular endothelial growth factor (VEGF)[13, 71]. In the latter case, the effects of NOX1/4 were attenuated by overexpression of catalase and glutathione peroxidase[71]. Several NOX enzymes have also been shown to play a role in cellular invasion and metastasis (NOX1/4), redox based control of the Src kinase (NOX1,2, and 4) and/or NF-κB signaling (NOX1)[13]. Additionally, in lung and breast epithelial cells, expression of oncogenic mutant p53, but not WT-p53, upregulates NOX4 expression, ROS

generation, and cell migration[72]. Activation of NOX4 has also been described to promote pancreatic ductal adenocarcinoma (PDAC) cell growth by oxidation of NADH and supporting increased glycolysis[73]. Recently, overexpression of NOX4 in colorectal cancer was found to be significantly associated with poor prognosis and tumor progression[74]. The lack of NOX5 from the rodent genome has limited our understanding of its biological role, but several recent studies have highlighted the potential importance of NOX5, based on findings of increased NOX5 expression and/or NOX5-derived ROS in various cancers, e.g. prostate [75, 76], hairy cell leukemia[77], esophageal [78], ALK lymphoma [79], and others[80, 81]. NOX2 has also been implicated in promoting the oncogenic phenotype in various cancers such as breast cancer[82], myelomonocytic leukemia[83], rectal cancer cells[84], prostate[85], and expression of NOX2 mRNA or protein was found to correlate with an invasive phenotype and poor prognosis in gastric cancer[86]. However, the explicit role for NOX-generated ROS in cancer-related outcomes is typically not demonstrated. There is almost no literature on NOX3, likely because of its highly limited distribution, but a recent study has linked NOX3 to development of hearing loss in cancer patients receiving cisplatin treatment[87].

Understanding the relevance of global changes in NOX expression and/or activity in cancer would also require insight into their subcellular localization which likely determine their distinct and/or unique roles in cancer biology. For example, NOX1/4 have been shown to localize to invadopodia and/or leading edges of invading cells, and RNAi mediated knockdown of NOX4 or strategies to inhibit global NOX activity effectively suppressed invadopodia formation[88–91]. NOX enzymes typically associate with complexes at the plasma membrane, e.g. in caveolae or invadopodia [92–95], and also can be internalized to endosomal compartments (e.g. NOX1/2[96, 97]) to enhance specificity in signaling[98]. Perturbed redox signaling due to altered NOX expression and/or activity therefore likely impacts on cell-cell communication, focal adhesions, or cellular migration [90, 92, 99]. NOX4 has also been detected in other cell compartments, including endoplasmic reticulum where it mediates oxidative inactivation of protein tyrosine phosphatase 1B (PTP1B) [100], or in mitochondria where it supports oncogenic features [101, 102], and in nuclei where it mediates O_2^- generation and thus likely contributes to oxidative DNA damage [103]. For example, in HRAS transformed thyroid cells, NOX4-derived H_2O_2 was found to enhance DNA replication rate as well as induce DNA damage[104]. NOX4 signaling in the nucleus could also have implications for regulation of redox sensitive transcription factors such as AP-1, NF- κ B, Nrf2, p53, among others, which have been implicated for their specific roles in cancer biology[99]. NOX4 localized to the mitochondria may impact on mitochondrial DNA, which is highly vulnerable to oxidative modification since mitochondria do not contain histones[13, 33].

Overall, although increased expression or activation of NOX enzymes may contribute to various aspects of cancer biology, the precise subcellular localization and contribution of the various NOXes is still incompletely understood, and published findings sometimes appear to be conflicting, likely due to the fact that they have diverse cell-specific actions in different cell types, which may have opposing consequences for tumor development[13, 14].

Specific Roles for DUOX1 and 2 in Epithelial Cancers

The most common type of cancer involves carcinomas that originate from epithelial cell types that are commonly exposed to environmental factors and carcinogens, such as in the lung, skin, or gastrointestinal tract. These mucosal surface cell types typically express high levels of unique NOX isoforms, the dual oxidases DUOX1 and/or DUOX2. Analogous to other NOXes, several studies have highlighted that DUOX2 mRNA and protein is often overexpressed in colorectal cancers[105, 106], pancreatic adenocarcinoma [107, 108], Barrett esophagus [105], and gastric cancers[105]. Using a newly developed monoclonal antibody against DUOX2, increased immunohistochemical staining was observed in various tumor types, including prostate, lung, breast, and colon [109]. These observations suggest that DUOX2-derived H_2O_2 plays an important role in carcinogenesis and tumor progression, which is supported by a recent report demonstrating that DUOX2 upregulation in colorectal cancer correlates with poor prognosis[110]. Analysis of data available from the NIH funded Tissue Cancer Genome Atlas Project (TCGA) (<http://cancergenome.nih.gov/>) further confirms that DUOX2 is overexpressed especially in colon and esophageal cancers, although expression levels are highly variable and DUOX2 is not significantly altered in other cancers (Fig. 3). Contrasting these findings, Knaus and colleagues originally reported that expression of both DUOX1 and DUOX2 is typically suppressed in lung cancer cell lines compared to normal epithelial cells [52], and subsequent studies confirmed that DUOX1 is frequently suppressed in both lung and hepatic cancers[13, 53, 111]. Analysis of TCGA data confirms that DUOX1 is commonly suppressed in various cancers, primarily those of lung and skin, compared to adjacent healthy tissues (Fig. 3). DUOX1 suppression in cancers was also confirmed by immunohistochemical (IHC) analyses, as reported by Luxen et al. [52] or data deposited in the Human Protein Atlas (<http://www.proteinatlas.org/ENSG00000137857-DUOX1/cancer>), which indicate reduced DUOX1 protein expression in many cancers compared to expression in corresponding healthy tissues. Further comparison of DUOX1 mRNA expression in non-small cell lung cancers (NSCLC, the vast majority of lung cancers) compared to corresponding matched normal lung tissues (from the TCGA repository) indicates that DUOX1 silencing is observed in both squamous cell carcinomas as well as adenocarcinomas (the 2 most frequent types of NSCLC), suggesting the potential broad relevance of DUOX1 silencing in cancer biology (Fig. 4). Indeed, ectopic expression of DUOX1 in liver cancers was found to correlate strongly with overall patient survival and thus was proposed as a marker for patient prognosis [53], suggests that DUOX1 silencing may be of similar importance in progression of others cancers including NSCLCs.

Observations of DUOX1 silencing in cancer are counterintuitive based on the common view that cancers are typically characterized by enhanced cellular ROS. Indeed, some studies have also suggested a contributing role for DUOX1-derived H_2O_2 in carcinogenesis. For example, ionizing radiation (IR), a strong risk factor for the development of thyroid cancer, was found to cause strong induction and activation of DUOX1 in thyrocytes and induction of genomic instability by DUOX1-derived H_2O_2 that was inhibited by pretreatment with catalase [112]. Moreover, patients with thyroid tumors that were previously exposed to IR displayed elevated DUOX1 mRNA expression and immunohistochemical DUOX1 staining compared corresponding normal thyroid tissues[112], suggesting that DUOX1 may be playing an

important role in radio-induced thyroid tumorigenesis[113]. DUOX1 as well as DUOX2 have also been detected in human neuroblastoma cells, which were attributed to ROS-mediated upregulation[114], although relevance for brain cancer is not clear. Finally, although DUOX1 is often suppressed in various cancers (Fig. 3), it was also found to be overexpressed in some colon and prostate cancers[115]. It is conceivable that DUOX1 may play different roles in cancer depending on the type and stage of cancer, with DUOX1 playing a potential contributing role in carcinogenesis (e.g. by mediating ROS-induced genomic instability) whereas DUOX1 silencing in established cancers may be more relevant for e.g. tumor progression or metastasis, thus explaining its association with poor prognosis in e.g. HCC [53].

Functional aspects of DUOX enzymes

To understand the potential functional consequences of DUOX1 silencing in epithelial cancers, it is important to first overview the known functional aspects of DUOX1 in epithelial biology. DUOX1 and 2 are highly homologous (sharing more than 85% sequence identity at the amino acid level) and both include an internal domain with two calcium binding EF-hands, an additional transmembrane spanning sequence and extracellular N-terminal peroxidase homology (PHD) domain. Sequence alignment of the two DUOX isoforms indicates relatively low sequence identity within regions of their PHD, EF-hand containing domains, and DUOX α -helical regions, suggesting that isoform-specific functions may largely depend on unique regulatory elements within these regions (Fig. 5). Activation of DUOX proteins to generate H_2O_2 as their primary product relies on Ca^{2+} signaling. Although DUOX enzymes do not require any additional co-factors for activation, other regulatory mechanisms have been identified [116, 117]. DUOX proteins are expressed primarily at the apical surface of differentiated epithelial cell lineages, which relies largely on the co-expression of DUOX maturation factors, DUOX1 and DUOX2[118–121], which control their transport from the endoplasmic reticulum to the plasma membrane, resulting in a mature enzymatic complex. As will be discussed later in this review, alternative roles for DUOX1 independent of DUOX2 have been described that may be relevant for cancer biology. Mammalian DUOX1 and 2 were first cloned from thyrocytes and were originally characterized as thyroid oxidase for its role in thyroid hormone synthesis[122, 123], but are now known to be more widely expressed in various epithelial tissues in the lung, pancreas, placenta, gastrointestinal tract, testis, liver, prostate, salivary glands, skin, and brain[53, 111, 124–126].

Based on analogous roles of phagocyte oxidase (NOX2), the main function of DUOX at mucosal surfaces (e.g. in the lung) is thought to be innate host defense against infection, by promoting direct luminal oxidative antimicrobial mechanisms catalyzed by locally secreted lactoperoxidase[48, 49, 127, 128]. A similar concerted action of DUOX2 with thyroperoxidase in thyrocytes is responsible for generation of thyroid hormone[50]. However, the precise isoform-specific roles for DUOX1 and DUOX2 in host defense mechanisms are less well understood, and current evidence most strongly supports a role for DUOX2 rather than DUOX1 in antimicrobial or antiviral defense in the airway[129], consistent with the high inducibility of DUOX2 in response to viral infection [130–132]. Interestingly, although both DUOXes are thought to depend on Ca^{2+} signaling, studies with

cultured airway epithelial cells indicate that basal extracellular H₂O₂ production under non-stimulated conditions primarily originates from DUOX2 [133, 134], whereas DUOX1 appears to be primarily responsible for enhanced H₂O₂ production in response to Ca²⁺-mobilizing stimuli[134]. This would suggest that DUOX2 possesses basal activity and is less strictly dependent on Ca²⁺ signaling.

Influx or intracellular mobilization of Ca²⁺ in response to diverse mechanical or biological stimuli commonly involve the initial cellular release of the cellular damage signal ATP[134–137], which mediates critical cell responses through activation ionotropic and metabotropic purinergic receptors at the cell surface, as a highly conserved cellular mechanism in response to injury and inflammation[135, 137, 138]. Based on the known importance of ATP-dependent purinoceptor signaling in epithelial wound responses, and the ability of ATP to activate DUOX1[133, 139], our group considered a potential role of DUOX1 epithelial wound responses, and discovered that DUOX1 is indeed important in mediating epithelial cell migration and induction of various wound response genes [134, 139, 140]. Subsequent studies also highlighted a role for epithelial DUOX1 in epithelial production of cytokines/chemokines [141–144] and neutrophil recruitment[142, 145, 146]. DUOX1 was also found to play a potential role in T-cell receptor signaling[147]. These various actions of DUOX1 are largely mediated by H₂O₂-dependent regulation of redox-dependent cell signaling pathways, through inactivation of protein tyrosine phosphatases[147] and/or direct oxidative activation of tyrosine kinases such as the non-receptor kinase Src and epidermal growth factor receptor (EGFR) [134, 142, 144, 146, 148]. Similar roles of DUOX in epidermal wound responses were subsequently also demonstrated in other organisms [149, 150], collectively highlighting the importance of DUOX1 in maintaining epithelial homeostasis. More recent studies also demonstrated the importance of DUOX1 in epithelial secretion of the alarmin IL-33 in response to airborne protease allergens or other non-microbial injurious stimuli[144]. Epithelial secretion of IL-33 forms an important component of type 2 immunity as a critical host defense mechanism against non-microbial triggers, such as parasites, allergens, etc, which is largely mediated by stimulation of mucus proteins and epithelial repair pathways to enhance resistance or tolerance to such non-microbial insults. Moreover, allergen-induced IL-33 secretion was also found to involve H₂O₂-mediated activation of the tyrosine kinases Src and EGFR[144]. Based on the notion that exaggerated type 2 responses can contribute to development of allergic diseases such as asthma[144], our group subsequently observed that allergic asthma is indeed associated with enhanced airway DUOX1 expression and that DUOX1 contributes to various critical features of asthma pathology[146].

Mechanisms of DUOX Regulation

The transcriptional or post-transcriptional mechanisms that regulate DUOX expression are still poorly defined. Much of our understanding of DUOX transcriptional regulation comes from organisms that only harbor one DUOX gene[1, 151], such as *Drosophila melanogaster*, which highlighted transcriptional regulation of DUOX by elements of the MAPK pathway[152]. The two mammalian DUOX genes and corresponding DUOXA genes (which encode for specific partner proteins, also known as DUOX maturation factors) are oriented in a head-to-head fashion, in tandem on chromosome 15[116]. Upon initial discovery of

DUOX within the thyroid and its participation in thyrocyte differentiation, it was hypothesized that the DUOX genes were controlled by thyroid-specific transcription factors (TTF-1, Pax8), but this was found not to be the case[153]. More recent studies indicate that DUOX2 promoter activity was enhanced by Nkx2.5 and coactivator TAZ, transcriptional activators unrelated to thyroid-specific hormone production[154]. In lung epithelia, DUOX1 expression is also strongly enhanced during epithelial differentiation, and DUOX1 expression also increases strongly during alveolar maturation[155]. DUOX1 expression in fetal lung cells could also be enhanced by hormone mixtures that promote alveolar type II differentiation, although the precise transcriptional mechanisms were not defined[155]. However, other studies of in vitro differentiation of primary tracheobronchial epithelial cells using all-trans retinoic acid indicated strong induction of DUOX2 rather than DUOX1[156]. The DUOX1/DUOX1A1 promoter region harbors several Sp1 binding sites, while DUOX2/DUOX2A2 promoter segment contains putative initiator elements (Inr) and a TATA box, for initiation of gene transcription[116]. Moreover, although such bidirectionally promoted gene pairs have a higher probability of coordinated expression, studies using bidirectional reporter constructs of the DUOX2/DUOX2A2 promoter revealed regulator regions necessary for bidirectional transcriptional activity, while other regions were found to control unidirectional activity [157]. The differential regulation of DUOX1 and DUOX2 in airway epithelia is also evident from different responses to pro-inflammatory cytokines or environmental stimuli. Indeed, whereas DUOX1 is largely expressed constitutively and somewhat inducible by Th2 cytokines such as IL-4 and IL-13, DUOX2 expression was strongly inducible by Th1 cytokines (IL-1 α , IL-1 β , and IFN- γ) or viral stimuli[130, 158], suggesting unique and independent roles for DUOX1 vs DUOX2. Indeed, single nucleotide polymorphisms (SNPs) observed within the DUOX2/DUOX2A2 promoter were found to significantly affect promoter activity in response to exogenous dsDNA [157]. The fact that DUOX2 is far more inducible by environmental or proinflammatory stimuli compared to DUOX1 would suggest that DUOX2 may be more involved in pathological responses, whereas DUOX1 more likely participates in more homeostatic roles within the epithelium [130, 159], which will be discussed in more detail below.

Given our incomplete understanding of transcriptional and post-translational DUOX regulation, it is not surprising that the mechanisms involved in enhanced DUOX2 expression in cancer are also poorly understood. Overexpression of DUOX2 and DUOX2-dependent ROS generation in HCC has been attributed to post-transcriptional upregulation of DUOX2 by protein kinase C α (PKC α), which is also commonly overexpressed in HCC and associated with poor prognosis[160]. Observations of DUOX2 overexpression in pancreatic cancer are thought to be linked to pancreatitis and associated pro-inflammatory cytokines, and studies in pancreatic cancer cell lines indeed confirm induction of DUOX2 in response to bacterial lipopolysaccharide (LPS) or the inflammatory cytokine IFN- γ , due to concerted actions of transcription factors STAT1 and NF- κ B [108, 161]. Similarly, pro-inflammatory cytokines IL-1 α and IL-1 β can also induce DUOX2 expression (as mentioned previously) and also have important contributing roles in tumor progression [162–166], which could potentially involve increased DUOX2-dependent ROS production. DUOX2 activation has been implicated in chronic inflammatory diseases such as ulcerative colitis [167, 168] or chronic airway inflammation [169], and may thus be key in cancer development or

progression associated with chronic inflammation. Similarly, the potential roles of IL-4/IL-13 in tumor progression or immunosurveillance[170] may be related to their ability to induce DUOX1 activity within the tumor microenvironment in some cases.

Epigenetic Silencing of DUOX in Cancer

Upon initial discovery of DUOX silencing in lung cancer [52], it was recognized that this is primarily due to increased DNA methylation within the DUOX promoter regions. DNA hypermethylation has been well characterized as a signature mode of regulation for cancer cell[171–174]. DNA methylation typically involves methylation of cytosine within CpG-rich regions that are prominently present in promoter regions of tumor suppressor genes such as cyclin-dependent kinase inhibitor 2A (CDKN2A) and BRCA1[172, 175], and typically results in reduced expression of these genes. Conversely, DNA methylation is often suppressed in oncogenes and thereby permit their expression to promote cellular transformation[175, 176]. The intergenic promoter regions of DUOX1/DUOXA1 and DUOX2/DUOXA2 were found to be rich in CpG islands, and their hypermethylation in cancer results in reduced expression[52, 54, 111, 177], likely due to interference with binding of transcription factors such as Sp1 and Sp3[178, 179]. Therefore, DUOX1 has been considered to function as a tumor suppressor [52]. In addition to DNA methylation, other epigenetic modifications such as methylation or acetylation of histones serve to control their interactions with DNA and thereby regulate gene expression. The importance of histone methylation or acetylation for DUOX1 expression is not clear, and DUOX1 silencing in lung cancer cells could not be restored by the histone deacetylase (HDAC) inhibitor trichostatin A[52]. Recent studies indicate that these epigenetic regulatory mechanisms can regulate other NOX isoforms such as NOX4[180, 181], and suggest that DUOX1 expression is also subject to similar regulation by histone modifications.

DNA methylation is carried out by DNA methyltransferases (DNMT), and inhibitors of DNMT have therefore been explored for potential anticancer therapy. Indeed, DNMT inhibitors such as 5'-aza-deoxycytidine (AzadC) have been FDA approved for the treatment of the preleukemic disorder myelodysplastic syndrome [182] and have shown success treating various neoplasms, suggesting promise in other forms of cancer[183–185]. The precise mechanism of action of AzadC remains unclear and may also include actions independent of DNA methylation [186]. Other subsequently developed DNMT inhibitors such as zebularine also displays similar cytotoxic side effects, thus limiting their use therapeutically[186]. As might be expected, DNMT inhibition was found to be capable of restoring expression of DUOX1 in lung cancer cells[52, 54, 111], and suggest that the beneficial actions of DNMT inhibition might be related to recovering DUOX1 activity. Importantly, DNA hypermethylation in cancer is not unique to DUOX genes, but is also observed in other NOX isoforms such as NOX5[47]. More intriguingly, various cancers are also characterized by increased DNA methylation of other genes involved in redox regulation, such as extracellular superoxide dismutase (SOD3), a secreted antioxidant enzyme that is highly expressed in various tissues and is critical for regulation of ROS-mediated alterations of extracellular matrix biology [187, 188]. Indeed, SOD3 is frequently suppressed in pancreatic ductal adenocarcinoma [189], breast cancer[190], and lung cancer[191], and overexpression of SOD3 or recovering SOD3 expression by DNMT

inhibitors can suppress tumorigenic outcomes [189–192], suggesting the functional importance of SOD3 silencing for cancer progression. It is, however, unclear how silencing of DUOX1 as well as SOD3, which often occur in the same cancers, are mechanistically linked. DUOX1 (unlike other NOX isoforms) does not generate $O_2^{\bullet-}$ but only produces H_2O_2 , which would therefore not be affected by superoxide dismutases such as SOD3. However, the fact that both DUOX and SOD3 could contribute to increased extracellular H_2O_2 production, might imply that suppression of such extracellular H_2O_2 -generating mechanisms may be beneficial for tumor development.

The DNA methyltransferases (DNMT) form a family of enzymes with isotype-specific modes of action, with DNMT3A/B being largely responsible for methylation of unmethylated DNA with important roles in development whereas DNMT1 primarily acts on hemimethylated DNA and participates in tissue-specific methylation patterns[193]. DNMTs are subject to regulation by e.g. phosphorylation, e.g. by cyclin-dependent kinases (CDK1/2/5) which stimulate their enzymatic activity [194]. Increased expression or activation of DNMTs are observed as a result of oncogenic signaling programs related to constitutive activation of KRAS or epithelial-to-mesenchymal transition (EMT) [172, 176]. Moreover, DNMT activity and DNA methylation status are also subject to change due to environmental factors, and might also occur as a result of ROS production by NOX enzymes[47, 195]. Therefore, the fact that NOXes can both contribute to promoting epigenetic alterations, and in turn also be downregulated by such alterations, further illustrates the complexity of NOX biology in the context of cancer.

Countering the actions of DNMTs (which are considered to be ‘epigenetic writers’ able to edit the genome) are the actions of DNA demethylases (which serve as ‘epigenetic erasers’ and remove signatures made by the ‘writers’) [196]. Rao et al. first identified the ability of a ten eleven translocation (TET) enzyme, TET1, to catalyze the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) as a critical step in DNA demethylation[197, 198]. Two other TET family enzymes were subsequently identified[199]. Interestingly, TET enzymes are often downregulated in various cancers by epigenetic mechanisms [200–203]. Therefore, alterations of DNA methylation in cancer is not only mediated by altered or enhanced DNMT activity, but also appear to involve suppression of these ‘erasers’. A recent report demonstrated that oncogenic EGFR signaling represses various tumor suppressor genes through inhibition of TET1 activity, which could be restored by EGFR inhibition [202]. Moreover, TET1 overexpression inhibited tumor growth, whereas TET1 silencing enhanced resistance to EGFR-targeted therapies, collectively defining a unique dynamic between oncogenic EGFR activation and DNA methylation patterns[202]. Recent studies also suggest important consequences of metabolic alterations associated with ROS for TET enzymatic function. For example, ascorbate (vitamin C) is a critical co-factor for TET enzymes[204, 205] and may be depleted in cancer cells associated with increased ROS levels. Moreover, alterations in cellular metabolism due to e.g. disruption of the tricarboxylic acid (TCA) cycle, potentially associated with ROS, can give rise to accumulation of so-called oncometabolites such as succinate, which can reshape epigenetics by competitive inhibition of e.g. TET enzymes or histone demethylases[206, 207].

Functional Consequences of DUOX1 Silencing

Given the observed role of DUOX1 in innate epithelial wound responses and homeostasis, it would seem plausible that silencing of DUOX1 in various cancers may be associated with loss of functional epithelial characteristics. Indeed, our recent studies demonstrated that loss of DUOX1 in a panel lung cancer cell lines strongly corresponded to loss of the epithelial marker E-cadherin[54]. Subsequent studies showed that persistent silencing of DUOX1 using RNAi strategies, in normal epithelial cells or in the lung cancer cell line H292 (which maintains epithelial characteristics and expresses DUOX1), results in progressive loss of epithelial characteristics and acquisition of features representative of epithelial-to-mesenchymal transition (EMT)[54]. The process of EMT involves the loss of epithelial cell-cell contacts and epithelial molecular signatures (such as E-cadherin) and the gradual acquisition of a more motile, mesenchymal cell phenotype and expression of characteristic mesenchymal markers (e.g. vimentin, collagen, etc.). The importance of EMT in cancer biology is widely appreciated for its critical contribution to tumor invasion and metastatic behavior, and is strongly associated with negative prognosis[208, 209]. Our recent studies indicated that suppression of DUOX1 in H292 cells markedly enhanced their migratory and invasive capacity, both in vitro and in vivo[54]. Further supporting the functional importance of DUOX1 silencing to drive features of EMT are findings that overexpression of DUOX1 in invasive lung cancer cell lines such as A549 or H187 (which normally lack DUOX1) was found to promote the reverse process of mesenchymal-to-epithelial transition (MET), and diminish migratory capacity and restore epithelial characteristics[54].

The process of EMT in the context of cancer has also been associated with increased cancer cell stemness, which is highly relevant for cancer progression and negative prognosis since cancer stem cells (CSC's) are typically more resistant to anticancer therapies such as radiation or chemotherapies[210]. Thus, even if tumor size can be successfully reduced using e.g. targeted therapies, the presence of surviving CSCs typically gives rise to rapid tumor recurrence. Interestingly, our recent studies indicate that DUOX1 silencing in the cancer cell line H292 enhances the proportion of CSCs, based on analysis of CSC markers CD133 and ALDH[54], and thus may suggest that DUOX1 silencing in cancers may pertain primarily to the most poorly differentiated cancer cells with increased CSC properties.

The molecular mechanisms by which DUOX1 silencing promotes these various features are still unclear. On one hand, they may be related to reduced local DUOX-dependent H_2O_2 production, thereby potentially modulating localized redox-dependent cellular signaling events that control epithelial differentiation or motility. However, since these effects of DUOX1 silencing were observed in the absence of known DUOX1-activating stimuli, it is also possible that they may point to actions of DUOX1 that are independent of its NADPH oxidase activity. In the next paragraphs, we will address potential mechanistic consequences of DUOX1 silencing, based on known roles of DUOX1 in epithelial signaling.

DUOX1 Silencing, EMT, and Altered EGFR Dynamics: A Dangerous Triad?

Our studies of DUOX1-dependent redox signaling have highlighted an intricate relationship with the tyrosine kinases Src and EGFR as critical mediators of many of its functional

properties [134, 146, 148, 211] (Fig. 6A). Indeed, these kinases may directly interact with DUOX1 in response to cell stimulation by e.g. ATP[134], and are therefore subject to direct oxidation of redox-sensitive cysteines within their kinase domains, thereby controlling kinase activity (see below). EGFR is a member of the ErbB (HER) family of receptor tyrosine kinases, which are transmembrane proteins that are activated via its interaction with their cognate ligands to induce homo- or hetero-dimerization, thereby activate intrinsic tyrosine kinase activity and a range of signaling cascades that regulate a diverse array of cellular functions, such as differentiation, cytoskeletal arrangement, and cell migration[212]. Although EGFR is expressed in normal polarized epithelia, e.g. in the airway, it is localized primarily to the basolateral compartment[213], directed by its recognized basolateral sorting signals [214, 215], and is thus segregated from its ligands that are primarily localized and secreted apically. Hence, in healthy epithelia, EGFR is largely inactive and only becomes activated upon epithelial injury or other events that breach epithelial barrier activity [216]. Loss of epithelial polarity as a result of EMT diminishes this spatial control of EGFR signaling, for example by minimizing its interactions with E-cadherin[217], and thus permits the unabated interactions between the receptor and its ligands, promoting persistent activation and features such as proliferation and cell migration. Loss of DUOX1 expression and resulting EMT features may therefore have consequences for regulation of epithelial EGFR signaling (Fig. 6B). Cellular localization and activation of Src may also be dysregulated in cancer, which recent findings showing an imbalance in shuttling between caveolin-rich lipid rafts and focal adhesion complexes[218]. Given our findings linking DUOX1 with Src in epithelial signaling presumably in lipid rafts [134], DUOX1 silencing in lung cancer may have consequences for cellular Src distribution as well.

EGFR signaling has long been appreciated for its role in cancer biology, primarily because of its ability to promote cellular events related to oncogenic cellular behavior, such as proliferation, cell cycle progression, and migration[219–221]. EGFR is frequently overexpressed or dysregulated in various human carcinomas, such as head and neck, bladder, breast, ovarian, renal, colon, and lung cancer[222, 223]. Cancers that rely on the overexpression of growth factors and their receptors to promote cell growth, such as EGF and the EGFR, are often classified to have “oncogene addiction”. Dysregulated EGFR signaling can promote enhanced or prolonged signals that can lead to persistent activation of downstream effectors such as the MAPK and PI3K/Akt pathways[224]. Upon EGFR activation, the receptor complex is normally internalized into endosomal sorting complexes and can be recycled back to the cell surface, or targeted for lysosomal degradation, or towards other intracellular organelles[225, 226]. In cancer cells, EGFR activation may results in alternative intracellular distribution (Fig. 6C), for example by enhanced targeting to the nucleus where it promotes expression of genes that are involved in proliferation and cell cycle progression [227, 228]. In addition, EGFR can also be target to mitochondria in non-small cell lung cancers (NSCLC) and thereby alter mitochondrial dynamics, which may have implications for metastatic potential[229].

Because of the prominent role of EGFR signaling in lung cancer, approaches to inhibit EGFR have been developed for use in anticancer therapy, especially in NSCLC patients that harbor EGFR activating mutations, e.g. involving a small deletion in exon 19 or an L858R mutation in exon 20[222, 224]. These include tyrosine kinase inhibitors (TKIs), such as

erlotinib and gefitinib, that compete with ATP for binding the kinase domain, or EGFR-targeted monoclonal antibodies such as cetuximab and panitumumab that block ligand binding, and both have shown effectiveness in various settings[223, 224]. However, in spite of initial efficacy of these EGFR-targeted therapies, patients invariably develop acquired resistance to these inhibitors, either due to additional EGFR mutations (e.g. EGFR S492R, T790M) that render it more resistant to these TKIs, or to other cellular events (e.g. C-MET amplification, KRAS, others) or development of EMT[230, 231]. Intriguingly, analyses of a broad panel of lung cancer cell lines has shown that sensitivity to EGFR TKI (even in cells containing wild-type EGFR) is inversely related to the extent of EMT[232]. Moreover, our recent studies indicated that acquired resistance of H292 lung cancer cells to erlotinib is associated with a loss of DUOX1 and, conversely, that DUOX1 knockdown within H292 cells was sufficient to promote resistance to erlotinib [54]. Conceivably, these changes in EGFR TKI sensitivity may be related to altered subcellular trafficking, e.g. towards nuclei [215, 228, 233]. In spite of the suggested association of DUOX1 silencing with EGFR TKI resistance in lung cancer, it is unclear whether DUOX1 status relates to the presence of activating EGFR mutations in lung cancers. For example, although EGFR mutations are more common in adenocarcinoma patients (about 30%) compared to non-adenocarcinoma NSCLC patients (about 2%)[234], TCGA data analysis suggests that DUOX1 silencing is equally common among both NSCLC types (Fig. 4).

The process of EMT has been strongly associated with altered or increased ROS from multiple sources[235], in spite of our studies indicating that one specific source of ROS, DUOX1, is in fact downregulated. This could have direct consequences for EGFR signaling as it is highly subject to redox regulation. Indeed, various recent studies indicated that oxidation of a conserved cysteine near the ATP-binding site within the EGFR tyrosine kinase domain, C797, is susceptible to oxidation, and research by Kate Carroll and co-workers has indicated that oxidation of EGFR C797 to a sulfenic acid (–SOH) in response to ligand stimulation contributes to enhanced tyrosine kinase activity [236–238]. Moreover, the extent of EGFR C797 oxidation varied between cancer cells with WT EGFR or containing oncogenic mutants (e.g. L858R/T790M, 746–750) with the latter showing more extensive EGFR oxidation. In addition to oxidation to –SOH, our recent studies show that EGFR is also subject to *S*-glutathionylation, which may not necessarily affect kinase activity [134, 148, 239] but likely impact on the efficacy of EGFR TKI that are covalently targeted to C797 in an attempt to overcome TKI resistance[238, 240, 241]. Although oxidation of EGFR C797 to –SOH may enhance tyrosine kinase activity [148, 238], the significance of other modifications (e.g. *S*-glutathionylation) for EGFR regulation is unknown, and it is plausible that these redox modifications might also affect subcellular EGFR trafficking. The cellular mechanisms that mediate EGFR (and other protein) cysteine oxidation are not fully understood[242]. While original studies suggested the involvement of NOX2 in the context of ligand-dependent stimulation, alternative mechanisms of EGFR transactivation involve DUOX1 [148, 243]. Hence, the oxidative events that mediate EGFR oxidation, as well as its downstream consequences, could be highly context-dependent and are likely dysregulated in cancer biology, which may also have important consequences for the efficacy of EGFR-targeted therapies[238, 240, 241]. Intriguingly, recent studies have shown that the use of third-generation EGFR TKI such AZD9291[244], designed to covalently target the redox-

sensitive C797 and to overcome TKI resistance associated with T790M mutations, leads to additional resistance mechanisms that are mediated mutation of C797 to serine [245]. C797 is not essential for EGFR tyrosine kinase activity, and observations of T790M/C797S mutations have spurred the development of additional fourth-generation EGFR inhibitors that do not target the ATP-binding site but selectively inhibit these mutants while not affecting WT-EGFR activity by an allosteric mechanism[246].

Collectively, while DUOX1 silencing in lung cancer may be associated with EMT and altered dynamics and trafficking mechanisms of the EGFR, it is not yet clear how this relates to redox-mediated EGFR regulation, and the importance of dysregulated ROS mechanisms that may be associated with DUOX1 silencing for EGFR activation and trafficking is still unknown. Further studies are clearly needed to clarify the mechanistic consequences of specific redox modifications within EGFR for spatiotemporal control of EGFR activation or for efficacy of EGFR-targeted inhibitors, as well as the specific ROS sources that mediate redox-dependent EGFR regulation in different cell compartments. Finally, it is also important to consider the importance of tyrosine kinase-independent functions of EGFR in cancer biology[247], which may also be controlled by such redox-dependent mechanisms.

DUOX and Their Maturation Factors: Partners or Enemies?

As mentioned in previous sections, DUOX proteins are typically expressed in a coordinated manner with their maturation factors, the DUOXA proteins. Both DUOXA1 and DUOXA2 consist of five alpha helical transmembrane regions, with extracellular loops and an intracellular tail (Fig. 5), and associate with immature DUOX within in the endoplasmic reticulum to promote its proper folding and glycosylation and transport to the plasma membrane to generate a mature, fully functional enzyme[120, 121, 248]. However, immature DUOX1 still has intrinsic catalytic activity as well[249]. Recent studies indicate that DUOX-DUOXA protein interactions also potentially involve intramolecular disulfide bridges between their extracellular regions[118, 250]. Additionally, it was recently recognized that the specificity of ROS release, either O_2^- as an intermediate or H_2O_2 as its final product, is determined by sequences in the extracellular A loop of the DUOX proteins, which bind the N-termini of DUOXA proteins[119]. DUOXA1 is alternatively spliced, thereby yielding several splice variants and little is known regarding their individual roles[121]. Intriguingly, it has been previously reported that certain splice variants of DUOXA1 can also partner with DUOX2, which permits the generation of H_2O_2 at nearly the same levels as DUOX2-DUOXA2 heterodimers, although this was not found to be true upon mispairing DUOX1 with DUOXA2[119, 121].

In addition to partnering with corresponding DUOX proteins, DUOXA proteins may also have functions independent of DUOX. Indeed, DUOXA was originally identified in *Drosophila melanogaster*, as a binding partner to a master regulator of the Notch cell differentiation pathway, Numb, and was therefore named Numb Interacting Protein (NIP) [120, 251, 252]. Sequence characteristics in the NIP proteins suggested they are membrane associated proteins, and as such, it was hypothesized that NIP regulates the subcellular localization of Numb by sequestering Numb to the plasma membrane during asymmetric cell division[251]. Mammalian NIP proteins were later recognized for their ability to

regulate DUOX-dependent ROS production, and thus were denoted as DUOX activators, DUOX1 and DUOX2[120, 251]. Given the importance of the Notch signaling pathway as a master controller of cellular differentiation, altered expression of DUOX1 could have significant downstream effects on the Notch pathway, thereby leading to abnormalities in cell differentiation and/or tumor progression. Indeed, aberrant Notch signaling has been observed in a variety of cancers, with its signaling or expression either being pro- or anti-tumorigenic, highly dependent on the tissue and cellular context[253–255]. Interestingly, oncogenic EGFR signaling and Notch signaling have been considered mutual or correlative[256], which could potentially be related to a common role for DUOX1/DUOX2 on these processes. Beyond its role as a maturation factor for DUOX1, or in regulating the Notch pathway, DUOX1 may also have other independent functional roles. For example, recent studies implicated DUOX1 in cellular differentiation in muscle satellite cells[257] and neuronal cells[258, 259], which appears to involve interactions with p53, well-appreciated for its tumor suppressor function and its roles in cell cycle dynamics and differentiation[258]. Also, DUOX1 was found to harbor roles in various tissues such as the brain[258, 259] and breast[260], which appear unrelated to DUOX1.

Since the bidirectional promotor region of DUOX and DUOX2 is rich in CpG islands, both DUOX and DUOX2 genes were found to be suppressed in lung cancer cell lines due to hypermethylation[52]. Analysis of TCGA data of DUOX1 mRNA expression indeed indicates that DUOX1 is downregulated in various cancers, similar to DUOX2, whereas this seems to apply less to DUOX1 and DUOX2 (Fig. 7). The functional significance of such DUOX1 downregulation was suggested by studies in breast cancer cells, which showed reduced DUOX1 expression in metastatic cell lines, and increased proliferation, altered cell adhesion, and susceptibility to doxorubicin after transient overexpression of DUOX1, but not DUOX2 [260]. Interestingly, our recent studies in lung cancer cells indicate that overexpression of DUOX1, but not DUOX2, can restore epithelial characteristics and reduce migratory and invasive properties [54]. Hence, the functional importance of DUOX1 in cancer still needs further clarification, but is likely independent of DUOX2. Moreover, in contrast to common silencing of DUOX1 appears in many cancers malignancies based on both TCGA data and IHC data in Human Protein Atlas, these data often show positive DUOX1 protein expression in various cancer tissues (e.g. neuroendocrine, head and neck, testicular cancers) as well as mixed IHC protein staining in various other cancers. In addition, results from TCGA analysis show DUOX1 mRNA expression is also enhanced in thyroid cancers (Fig. 7A). Collectively, these observations suggest that DUOX1 and DUOX2 may have independent functional properties, and alterations of DUOX1 expression in cancer may affect cancer biology by DUOX1-independent functions, potentially related to interactions with p53 or Numb/Notch pathways, in addition to regulating the actions of DUOX1.

Clinical Implications and Concluding Remarks

In summary, while the role of ROS in cancer biology is still incompletely understood, and various NOX enzymes are variably overexpressed in cancer, common observations of DUOX1 silencing in many carcinomas suggest that it may represent an important prognostic marker. Indeed, DUOX1 expression levels has been successfully linked with positive

prognosis in patients with hepatocellular carcinoma [53], although it is presently unclear whether this is also true for other epithelial-derived cancers. However, our recent studies highlighting a direct link between DUOX1 loss and EMT, CSC characteristics and resistance of EGFR-targeted therapy [54] suggests that DUOX1 expression status may also be an important prognostic indicator in lung cancer. The mechanistic consequences of DUOX1 silencing in cancer remain to be further elucidated, and may involve dysregulated redox signaling but may also reveal redox-independent functions of DUOX1. So far, the importance of DUOX1 silencing in cancer has not been addressed in any mouse models. Newly developed mouse models of conditional DUOX1 deletion (e.g. using CRISPR/Cas9 technologies), combined with chemical or genetic models of e.g. lung cancer, will be critical to address this more directly. Alternatively, approaches to conditionally overexpress DUOX1 in preformed tumors in animal models would be useful to further address the relevance of DUOX1 silencing for cancer progression or responses to anticancer therapies. Since DUOX1 suppression in cancer is primarily dictated by hypermethylation of its promoter [52, 54, 111, 177], attempts to recover DUOX1 expression would need to revolve around inhibition of DNA methyltransferases (DNMTs) or potentially approaches to minimize TET suppression. Clinical trials have been performed attempting to target DNMTs and have shown some efficacy [261], although specific recovery of DUOX1 by DNMT inhibition in a pre-clinical or clinical setting has not been explicitly examined. Additional insights are needed into other epigenetic modifications, such as histone acetylation or methylation patterns, that may regulate DUOX1 and may offer additional strategies to restore DUOX1 expression in the context of cancer. Moreover, since Th2 cytokines such as IL-4 and IL-13 can enhance DUOX1 expression and/or activity [262–264], they could potentially be exploited as an alternative therapeutic approach to recover DUOX1. Another important gap in this field is the lack of information regarding existing DUOX1 polymorphisms and lung cancer. Whereas abundant literature has connected DUOX2 SNPs to hypothyroidism [265, 266], and several reports suggest that the development of hypothyroidism is a risk factor for the onset of cancer [267–269], information on DUOX1 SNPs are scarce, with only one report identifying two SNPs on exons 27 and 28, although no association with lung disease was observed [270]. Potential associations of DUOX1 SNPs with increased cancer incidence or progression could offer important additional insights into the overall importance of DUOX1 in cancer biology.

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Abbreviations

Akt	protein kinase B
ALDH	aldehyde dehydrogenase
AzaC	5'-aza-deoxycytidine

BRCA1	breast cancer 1 gene
CDK	cyclin dependent kinase
CDKN2A	cyclin dependent kinase inhibitor 2A
CSC	cancer stem cell
DHA	dehydroascorbic acid
DNMT	DNA methyltransferase
dsDNA	double stranded DNA
DUOX	dual oxidase
DUOXA	dual oxidase maturation factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
FAD	flavin adenine dinucleotide
GBM	glioblastoma
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
IFN-γ	interferon gamma
IHC	immunohistochemistry
IL	interleukin
Inr	putative initiator elements
IR	ionizing radiation
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MET	mesenchymal-to-epithelial transition
C-MET	tyrosine protein kinase Met or hepatocyte growth factor
NAC	N-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa beta
NIP	numb interacting protein
Nkx2.5	homeobox protein Nkx2.5

NOX	NADPH oxidase
NSCLC	non-small cell lung cancer
Pax8	paired box gene 8
PDAC	pancreatic ductal adenocarcinoma
PHD	peroxidase homology domain
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKCα	protein kinase C alpha
PTP1B	protein tyrosine phosphatase 1B
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
SOD3	extracellular superoxide dismutase
Sp1	specificity protein 1
TAZ	transcriptional coactivator with PDZ-binding motif
TCGA	tissue cancer genome atlas
TET	ten eleven translocation enzyme
Th2	T-helper 2
TKI	tyrosine kinase inhibitor
TTF-1	thyroid transcription factor 1
VEGF	vascular endothelial growth factor

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Highlights

- Expression and activation of NOX enzymes is often dysregulated in cancer, and increased ROS production by NOX enzymes may promote features of cancer biology.
- Several NOXes, especially DUOX1, are often suppressed epithelial derived cancers by epigenetic mechanisms.
- Epithelial silencing of DUOX1 promotes epithelial alterations and invasive properties, and DUOX1 silencing in cancer may be associated with worse prognosis.

Figure 1. Importance of redox homeostasis for cellular function

Cellular production of reactive oxygen species (ROS) from various sources is controlled by various antioxidant networks. Dysregulated ROS production or antioxidant systems can disturb cellular redox homeostasis, and contribute to pathology.

A - DNA Oxidation

B - Lipid Peroxidation

C - Protein Oxidation

Figure 2. Biochemical reactions associated with oxidation-dependent contributions to cancer biology

Enhanced levels of reactive oxygen species (e.g. O_2 , H_2O_2) promotes oxidation events such as direct DNA oxidation (e.g. 8-oxo-guanine) (A), or lipid peroxidation (B), which can both lead to genomic instability and drive tumor initiation and/or progression. ROS imbalance can also perturb redox dependent cellular processes through oxidation of sensitive amino acid residues such as methionine or cysteine (C), leading to potential alternative protein function and/or dysregulated cell signaling.

Figure 3. DUOX1 and DUOX2 display differential mRNA expression in various epithelial derived cancers

Dot blots represent mRNA expression of DUOX1 (A) or DUOX2 (B) extracted from either normal (N) healthy tissue (blue symbols), or tumor (T) tissue (red symbols). Data extracted from Tissue Cancer Genome Atlas (TCGA); results shown as AVG $\pm$ SEM. Statistical significance determined by one-way ANOVA, n.s. = not significant; * p-value<0.05; ***p-value<0.001. RPKM = Reads Per Kilobase of transcript per Million mapped reads.

Figure 4. Comparison of DUOX1 mRNA expression lung squamous cell carcinomas (SCC) and lung adenocarcinomas (AC) compared to corresponding matched controls
 RNASeq data for SSC (n=50; A) and AC (n=58; B) and matched controls were downloaded from the Genomic Data Commons Data Portal (<https://portal.gdc.cancer.gov/>) and converted to z-scores as described: *Cheadle C, Vawter MP, Freed WJ, Becker KG. Analysis of Microarray Data Using Z Score Transformation. The Journal of molecular diagnostics: JMD. 2003;5(2):73–81. *P<0.0001 based on Wilcoxon matched-pairs signed rank test (Prism v7.01).*

Figure 5. Topology of DUOX1-DUOX1A1 proteins

DUOX1 utilizes reducing equivalents from NADPH to transfer electrons through the two heme molecules, reducing molecular O₂ to H₂O₂. Regions highlighted in RED and YELLOW indicate regions with relatively contain low sequence homology between DUOX1 and DUOX2 and between DUOX1A1 and DUOX2A2.

Figure 6. Loss of DUOX1 and local redox signaling has potential implications for EGFR regulation and signaling

(A) Schematic diagram of DUOX1-dependent redox signaling in response to epithelial stimuli. DUOX1-derived H_2O_2 oxidizes target protein cysteines (e.g. Src/EGFR) in a localized fashion near or at the plasma membrane, and promotes features consistent with normal epithelial redox signaling. (B) Loss of DUOX1 in cancer removes a local oxidant source, and is associated with oncogenic EGFR signaling, potentially due to perturbed redox signaling mechanisms e.g. contributions from other NOXes or mitochondria. (C) DUOX1 suppression promotes EMT, which is accompanied with altered EGFR spatial distribution, although how altered redox regulation contributes to these consequences are unknown. (D) Associations between altered EGFR subcellular localization and oncogenic outcomes.

Figure 7. DUOXA1 and DUOXA2 mRNA expression in various epithelial derived cancers
Dot blots represent mRNA expression of DUOXA1 (A) or DUOXA2 (B) extracted from either normal (N) healthy tissue (blue symbols), or tumor (T) tissue (red symbols). Data extracted from Tissue Cancer Genome Atlas (TCGA); results shown as AVG $\pm$ SEM. Statistical significance determined by one-way ANOVA, n.s. = not significant; * p-value<0.05; ***p-value<0.001. RPKM = Reads Per Kilobase of transcript per Million mapped reads.

Table 1

Associations to cancer biology among various NOX enzymes.

NADPH Oxidase	Cell/Tissue; Cancer Type	Contribution to Cancer Phenotype	Ref
NOX1	Breast Cancer	Invasiveness; Growth/Proliferation	[42, 68]
	Colorectal Cancer	Overexpression	[65]
	Fibrosarcoma	Invasiveness; Growth/Proliferation	[42, 64]
	Head/Neck Squamous Cell Carc.	Invasiveness	[42]
	Melanoma; Skin Cancer	Invasiveness; Impaired DNA Repair	[42, 67]
	Oral Squamous Cell Carcinoma Renal Cell Carcinoma (HRAS transformed cells)	Resistance to Apoptosis	[69]
		Invasiveness	[43]
NOX2	Breast Cancer	Growth/Proliferation	[82]
	Colorectal Cancer	Overexpression and Invasion; Growth/Proliferation	[66, 84]
	Gastric Cancer	Tumor Invasiveness; Correlates with Poor Prognosis	[86]
	Myelomonocytic Leukemia	Immunosuppressive	[83]
	Prostate Cancer	Growth/Proliferation	[85]
NOX4	Breast Cancer	Migration	[72]
	Colorectal Cancer	Poor Prognosis	[74]
	Lung Cancer	Migration	[72]
	Ovarian Cancer	Angiogenesis	[71]
	Pancreatic Ductal Adenocarcinoma	Glycolytic Switch (Altered Metabolism)	[73]
	Prostate Cancer	Angiogenesis	[71]
	Thyroid (HRAS transformed cells)	DNA Damage; Enhanced DNA Replication	[104]
NOX5	ALK Lymphoma	Proliferation/Survival	[79]
	Esophageal Cancer	Overexpression, Proliferation/Survival	[78]
	Leukemia	Unknown	[77]
	Prostate Cancer	Proliferation/Survival	[75, 76]
DUOX1	Colorectal Cancer	Overexpressed	[115]
	Hepatocellular Carcinoma	Expression Silenced; Correlates with Positive Prognosis	[53, 111, 271]
	Lung Cancer	Expression Silenced; Loss Promotes EMT	[52, 54]
	Neuroblastoma	Unknown	[114]
	Prostate Cancer	Overexpressed	[115]
	Thyroid Cancer (Radiation-Induced)	Genomic Instability	[112]
DUOX2	Breast Cancer	Overexpressed	[109]
	Colorectal Cancer	Overexpressed; Correlates with Poor Prognosis	[105, 106, 109, 110]
	Gastric Cancer	Overexpressed	[105]
	Lung Cancer	Overexpressed	[109]
	Pancreatic Cancer	Overexpressed; Angiogenesis	[107, 108]
	Prostate Cancer	Overexpressed	[109]