Duox NADPH oxidases in the airways: regulation in health and malignancy

Inaugural-Dissertation
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vorgelegt von
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meiner Familie
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<tr>
<td>2D</td>
<td>two dimensional</td>
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<td>three dimensional</td>
</tr>
<tr>
<td>3'</td>
<td>three prime end of DNA sequences</td>
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<td>five prime end of DNA sequences</td>
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<td>aa</td>
<td>amino acid</td>
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<td>ALI</td>
<td>air-liquid interphase</td>
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<td>ammonium persulfate</td>
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<td>airway surface liquid</td>
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<td>bronchial epithelial growth media</td>
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<tr>
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<td>base pair</td>
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<td>H₂O₂</td>
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<td>HEPES</td>
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<td>His</td>
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<td>hour</td>
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<td>horseradish peroxidase</td>
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<td>immunofluorescence</td>
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<td>interleukin</td>
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<tr>
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<td>NEM</td>
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<td>platelet-derived growth factor</td>
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<td>SCN</td>
<td>thiocyanate</td>
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<td>sodium dodecyl sulfate</td>
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<tr>
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<td>small/short hairpin RNA</td>
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<td>single-stranded</td>
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<td>Pol polymerase from <em>Thermus aquaticus</em></td>
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<td>TBS</td>
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<tr>
<td>TBS-T</td>
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<td>Tris-EDTA buffer</td>
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<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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1 Introduction

1.1 Reactive Oxygen Species in the Lung

The lung is a highly specialized organ and plays fundamental roles for the host organism. Based on its distinctive structure, it provides an enormous surface area to the ambient air, thereby allowing efficient gas exchange of vital oxygen (O$_2$) and the metabolic waste product carbon dioxide (CO$_2$). However, the lung does not only provide an entry for oxygen; its epithelium faces harmful oxidants, toxicants, pollutants as well as airborne pathogens. Many of these inhaled environmental challenges give rise to reactive oxygen species (ROS), which have been identified as major contributors to oxidative stress (Azad et al., 2008; van der Vliet, 2008).

ROS include radical components, such as superoxide (O$_2^•$) or hydroxyl radicals (OH$^•$), and non-radical components, such as hydrogen peroxide (H$_2$O$_2$), hydrochlorous acid (HOCI), ozone (O$_3$) or peroxynitrate (ONO$_2$) (Henricks and Nijkamp, 2001). ROS are generally highly reactive and have the ability to oxidize intracellular macromolecules, such as proteins, nucleic acids, lipids and membranes (Stadtman, 2006). Thus, ROS have been linked to DNA mutations, protein oxidation, lipid peroxidation and cell damage (Lambeth, 2004; Ryter et al., 2007). During inflammation excessive endogenous ROS levels are responsible for tissue injury, as they generate stable oxidation products. In the airways, ROS have been linked to acute respiratory distress syndrome and acute lung injury by either directly damaging the pulmonary structures or indirectly by initiating the release of other inflammatory mediators (Henricks and Nijkamp, 2001; Mills and Higgins, 1997). Prolonged oxidative stress can cause chronic lung diseases such as asthma, idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease (COPD) (Barnes, 2000).

Environmental ROS are largely derived from air pollutants as well as cigarette smoke. In addition, ROS can also be derived endogenously. Low amounts of ROS were identified as accidental by-products of the mitochondrial respiratory chain; however, the mitochondria also can produce high ROS levels during hypoxia (Chandel et al., 1998; Chandel et al., 2000; Raha and Robinson, 2000). As a result, ROS have been viewed as harmful, yet unavoidable compounds.

Recent studies have provided evidence that small localized concentrations of ROS are generated deliberately by ROS-producing enzymes, such as NADPH oxidases. ROS act as secondary messengers in intracellular signaling cascades, which are implicated in proliferation and aging as well as anti-tumorigenic processes, such as senescence and apoptosis (Valko et al., 2006). This is accomplished by reversible inhibition of protein or lipid phosphatases (PTPs) such as PTP1B, SHP-1, SHP-2 or PTEN (Finkel, 1999). They all contain an invariant catalytic cysteine residue that is highly susceptible to oxidative inactivation through transiently produced H$_2$O$_2$, leading to the formation of intermolecular disulfides (R-SOH → R-SS-R). As a consequence, receptor tyrosine kinases (RTKs) are susceptible for activation by their ligands (EGF, PDGF), leading to downstream signaling (FIGURE 1).

Endogenous ROS has further been identified during inflammation, which was generated by neutrophils and macrophages. However, the best understood mechanism of inducible local ROS generation is a process known as respiratory burst. In this case, as part of an innate immunity defense mechanism, phagocytes produce superoxide in order to kill invading microorganisms (Babior, 1999).
1 INTRODUCTION

![Diagram](Image)

**Figure 1:** NADPH oxidase-derived ROS mediates intracellular signaling. Protein tyrosine phosphatases (PTPs) block downstream signaling of receptor tyrosine kinases (RTKs), such as epidermal growth factor-receptor (EGF-R). Transient production of $H_2O_2$ by NADPH oxidases leads to reversible oxidative inactivation of the PTP, through oxidation of an invariant catalytic cysteine residue. Consequently, downstream signaling of the RTKs is not blocked anymore and downstream affected pathways are activated. Adapted from (van der Vliet, 2008).

1.2 Family of NADPH Oxidases

The membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) complex of phagocytes reduces molecular oxygen to superoxide through oxidation of NADPH. This oxidase complex consists of two membrane-bound proteins, the glycosylated catalytic component gp91phox (Nox2) and the non-catalytic subunit p22phox. They form a non-covalent dimer that is referred to as flavocytochrome b$_{558}$ (Wallach and Segal, 1996). The so-called “Nox-domain” of Nox2 comprises 6 transmembrane (TM) helices and contains subregions which form cavities for the binding of flavin adenine dinucleotide (FAD) and NADPH. In addition, Nox2 possesses two hemes, which are bishistidine-ligated in the parallel transmembrane helices 3 and 5, coordinated with His$^{101}$-His$^{115}$ and His$^{208}$-His$^{222}$, respectively (Biberstine-Kinkade et al., 2001). The enzyme functions as an electron transferase by facilitating the transport of NADPH-derived electrons across the hemes towards extracellular molecular O$_2$, thus forming superoxide (Nauseef, 2008).

Nox2 activity is regulated by compartmentalization, as this enzyme has to assemble with 2 additional protein complexes in order to be fully active (Figure 2). These complexes reside in the cytoplasm and translocate to the membrane upon stimulation (Thrasher et al., 1994). The first complex consists of the three proteins p40phox, p47phox and p67phox. p47phox does not possess any intrinsic catalytic activity and acts as an organizing adaptor protein. p67phox functions as an activating cofactor based on its domain that regulates the reduction of FAD by NADPH (Nisimoto et al., 1999). p40phox interacts with p67phox and participates in ROS production (Bissonnette et al., 2008). The second complex comprises the small GTPase Rac2, which is bound to RhoGDI, a GDP dissociation inhibitor for Rho GTPases. Activation of those two complexes leads to phosphorylation of p47phox and RhoGDI (Nauseef, 2008). As a consequence, p47phox can bind to p22phox and RhoGDI dissociates from
Rac-GDP. Catalyzed by guanine nucleotide exchange factors (GEFs), GDP is exchanged with GTP, resulting in active Rac-GTP that independently translocates to the plasma membrane.

**FIGURE 2:** Assembly and activation of the phagocytic NADPH oxidase Nox2. The resting enzyme consists of the two membrane-bound components gp91phox and p22phox, which form the heterodimeric complex flavocytochrome b558. In order to be fully functional, the enzyme has to assemble with the cytosolic subunits p40phox, p47phox, p67phox and Rac2. Upon activation, the cytosolic components translocate to the membrane, where they assemble with b558. Phosphorylation of p40phox enables binding to p22phox, while phosphorylation of RhoGDI leads to dissociation from inactive Rac (Rac-GDP). Rac-GDP is then activated (Rac-GTP), which promotes binding to p67phox to form an active complex. Adapted from (Lambeth, 2004).

Mutations in any of these components (except of p40phox) result in chronic granulomatous disease (CGD), a genetic immune disorder that results in an impaired ability of phagocytes to kill invading microorganisms (Kawahara et al., 2007; Lambeth, 2004; Nauseef, 2008; van der Vliet, 2008).

Low amounts of ROS have also been found in non-phagocytic cells and tissues. Inhibitor studies indicated that these ROS were not by-products of the mitochondrial respiratory chain, but actually flavoprotein-derived (Lambeth, 2004). Six other homologues of Nox2 were identified (Nox1, Nox3, Nox4, Nox5, Duox1, Duox2), which all possess the Nox-domain and now constitute the family of NADPH oxidases (**FIGURE 3**). Members can be divided into three groups, based on their similarity with gp91phox. Nox1, Nox3 and Nox4 feature the same topology as Nox2. The slightly larger Nox5 possesses an N-terminal calmodulin-like domain, which contains four EF-hands. The EF-hand motif is ubiquitous in the genomes of eukaryotes and prokaryotes. It consists of a characteristic 30 amino acid long helix-loop-helix (HLH) structure and allows the binding of calcium. The two largest members of the Nox family, Duox1 and Duox2, build onto the structure of Nox5. These enzymes feature an additional amino terminal domain, which is homologous to peroxidases. This domain is connected to the intracellular calcium-binding region, which encodes two canonical EF-hands and an additional HLH-structure by a transmembrane α-helix (De Dekens et al., 2000; Schwarzer et al., 2004).
Not all members of the Nox family are regulated similar to Nox2. Only Nox1, Nox3 and Nox4 form a heterodimer with p22phox, while Nox5, Duox1 and Duox2 do not require this subunit for function. Nox1 and Nox3 also associate with cytosolic factors, which are homologous to p47phox and p67phox and are termed Nox organizing (Noxo1 or p41) and Nox activating (Noxa1 or p51) protein, respectively. While both Nox1 and possibly Nox3 also need the GTPase Rac for function, Nox4, Nox5, Duox1 and Duox2 are Rac-independent (Kawahara et al., 2007; Nauseef, 2008). Nox5 function is entirely dependent on the elevation of intracellular calcium concentrations, resulting in phosphorylation of Thr494 and Ser498 and in conformational changes. The latter in turn leads to an interaction between the N-terminus and an unidentified C-terminal domain, facilitating electron delivery and superoxide production (Banfi et al., 2004; Jagnandan et al., 2007). Duox1 and possibly Duox2 are also predicted to undergo conformational changes via increase of intracellular calcium levels, resulting in ROS production upon dissociation from regulatory subunit Noxa1 (Pacquelet et al., 2008).

So far, the Nox family members have been divided into three groups, based on their similarity with gp91phox. However, a recent study suggested a different classification (Kawahara et al., 2007). As Nox members are widely distributed from plants to mammals, 7 subfamilies of Nox/Duox enzymes were identified based on molecular taxonomy. No Nox-coding sequences were found in the genomes of prokaryotes, while – except for yeast – most eukaryotes (such as vertebrates, urochordates, echinodermates, nematodes, insects, fungi, plants, amoeba and red algae) encoded those genes. Nox5 and Duox are related to plant Nox, which also possess EF hands, demonstrating that the acquisition of calcium-binding domains occurred early in evolution. Indeed, the EF-hand-containing subfamilies comprise more than 50% of the taxonomic tree (Bedard et al., 2007; Kawahara et al., 2007; Torres et al., 1998).
Nox and Duox generate ROS in a regulated manner, producing reactive oxygen in various cells and tissues in response to growth factors, cytokines and Ca\(^{2+}\)-dependent signals. The expression pattern of these enzymes (Table 1) reveals tissue specificity and provides evidence that ROS generation by NADPH oxidases is a general feature of many, maybe all cells (Lambeth, 2004).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Highest level of expression</th>
<th>Known regulatory factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox1</td>
<td>colon, uterus, prostate, vascular smooth muscle cell</td>
<td>Noxo1, Noxa1, p22(^{phox}), Rac</td>
</tr>
<tr>
<td>Nox2 (gp91)</td>
<td>phagocytes</td>
<td>p47(^{phox}), p67(^{phox}), p40(^{phox}), p22(^{phox}), Rac</td>
</tr>
<tr>
<td>Nox3</td>
<td>inner ear, fetal kidney</td>
<td>p22(^{phox}), Nox1, p47(^{phox}), p67(^{phox})</td>
</tr>
<tr>
<td>Nox4</td>
<td>kidney, ovary, osteoclasts, heart, endothelial cells</td>
<td>p22(^{phox})</td>
</tr>
<tr>
<td>Nox5</td>
<td>spleen, sperm, mammary gland, cerebrum</td>
<td>Calcium</td>
</tr>
<tr>
<td>Duox1</td>
<td>airways, thyroid, testis, tongue, cerebellum</td>
<td>Calcium, Noxa1</td>
</tr>
<tr>
<td>Duox2</td>
<td>thyroid, airways, colon, pancreatic islets</td>
<td>Calcium</td>
</tr>
</tbody>
</table>

Table 1: Tissue distribution and known regulatory factors of human Nox/Duox. Adapted from (Lambeth, 2004).

1.3 Duox1 and Duox2, novel members of the Nox family

Dual oxidases were first cloned from cDNA libraries of thyroid cells (De Deken et al., 2000; Dupuy et al., 1999) and were initially called Thox (throid oxidase) or Lnox (large NADPH oxidase). Duox1 was later also identified in the epithelia of the whole lung, trachea, testis, cerebellum and tongue, while Duox2 was also expressed in salivary gland, parotid gland, trachea, colon, prostate, lung and pancreas (Caillou et al., 2001; Edens et al., 2001; Forteza et al., 2005; Geiszt et al., 2003). The genes for both enzymes are located on the long arm of chromosome 15, at location 15q15.3, and are arranged head-to-head, divided by a 16 kb region (Pachucki et al., 2004). The gene for DUOX1 is located more telomeric and spans 36 kb, encoding 35 exons, of which the first two are non-coding. The DUOX2 gene consists of 34 exons, of which only the first exon is not coding and the whole sequence spans over 22 kb. Exons 4, 5 and 6 of DUOX1 and DUOX2 are more than 99% similar and the translated proteins, with respective open reading frames of 1551 and 1548 amino acids, display an 83% sequence similarity (Donko et al., 2005). The promoter regions of DUOX1 and DUOX2 are dissimilar. The Duox1 promoter lacks a TATA-box and features three putative binding sites for the transcription factor SP-1. Those sequences are commonly found within 100 bp of the transcriptional initiation site and are also referred to as a GC-box. While no such sequences are present in the DUOX2 promoter, it exhibits a reverse TATA-box as well as a putative ATF/CRE-binding site, a stress-responsive element.

The Nox domain of Duox proteins is 47% similar to Nox2. In addition, these enzymes possess an N-terminal extracellular peroxidase-homology domain. Although this domain is 43% homologous with thyroperoxidase (TPO) and shares 25% identical amino acids with myeloperoxidase (MPO), its function is still a matter of controversy. Animal peroxidases contain hemes, which are bound via their iron to invariant histidine residues that provide proximal and distal axial bonds. However, the Duox peroxidase-homology domain lacks those key amino acids and encodes for serines instead (Donko et al., 2005; Nauseef, 2008). Surprisingly, when expressed in E. coli it could still form di- and trityrosines, a classical peroxidase feature (Edens et al., 2001). It has been suggested that this domain could also have a superoxide dismutase-like activity to promote exclusive H\(_2\)O\(_2\) production (Morand et al., 2004), or that it functions as a docking site for secreted peroxidases, which use the Duox-based H\(_2\)O\(_2\).
(Wong et al., 2004). A long intracellular loop, which contains ER-retention signals (aa 596 to 685) and two EF-hands, links the first transmembrane domain with the Nox-domain. The latter contains, in accordance with the other members of the Nox-family two binding sites for FAD and four binding sites for NADPH (Figure 4).

Both Duox1 and Duox2 are glycoproteins. When expressed in thyroid cells, they possess a molecular weight of 190 kDa, while a 180 kDa form is found in non-thyroid cells. Glycosylation accounts for about 10-20 kDa. Deglycosylated proteins have a molecular weight of about 160 kDa and are non-functional. It appears that glycosylation is an important step in folding and targeting the proteins to the proper compartment of the cell, thus allowing for full enzymatic activity of Duox (Caillou et al., 2001; De Deken et al., 2000).

Based on the presence of EF-hands, Duox1, as well as Duox2 and Nox5, require calcium for activity and therefore can be stimulated by ionomycin or thapsigargin. Both compounds increase intracellular calcium levels; thapsigargin primarily leads to intracellular calcium store-depletion while ionomycin works as an ionophore, allowing extracellular calcium to transit into the cell. ATP was also shown to stimulate Duox function via paracrine signaling, resulting in intracellular Ca\(^{2+}\) increase.

1.3.1 DuoxA1 and DuoxA2, maturation factors for Duox1 and Duox2
Attempts have been made to transiently reconstitute either form of Duox in non-thyroid cell lines. However, the proteins remained as an immature form in the ER rather than being translocated to the plasma membrane (Ameziane-El-Hassani et al., 2005; De Deken et al., 2002). A yeast two-hybrid screen identified EF hand-binding protein 1 (EFP1), a TPO-interacting thioredoxin-related protein, as a binding partner of Duox. However, co-expression of both EFP1 and Duox did not induce membrane targeting or Duox-dependent H\(_2\)O\(_2\) production (Wang et al., 2005). Recently, maturation factors for Duox1 and Duox2, termed DuoxA1 and DuoxA2, were identified (Grasberger and Refetoff, 2006). They are located head-to-head with their respective oxidase in the 16 kb stretch between both DUOX genes (Figure 5A). This intergenic region was initially annotated as “homolog of Drosophila Numb-interacting protein (NIP)”. A bidirectional promoter drives the transcription of Duox1/DuoxA1 and Duox2/DuoxA2, respectively. Bidirectional promoters account for 10% of all transcriptional units and
increase the probability of coordinated expression (Trinklein et al., 2004). Both DuoxA1 and DuoxA2 are integral membrane proteins and are predicted to possess 5 membrane-spanning α-helices. They also feature an extended extracellular loop between transmembrane domain 2 and 3, which contains 3 putative N-glycosylation sites (FIGURE 5B). Co-expression of Duox1 or Duox2 with their respective maturation factor allowed Duox proteins to exit the ER and localize to the plasma membrane, resulting in inducible production of H₂O₂ (Grasberger and Refetoff, 2006).

**FIGURE 5**: Genomic arrangement of DUOX/DUOXA and topology of DuoxA maturation factors. (A), DUOX1 and DUOX2 are arranged head-to-head on chromosome 15, at position q15. Both maturation genes for DUOX1 and DUOX2 are located in the intergenic region, located head-to-head with their respective oxidase. (B), DuoxA1 and DuoxA2 are integral membrane proteins. They feature the same topological structure, encompassing 5 transmembrane (TM) domains, and contain 3 putative N-glycosylation (Y) sites in the extended extracellular loop between the second and the third TM domain. Adapted from (Grasberger and Refetoff, 2006).

### 1.3.2 Tissue localization of Duox

Although both isoforms can be found in the thyroid and in the lung, Duox1 and Duox2 are differentially expressed and regulated. In the thyroid Duox2 is up to six times more abundant than Duox1 (Pachucki et al., 2004). Duox2 co-localizes with thyroperoxidase (TPO) at the apical poles of thyrocytes and serves as a supplier of H₂O₂ for TPO-mediated oxidation of iodide and thyroxine synthesis in the thyroid (Donko et al., 2005; Geiszt and Leto, 2004). Duox1 is the major oxidase apically expressed in mucosal surfaces of the trachea, bronchi and large airways (Geiszt et al., 2003; Shao and Nadel, 2005). Duox1 is dramatically upregulated during alveolar maturation in the late gestational period in fetal lung epithelium and expression levels are then maintained in the adult lung (Fischer et al., 2007). In addition, other studies demonstrated that in lung epithelial cells message of Duox1 was modestly increased by Th2-specific cytokines interleukin (IL)-4 and IL-13, while the Th1-specific cytokine IFN-γ as well as bacterial infection markedly increased Duox2 transcripts (Ha et al., 2005; Harper et al., 2005).

In the airways a similar H₂O₂-providing role has been suggested for Duox1 and Duox2 in conjunction with lactoperoxidase (LPO). LPO, which is largely found in milk, saliva, tears and mucosal secretions, can utilize H₂O₂ to oxidize the pseudohalide thiocyanate (SCN⁻) into hypothyocyanate (OSC(N)⁻), an oxidant which is highly effective against Gram-positive and Gram-negative bacteria (Forteza et al., 2005; Geiszt and Leto, 2004). Hence, like the phagocyte NADPH oxidase, Duoxs are thought to be involved in antimicrobial defense mechanisms in the lung. However, their roles may be
divergent, with Duox1 being the constitutive isoform monitoring long term changes and Duox2 as the inducible airway isoform, important for observation of pathologic changes (Harper et al., 2005).

1.3.3 Function of Duox in other species
NADPH oxidases have been conserved in many eukaryotes and Duox enzymes have been identified in other species. The murine isoform of Duox1 was associated with B-cell receptor (BCR) -dependent ROS generation and its suppression led to inhibition of the early ROS response, which is normally obtained upon triggering of the BCR (Singh et al., 2005). Urchin oxidase 1 (Udx1), a Duox isozyme found in sea urchins Strongylocentrotus purpuratus and Lytechinus variegatus, has a critical role during fertilization, as it supplies H$_2$O$_2$ that leads to cross-linking and therefore hardening of the fertilization envelope, thus preventing polyspermy and protecting the embryo (Wong et al., 2004). Another example of extracellular matrix-modifying activity of Duox enzymes is the process of protein cross-linking in Caenorhabditis elegans. Ce-Duox was shown to mediate oxidative cross-linking of tyrosine residues during cuticle biosynthesis, thereby stabilizing the extracellular matrix structure, which provides exoskeletal support for the nematode. A recent in vivo study demonstrated that the Drosophila melanogaster homologue of Duox1, termed dDuox, is indispensable for innate gut immunity. Silencing of dDuox in adult flies resulted in markedly elevated mortality rates, upon bacterial infection through contaminated food. Re-introduction of full-length dDuox reversed the phenotype. However, reconstitution of a dDuox-truncation variant lacking the extracellular peroxidase-homology domain did not augment the survival rates and flies were still as susceptible to infection as complete dDuox-deficient flies. ROS generated by dDuox was essential for the generation of an antimicrobial environment in the gut, thereby demonstrating for the first time that Duox is involved in oxidant-mediated antimicrobial response in mucosal barrier epithelia (Ha et al., 2005).

1.3.4 Potential functions for Duox in the airway epithelium
In analogy to the function of Ce-Duox, the human Duox enzymes might have a similar role in stabilizing the epithelial barrier. As they are abundantly expressed in the epithelial cells of the airways, the proteins might be able to oxidize matrix-proteins or other extracellular substrates through the peroxidase homology domain (Lambeth, 2004). Duox enzymes are further involved in homeostatic processes in the lung epithelium. Duox1 was recently linked to epithelial mucin expression, and accelerated cell migration and wound healing in vitro via a Duox1-H$_2$O$_2$-TACE-proligand-EGF receptor cascade (Koff et al., 2006; Kuwahara et al., 2007; Shao and Nadel, 2005; Wesley et al., 2007). Further, Duox1 was connected to acidification of the airway surface liquid (ASL) via epithelial generation of H$^+$ (FIGURE 6). The composition of the airway surface liquid and its pH (pH$_{\text{ASL}}$) are of critical importance for the normal function of the airways, including mucociliary clearing and microbial killing (Schwarzer et al., 2004). Abrogation of the pH$_{\text{ASL}}$ can negatively impact homeostatic functions in the epithelium, such as ciliary beating, ion transport and epithelial integrity. Activity of the NADPH oxidase domain generates intracellular H$^+$ accumulation, resulting in a gradient across the apical plasma membrane. This drives acid secretion and can therefore be utilized to fine tune the pH of the ASL (pH$_{\text{ASL}}$) (Schwarzer et al., 2004). Moreover, intracellular acidification activates Na$^+/H^+$ exchangers, NHE, which are ubiquitously expressed and regulate cell volume, shape, migration, adhesion and proliferation (van der Vliet, 2008). In addition, Duox might actively contribute to host defense by killing invading microorganisms which escaped the primary destruction in the airway surface liquid in a Nox2-similar manner (Kyle et al., 1990; Lambeth, 2004).
1.4 Duox in disease

Based on their ability to produce ROS, which in turn are involved in a multiplicity of cellular pathways, it is conceivable that a de-regulation of Duox enzymes could have severe consequences for a cell, tissue, organ or consequently, the host. Indeed, it has been demonstrated that Duox2 has an essential role in hormone biosynthesis. To date, 12 point mutations in the sequence of Duox2 have been identified, which all lead to nonsense mutations or premature stop codons, deleting the functional domains of the enzyme (Figueiredo et al., 2001; Moreno et al., 2002; Ohye et al., 2008; Pfarr et al., 2006; Varela et al., 2006; Vigone et al., 2005). The phenotype of these mutations is dominant and biallelic mutations of the gene result in severe hypothyroidism, while a heterozygous mutation still causes mild hypothyroidism. Similarly, homozygous mutation of DuoxA2, encoding for a truncated protein that lacks the fifth transmembrane domain and the cytoplasmic C-terminus, leads to hypothyroidism (Zamproni et al., 2008). This disease is solely attributable to inactivity of Duox2. Duox1 is not able to compensate for mutations of Duox2, suggesting that both enzymes have different roles in the thyroid.

Expression levels of both, Duox1 and Duox2, have not been found to be altered in other diseases of the thyroid, including Graves’ thyroid tissue, toxic adenoma, multinodular goiter and hypofunctioning adenoma. In thyroid carcinomas, however, Duox levels were variable and the proteins showed a cytoplasmic localization, in contrast to the apical membrane location in normal thyroid tissue. Duox expression was increased in papillary thyroid carcinoma, although the expression levels could not be linked to certain stages of the disease (Gerard et al., 2003; Lacroix et al., 2001).

No direct causality has yet been shown between Duox de-regulation and lung disease. However, several studies have shown that transcriptional levels of Duox1 and Duox2 are affected during the emergence of certain lung pathologies and diseases. Cystic fibrosis, which is also known as mucoviscidosis, is a hereditary disease characterized by thick mucus production and compromised airway immunity, resulting in recurrent lung infections. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a channel that facilitates the transport of anions such as chloride (Cl⁻) or thiocyanate (SCN⁻) across the membrane. Inactivating mutations of the CFTR channel lead to a defect in SCN⁻ secretion. Consequently, LPO cannot use Duox-based H₂O₂ to catalyze the oxidation of SCN⁻ into the powerful antimicrobial hypoiodite (OSCN⁻), resulting in impaired immunity in the lung (Moskwa et al., 2007). In addition, a recent study demonstrated a
significant downregulation of Duox2 in cystic fibrosis in vivo, thus further substantiating the relationship between impaired host defense and the Duox-LPO airway defense system (Wright et al., 2006). Defective Cl⁻ transport triggers deficient water transport into the airway surface liquid due to annihilated osmotic force. This causes thick and sticky mucus, which in turn slows clearance of the mucus and promotes formation of mucus plaques that provide nests for infection (Donaldson et al., 2006).

In asthma, levels of the Th2 cytokines IL-4 and IL-13 are upregulated, leading to enhanced Duox1 expression. A connection has been suggested between its expression and the disease, as Duox1 has been associated with airway acidification, enhanced EGFR activation and mucus hypersecretion, which are all characteristics of asthma and other inflammatory airway diseases, such as cystic fibrosis and COPD (van der Vliet, 2008). Chronic obstructive pulmonary disease (COPD) is an umbrella term for lung diseases such as emphysema, chronic bronchitis, bronchiectasis and refractory asthma, which are all delineated by permanent, incurable reduction of lung capacity. COPD is caused by smoking and characterized by shortness of breath, chronic cough, excessive production of phlegm (mucus), wheezing, recurring respiratory infections and damage to the lungs (Barnes, 2000). A direct impact of chronic smoking and the development of COPD has been shown, as both Duox1 and Duox2 are downregulated in COPD patients, which have been former smokers. Long-term exposure to cigarette smoke alone diminished only Duox1, while Duox2 was upregulated (Nagai et al., 2008). Cigarette smoke contains many cancerous and toxic substances, of which particularly thiol-reactive compounds were linked to H₂O₂ production in airway cells through activation of Duox (Jaimes et al., 2004; Lavigne and Eppihimer, 2005).

Owing to their ability to generate ROS, Duox de-regulation could lead to an increase of DNA strand breaks, mutations and deletions. As many chronic diseases of the respiratory tract are associated with increased oxidative stress and enhanced ROS production, Duox enzymes are an interesting candidate in connection with the onset of lung cancer and its progression.
1.5 Specific Aims

The family of NADPH oxidases has been associated with cell transformation, differentiation, inflammation and host defense. Based on their expression in the airway epithelium of the lung and their ability to generate ROS, Duox1 and Duox2 specifically have to be taken into consideration as mediators of host defense and pro-inflammatory effects. To date, not much is known about the spatio-temporal regulation of these enzymes, nor if lung malignancy involves Duox de-regulation. Therefore, this thesis focuses primarily on two aspects of Duox biology in the airway epithelium – Duox regulation in lung epithelial cells and its expression in lung cancer. Expression of Duox will also be analyzed in rodents, as particularly mice and rats have proven to be indispensable models for studies of human diseases. This study will include the following goals:

Objective 1: Function, regulation and expression of Duox enzymes in lung epithelial cells
To elucidate the localization and regulation of functional Duox proteins, human lung cancer cells will be utilized for exogenous expression of Duox and DuoxA protein. Further, primary lung epithelial cells will be used for analysis of endogenous expression of Duox in undifferentiated cells as well as highly differentiated, three dimensional systems.

Objective 2: Characterization of Duox expression in primary human lung cancer
To decipher if Duox expression is altered in human lung cancer, cancer cell lines and primary lung epithelial cells will be probed for expression of Duox and DuoxA. Using tissue samples derived from patients with primary lung carcinomas, expression will also be analyzed in vivo. If Duox expression is altered, its contribution to cell transformation will be investigated in vitro.

Objective 3: Evaluation of Duox expression and function in potential lung disease models
To evaluate if rodent models are applicable for studies of lung disease with respect to Duox expression and function, expression of the Duox and DuoxA homologs will be analyzed in the airways of mice and rats. Functional studies will allow correlation between the human and the rodent isoform. This might facilitate the generation of disease models, in which Duox function could be linked to certain stages or types of lung pathologies.
2 Materials and Methods

2.1 Chemicals and Reagents

Albumin Standard from BSA
Albumin bovine serum, fatty acid free, low endotoxin
Ampicillin (Na salt)
Aprotinin
APS (ammonium persulfate)
5-Aza-2'- deoxyctydine
β-mercaptoethanol
Bromophenolblue
Catalase
Collagen (human placenta, type VI)
Coverslips (BD Biocoat Collagen glass coverslips)
Coverslips (photoetched)
DAPI (4',6-diamidino-2-phenylidole)
DEPC (diethyl pyrocarbonate)
DMSO (dimethyl sulfoxide)
dNTP
DPI (Diphenylenedioninium chloride)
DSP (dithiobis(succinimidyl-propionate))
Ethanol
Ethidiumbromide
EDTA (ethylenediaminetetraacetic acid)
Fibronectin (bovine plasma)
Fluoromount G
Glacial acetic acid
Glycerol
Glycine
Goatserum
GoTaq DNA Polymerase
H₂O₂ (Hydrogen peroxide)
Homovanillic Acid
Horseradish peroxidase
Ionomycin
Leupeptin
Lipofectamine reagent (2mg/ml)
Mayer’s Hematoxylin
Methanol
MTT
Na-Cholate
Na-Citrate
NaCl (sodium chloride)
NaF (sodium fluoride)
NaNO₃ (sodium azide)
NaOH (sodium hydroxide)
Na₃VO₄ (Sodium orthovanadate)
NEM (N-ethylmaleimide)
NP-40 (Nonidet P-40)
NuSerum V

Pierce, Rockford, IL
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
BD Biosciences, San Jose, CA
Belco Glass, Vineland, NJ
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Promega, Madison, WI
Sigma-Aldrich CO, St. Louis, MO
Pierce, Rockford, IL
Aaper Alcohol, Shelbyville, KY
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Southern Biotech, Birmingham, AL
Fisher Scientific, Hampton, NH
Sigma-Aldrich CO, St. Louis, MO
J.T. Baker, Phillipsburg, NJ
Gibco Invitrogen, Carlsbad, CA
Promega, Madison, WI
Fisher Scientific, Hampton, NH
Sigma-Aldrich CO, St. Louis, MO
Calbiochem, San Diego, CA
Sigma-Aldrich CO, St. Louis, MO
Invitrogen, Carlsbad, CA
Sigma-Aldrich CO, St. Louis, MO
Fisher Scientific, Hampton, NH
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Fisher Biotech, Fair Lawn, NJ
Fisher Scientific, Hampton, NH
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Calbiochem, San Diego, CA
Sigma-Aldrich CO, St. Louis, MO
Calbiochem, San Diego, CA
BD Biosciences, San Jose, CA
Paraformaldehyde
PBS (phosphate-buffered saline)
PLUS Reagent (3 mg/ml)
PMA (Phorbol-12-myristate-13-acetate)
PMSF (Phenylmethylsulphonylfluoride)
Polybrene (hexadimethrine bromide)
Poly-L lysine
Protease inhibitor, complete mini, EDTA-free
ProtoGel
Restore Western Blot Stripping Buffer
Ponceau’s S
Sae Kem LE Agarose
Scott’s Tap water Substitute
SDS (sodium dodecyl sulfate)
SA (succinyl acetone)
Superoxidizedismutase (Marchetti et al.)
SuperSignal West Dura Extended Duration Substrate
TaqMan Universal PCR Master Mix (no AmpErase UNG)
TaqMan Fast Universal PCR Master Mix (no AmpErase UNG)
TEMED (N,N,N',N'-tetramethylethlenediamine)
Thapsigargin
TSA (Trichostatin A)
Trans-Blot, pure nitrocellulose
Tris base
Trizol
Triton X-100
Tween-20 (Polyoxyethylene-sorbitan-mono-laurate)
Xelenes

2.1.1 Kits
Aminoethyl carbazole (AEC) Staining Kit
BCA Protein Assay Reagent A+B
DNeasy Mini Kit
EZ DNA Methylation Kit
High Capacity cDNA Reverse Transcription Kit
PureLink Quick Gel Extraction Kit
QIAprep Spin Miniprep Kit
Qiashredder
Quick-Dip Stain
RNaseasy Mini Kit
SuperScript™ II Reverse Transcriptase
TOPO TA Cloning Kit

2.1.2 Molecular Weight Markers
100bp DNA ladder
Benchmarker Pre-Stained protein ladder
PageRuler Plus Prestained protein ladder

Electron Microscopy Sciences, PA
Gibco Invitrogen, Carlsbad, CA
Invitrogen, Carlsbad, CA
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Roche Diagnostics, Indianapolis, IN
Nat. Diagnostics, Atlanta, GA
Thermo Scientific, Waltham, MA
Fisher Scientific, Hampton, NH
Lonza Walkersville, Walkersville, MD
Fisher Scientific, Pittsburgh, PA
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Pierce, Rockford, IL
Applied Biosystems, Foster City, CA
Applied Biosystems, Foster City, CA
Sigma-Aldrich CO, St. Louis, MO
Invitrogen, Carlsbad, CA
Sigma-Aldrich CO, St. Louis, MO
Bio-Rad Laboratories, Hercules, CA
Calbiochem, San Diego, CA
Invitrogen, Carlsbad, CA
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Sigma-Aldrich CO, St. Louis, MO
Invitrogen, Carlsbad, CA
Invitrogen, Carlsbad, CA
Invitrogen, Carlsbad, CA
Invitrogen, Carlsbad, CA
Fermentas, Hanover, MD
### Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody diluent</td>
<td>50 ml 10x TBS, 5 g BSA, 15 ml goat serum, 1 ml Tween-20, ad 500 ml dH₂O</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>50 ml 10x TBS, 15 g BSA, 50 ml goat serum, 50 µl 20% NaN₃, ad 500 ml dH₂O</td>
</tr>
<tr>
<td>DEPC - H₂O</td>
<td>200 µl DEPC, ad 100 ml ddH₂O</td>
</tr>
<tr>
<td>HVA - solution</td>
<td>100 µM homovanillic acid, 4 U/ml horseradish peroxidase (HRP) in PBS with Ca²⁺, Mg²⁺</td>
</tr>
<tr>
<td>HVA - stop buffer</td>
<td>0.1 M NaOH, 25 mM EDTA, 0.1 M glycine, pH 12.0</td>
</tr>
<tr>
<td>IP-buffer (2x)</td>
<td>50 mM Tris/HCl (pH 7.4), 300 mM NaCl, 100 mM glycine, 1% TritonX-100</td>
</tr>
<tr>
<td>Laemmli buffer (4x)</td>
<td>40 ml 0.5M Tris-HCl, pH 6.8, 20 ml glycerol, 40 ml 10% SDS in dH₂O, 4 ml β-mercaptoethanol, pinch Bromophenolblue</td>
</tr>
<tr>
<td>Ponceau’s S</td>
<td>0.5 g Ponceau S, 1 ml glacial acetic acid, 100 ml dH₂O</td>
</tr>
<tr>
<td>Relaxation buffer</td>
<td>100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES (pH 7.4)</td>
</tr>
<tr>
<td>RIPA lysis buffer</td>
<td>150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Na-cholate, 1% NP-40, 0.1% SDS in ddH₂O</td>
</tr>
</tbody>
</table>
**2 MATERIALS AND METHODS**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SDS running buffer (10x)</strong></td>
<td>30 g Tris base</td>
</tr>
<tr>
<td></td>
<td>140 g Glycine</td>
</tr>
<tr>
<td></td>
<td>10 g SDS</td>
</tr>
<tr>
<td></td>
<td>ad 1000 ml dH₂O</td>
</tr>
<tr>
<td><strong>TAE (50x)</strong></td>
<td>242 g Tris base</td>
</tr>
<tr>
<td></td>
<td>57.1 ml glacial acetic acid</td>
</tr>
<tr>
<td></td>
<td>100 ml 0.5 M EDTA, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>ad 1000 ml ddH₂O</td>
</tr>
<tr>
<td><strong>TBS</strong></td>
<td>0.2 M Tris base</td>
</tr>
<tr>
<td></td>
<td>1.4 M NaCl</td>
</tr>
<tr>
<td></td>
<td>in dH₂O</td>
</tr>
<tr>
<td><strong>TBS-T (10x)</strong></td>
<td>0.2 M Tris base</td>
</tr>
<tr>
<td></td>
<td>1.4 M NaCl</td>
</tr>
<tr>
<td></td>
<td>0.5% Tween-20, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>in dH₂O</td>
</tr>
<tr>
<td><strong>TE</strong></td>
<td>1 M Tris-HCl (pH 7.5 or 8.0)</td>
</tr>
<tr>
<td></td>
<td>0.5 M EDTA</td>
</tr>
<tr>
<td></td>
<td>in dH₂O</td>
</tr>
<tr>
<td><strong>Transfer buffer (10x)</strong></td>
<td>250 mM Tris base</td>
</tr>
<tr>
<td></td>
<td>1.92 M glycine</td>
</tr>
<tr>
<td></td>
<td>in dH₂O</td>
</tr>
<tr>
<td><strong>Transfer buffer (1x):</strong></td>
<td>100 ml 10x transfer buffer</td>
</tr>
<tr>
<td></td>
<td>150 ml methanol</td>
</tr>
<tr>
<td></td>
<td>4 ml 10% SDS</td>
</tr>
<tr>
<td></td>
<td>750 ml ddH₂O</td>
</tr>
</tbody>
</table>
### 2.1.4 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tubulin (DM1)</td>
<td>Mouse</td>
<td>1:1000 (WB)</td>
<td>Sigma</td>
</tr>
<tr>
<td>β-tubulin IV</td>
<td>Mouse</td>
<td>1:200 (IF)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>Rabbit</td>
<td>1:500 (IF)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Alexa Fluor 568</td>
<td>Rabbit</td>
<td>1:500 (IF)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Alexa Fluor 568</td>
<td>Mouse</td>
<td>1:500 (IF)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>anti-rabbit HRP</td>
<td>Goat</td>
<td>1:10000 (WB)</td>
<td>Southern Biotech</td>
</tr>
<tr>
<td>anti-mouse HRP</td>
<td>Goat</td>
<td>1:10000 (WB)</td>
<td>Southern Biotech</td>
</tr>
<tr>
<td>Duox1/2 (aa 775-1026 of hDuox1)</td>
<td>Rabbit</td>
<td>1:200 (IF); 1:1000 (WB)</td>
<td>homemade</td>
</tr>
<tr>
<td>Duox1 (aa 988-1011 of hDuox1)</td>
<td>Rabbit</td>
<td>1:200 (IF); 1:1000 (WB)</td>
<td>homemade</td>
</tr>
<tr>
<td>Duox2 (aa 634-648 of hDuox2)</td>
<td>Rabbit</td>
<td>1:200 (IF); 1:1000 (WB)</td>
<td>homemade</td>
</tr>
<tr>
<td>DuoxA1 (aa 280-299 of hDuoxA1-2)</td>
<td>Rabbit</td>
<td>1:500 (IF); 1:5000 (WB)</td>
<td>OpenBiosystems</td>
</tr>
<tr>
<td>GFP</td>
<td>Mouse</td>
<td>1:100 (IP)</td>
<td>Genetex</td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit</td>
<td>1:1000 (WB)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>GST</td>
<td>Mouse</td>
<td>1:200 (IP)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>MUC5A (45M1)</td>
<td>Mouse</td>
<td>1:200 (IF)</td>
<td>Neomarker</td>
</tr>
<tr>
<td>c-myc (9E10)</td>
<td>Mouse</td>
<td>1:200 (IF)</td>
<td>Upstate</td>
</tr>
<tr>
<td>c-myc</td>
<td>Rabbit, mono</td>
<td>1:200 (IF); 1:1000 (WB)</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Biotinyl, IgG conjugate</td>
<td>Rabbit</td>
<td>1:200 (IHC)</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>Streptavidin, HRP conjugate</td>
<td>Rabbit</td>
<td>1:200 (IHC)</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

**Table 2: Antibody list**

### 2.1.5 Rodent primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' → 3')</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin Fwd (rat+mouse)</td>
<td>TGACGGGGTCACCCCCACACTGTGCCCACCTTA</td>
<td>60°C</td>
</tr>
<tr>
<td>β-actin Rev (rat+mouse)</td>
<td>CTAGAAGCATTTCGCGGGACATGGGAGGG</td>
<td>64°C</td>
</tr>
<tr>
<td>Mouse Duox1 Fwd</td>
<td>CTGTGACATTACAGGCGCTGGCTGGCTGGCTGG</td>
<td>60°C</td>
</tr>
<tr>
<td>Mouse Duox1 Rev</td>
<td>TGAGCCTGCCATCCTACTGAATATCCTGGAATC</td>
<td>60°C</td>
</tr>
<tr>
<td>Mouse Duox2 Fwd</td>
<td>CCCAGAAGGAAGGTTCCAGACAG</td>
<td>60°C</td>
</tr>
<tr>
<td>Mouse Duox2 Rev</td>
<td>GTAGCTATCAGAATACAGGACTGGAGGG</td>
<td>62°C</td>
</tr>
<tr>
<td>Mouse DuoxA1 Fwd</td>
<td>GCGCGCTACATACATCCTTC</td>
<td>62°C</td>
</tr>
<tr>
<td>Mouse DuoxA1 Rev</td>
<td>GTGCCTGAATGACTGGTGAAG</td>
<td>62°C</td>
</tr>
<tr>
<td>Mouse DuoxA2 Fwd</td>
<td>CGTGGACGAGGGGTGCTAC</td>
<td>62°C</td>
</tr>
<tr>
<td>Mouse DuoxA2 Rev</td>
<td>GCAGACCGACATGGGACTGAAC</td>
<td>62°C</td>
</tr>
<tr>
<td>Rat Duox1 Fwd</td>
<td>ATCAGGCAGATGGCAAGGGACTC</td>
<td>61°C</td>
</tr>
<tr>
<td>Rat Duox1 Rev</td>
<td>GGGGATGACGGCTGTCTCCG</td>
<td>61°C</td>
</tr>
<tr>
<td>Rat Duox2 Fwd</td>
<td>CCGGTTGGGAATCTATCCCTG</td>
<td>60°C</td>
</tr>
<tr>
<td>Rat Duox2 Rev</td>
<td>AGTTGGATGACGCTGGGACAC</td>
<td>60°C</td>
</tr>
<tr>
<td>Rat DuoxA1 Fwd</td>
<td>GCCTGCTACGCGGTGGTGAC</td>
<td>60°C</td>
</tr>
<tr>
<td>Rat DuoxA1 Rev</td>
<td>GCAGCGCGACGACATGGG</td>
<td>60°C</td>
</tr>
<tr>
<td>Rat DuoxA2 Fwd</td>
<td>GGCTGGAGGCGGCTGTTATAC</td>
<td>64°C</td>
</tr>
<tr>
<td>Rat DuoxA2 Rev</td>
<td>CTCAAGGACGACGCTGGTC</td>
<td>64°C</td>
</tr>
</tbody>
</table>

**Table 3: Rodent primer list**
### 2.1.6 Human Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin Fwd</td>
<td>TGA CGGG GTGCA CCCAC ACTGT GCCCCATCTA</td>
<td>60°C</td>
</tr>
<tr>
<td>β-actin Rev</td>
<td>CTA GAAG CATTTG CCGTG ACGAT GAGGG</td>
<td></td>
</tr>
<tr>
<td>Duox1 Fwd</td>
<td>CA AGC ATCA AACCCT GCAT CTCTC</td>
<td>65°C</td>
</tr>
<tr>
<td>Duox1 Rev</td>
<td>CTGC AATCT ACCAC ACCGAT CTGC</td>
<td></td>
</tr>
<tr>
<td>Duox2 Fwd</td>
<td>CCGC AATCT CATCT ATGAGGT</td>
<td>58°C</td>
</tr>
<tr>
<td>Duox2 Rev</td>
<td>TTTG ATGT ACGT ACCAG CCA</td>
<td></td>
</tr>
<tr>
<td>DuoxA1-1 Fwd</td>
<td>GACGT CAGT CACC AACCCT GATCA CA GAGG</td>
<td>63°C</td>
</tr>
<tr>
<td>DuoxA1-1 Rev</td>
<td>GACAG CTTG AACT ACCT GC CAC</td>
<td></td>
</tr>
<tr>
<td>DuoxA1-2 Fwd</td>
<td>GTC AGC ACAA AATTCT ACATCA AA GAGG</td>
<td>65°C</td>
</tr>
<tr>
<td>DuoxA1-2 Rev</td>
<td>CTGC ACAA AAGCC CCCAG AC</td>
<td></td>
</tr>
<tr>
<td>DuoxA1-3 Fwd</td>
<td>GTC AGC ACAA AATTCT ACATCA AA GAGG</td>
<td>59°C</td>
</tr>
<tr>
<td>DuoxA1-3 Rev</td>
<td>GAGAG CAGC ACGT TGGAGG</td>
<td></td>
</tr>
<tr>
<td>DuoxA2 Fwd</td>
<td>ACG CTT GCT CAT CTG CG</td>
<td></td>
</tr>
<tr>
<td>DuoxA2 Rev</td>
<td>TCC CAGG GCG TG GAGG</td>
<td></td>
</tr>
<tr>
<td>Alu Fwd</td>
<td>AGTTTT ATGGA ATTTGT GAAG</td>
<td>54°C</td>
</tr>
<tr>
<td>Alu Rev</td>
<td>ACCTA ACTCAA CAACTAAC</td>
<td></td>
</tr>
<tr>
<td>Duox1-Universal-a Fwd</td>
<td>AGTTTT ATGGA ATTTGT GAAG</td>
<td>64°C</td>
</tr>
<tr>
<td>Duox1-Universal-a Rev</td>
<td>ACCTA ACTCAA CAACTAAC</td>
<td></td>
</tr>
<tr>
<td>Duox1-MSP-a Fwd</td>
<td>CGTTAG ATTTT ATTTCC GTTTT ATGCTTG TGGTTTTC</td>
<td>64°C</td>
</tr>
<tr>
<td>Duox1-MSP-a Rev</td>
<td>GATAC CTCTCTA ACTCTA ACCTAC GGGAC</td>
<td></td>
</tr>
<tr>
<td>Duox1-USP-a Fwd</td>
<td>GAT GG AATTTTTT TATTTG TGTTTGT</td>
<td>58°C</td>
</tr>
<tr>
<td>Duox1-USP-a Rev</td>
<td>CCAA TACCT CACCTAC ACTAC ACCAA</td>
<td></td>
</tr>
<tr>
<td>Duox1-Universal-b Fwd</td>
<td>ATGG GATTTT GTG AAAG GTATTTG</td>
<td>60°C</td>
</tr>
<tr>
<td>Duox1-Universal-b Rev</td>
<td>CTTAT CT CACCT AACT-A ACT AAAC</td>
<td></td>
</tr>
<tr>
<td>Duox1-MSP-b Fwd</td>
<td>TGG TTT CCGG AGTTTT TC GTC</td>
<td>70°C</td>
</tr>
<tr>
<td>Duox1-MSP-b Rev</td>
<td>CTTA AC CGC AACC GAAG</td>
<td></td>
</tr>
<tr>
<td>Duox2-Universal-a Fwd</td>
<td>GATG AGT ACGA AAG TTTG TGT</td>
<td>54°C</td>
</tr>
<tr>
<td>Duox2-Universal-a Rev</td>
<td>CACT C TCAC CTCT TCTC</td>
<td></td>
</tr>
<tr>
<td>Duox2-MSP-a Fwd</td>
<td>GTA GTCT AGT TTTTTTT TTTTTG TC GTC</td>
<td>63°C</td>
</tr>
<tr>
<td>Duox2-MSP-a Rev</td>
<td>CTA AAAC CT ACCCT CCTCC ACTGTA</td>
<td></td>
</tr>
<tr>
<td>Duox2-USP-a Fwd</td>
<td>AGTAGT GGGAT GTG AAGTTTGT G</td>
<td>64°C</td>
</tr>
<tr>
<td>Duox2-USP-a Rev</td>
<td>ACT AA CTTA CT ACCAC ACTAC AT A</td>
<td></td>
</tr>
<tr>
<td>Duox2-Universal-b Fwd</td>
<td>TGGGT TG AGT TTG ATGGTTTG T</td>
<td>60°C</td>
</tr>
<tr>
<td>Duox2-Universal-b Rev</td>
<td>CCAAC TCA AAA ACC CCAACT AA AACT</td>
<td></td>
</tr>
<tr>
<td>Duox2-MSP-b Fwd</td>
<td>TTAG TC GG CAG CGA AAATA</td>
<td>70°C</td>
</tr>
<tr>
<td>Duox2-MSP-b Rev</td>
<td>CG TAAA A ACGGTG CCCA</td>
<td></td>
</tr>
<tr>
<td>BSP-Duox1 Fwd</td>
<td>GGTTG TGTT AAAG AAGGA</td>
<td>59°C</td>
</tr>
<tr>
<td>BSP-Duox1 Rev</td>
<td>CCCAAA ACTC CCCTCC AT TA TATAT</td>
<td></td>
</tr>
<tr>
<td>BSP-Duox2 Fwd</td>
<td>GAAAGG GGTT TGTG AG TATAG</td>
<td>58°C</td>
</tr>
<tr>
<td>BSP-Duox2 Rev</td>
<td>AA ACGCT TT AT TA CACA AACC CT CT CT C</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4: Human Primer List**
2.2 Technical Equipment

2100 Rainbow laser scanning confocal microscope
5415C Eppendorf Centrifuge
5810R Eppendorf Centrifuge
AB 104 Scale
AccuBlock Digital Dry Bath
Accumet AB15 pH meter
Alphaimager 2200
Autoclave Amsco Scientific series
Axiovert 100 Microscope
Beckman GS-6KR
Beckman J2-2A
Biological Safety Cabinets
Bio-Rad Power Pac 1000
Dry Bath Incubator
Fisher Stirring Hotplate
Fisher Vortex Genie 2
HE 33 mini horizontal submarine unit
Incubator, NAPCO CO2
Isotemp Incubator
IsoTemp 215 waterbath
LS55 Chemiluminescence Photometer
Microfuge 22R Centrifuge
Microplate Luminometer LB 96 V
NanoDrop ND-1000 Spectrophotometer
Nikon Eclipse E800
Nikon Eclipse TE2000-U
Olympus IX70 Microscope
Optima Microcentrifuge
PB3002 Scale
PCR-Express
ProteinPower Pac 200
Protein Bio-Rad Mini Protean II cell
PS 500 XT DC Power Supply
Rocker Platform
SRX-101A Medical Film Processor
STX2 Electrode
Synergy HT
Tissue Tearar
UV/Visible Spectrometer Ultrospec 3000
VERSAmex Microplatereader

Bio-Rad (Zeiss), Hercules, CA
Brinkman Instruments Inc, Westbury, NY
Brinkman Instruments Inc., Westbury, NY
Mettler Toledo, Switzerland
Labnet International Inc., Edison, NJ
Fisher Scientific, Hampton, NH
Alpha Innotech, San Leandro, CA
STERIS Corporation, Mentor, Ohio
Zeiss, Göttingen, Germany
Beckman Coulter, Fullerton CA
Beckman Coulter, Fullerton CA
Nuaire, Plymouth, MN
Bio-Rad, Hercules, CA
Fisher Scientific, Hampton, NH
Fisher Scientific, Hampton, NH
Fisher Scientific, Hampton, NH
Fisher Scientific, Hampton, NH
Perkin Elmer Instruments, Wellesley, MA
Beckman Coulter, Fullerton, CA
EG + G Berthold, Bad Wildbach, Germany
Nanodrop Technologies, Wilmington, DE
Nikon, Melville, NY
Nikon, Melville, NY
Olympus, Center Valley, PA
Beckman Coulter, Fullerton, CA
Mettler Toledo, Switzerland
Thermo Hybaid, Heidelberg, Germany
Bio-Rad, Hercules, CA
Amersham Bioscience, Uppsala, Sweden
Hoefer Scientific Instruments
Bellco Glass, Vineland NJ
Konica Minolta, Wayne, NJ
World Precision Instruments, Sarasota, FL
BioTek Instruments Inc., Winooski, VT
Biospec Products, Inc., Bartlesville, OK
Pharmacia Biotech, Cambridge UK
Molecular Devices, Sunnyvale, CA

2.2.1 Computer Programs

AxioVision AC 4.2
Bio-Rad LaserSharp 2000
EndNote X
Imaris 5
Image J
LSM Image Analyzer
Methamorph
Spectra Alias

Zeiss, Göttingen, Germany
Bio-Rad, Hercules, CA
EndNote, Carlsbad, CA
Bitplane Inc., Saint Paul, MN
NIH, Bethesda, MD
Zeiss, Göttingen, Germany
Molecular Devices Corp., Downingtown, PA
DeltaSoft/Biometallics, Princeton, NJ
2.2.2 Internet Programs

http://bisearch.enzim.hu/
http://ca.expasy.org/
http://cpgislands.usc.edu/
http://genome.ucsc.edu/cgi-bin/hgBlat
http://phobius.sbc.su.se/
http://psort.ims.u-tokyo.ac.jp/form2.html
http://symatlas.gnf.org/SymAtlas/
http://www.basic.northwestern.edu/biotools/oligocalc.html
http://www.ebi.ac.uk/Tools/clustalw2/index.html
http://www.ensembl.org/index.html
http://www.enzim.hu/hmmtop/index.html
http://www.urogene.org/methprimer/index1.html

2.3 Tissue Culture

2.3.1 Cell Culture Media

<table>
<thead>
<tr>
<th>Media Description</th>
<th>Supplier Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 with Glutamax</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>MEM</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>DMEM (4.5g/L D-glucose) with Glutamax</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>Ham’s F12 Medium</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>Trypsin-EDTA (1x) 0.05%</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>HEPES Buffer solution 1 M</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>Sodium pyruvate 100mM</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>Sodium bicarbonate solution 7.5%</td>
<td>Sigma-Aldrich CO, St. Louis, MO</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
<td>Gibco Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>SABM + SAGM single quots</td>
<td>Lonza Walkersville Inc., Walkersville, MD</td>
</tr>
<tr>
<td>BEBM + BEGM single quots</td>
<td>Lonza Walkersville Inc., Walkersville, MD</td>
</tr>
</tbody>
</table>

2.3.2 Cell Culture (2D)

Human lung cancer cell lines NCI-H69, NCI-H82, SHP-77, NCI-H292, NCI-H272, NCI-H441, UCLA-P3, NCI-H460, NCI-H661 and NCI-H157 were grown in RPMI 1640 media. Lung cancer cell lines Calu-3, A549 were cultured in MEM and Ham’s F12 media, in the presence of 1.5g/L sodium bicarbonate. HeLa and HEK293T cells were kept in DMEM media. All aforementioned media were supplemented with 10% FCS and 0.01mM HEPES. Human primary lung epithelial cells NHBE and SAEC as well as immortalized normal human lung epithelial cells BEAS-2B and SALE were cultured in SABM media, supplemented with SAGM SingleQuots. All cells were maintained in humidified tissue culture incubators at 37°C and 5% CO₂.
2.3.3 Polarized Airway System (3D)
NHBE and SAEC were cultured in SABM media supplemented with 30µg/ml bovine pituitary extract, 0.5µg/ml hydrocortisone, 0.5ng/ml human recombinant epidermal growth factor, 0.5µg epinephrine, 10µg/ml transferrin, 5µg/ml insulin, 0.1ng/ml retinoic acid, 6.5ng/ml triiodothyronine, 50µg/ml gentamicin, 50ng/ml amphotericin-B and 0.5mg/ml bovine serum albumin-fatty acid free (SingleQuots). Upon 80% confluency cells were split using the Reagent Pack (Lonza) and cells were resuspended in differentiation media, consisting of 50% DMEM and 50% BEBM media, supplemented with 200µg/ml bovine pituitary extract, 0.5µg/ml hydrocortisone, 0.5ng/ml human recombinant epidermal growth factor, 0.5µg epinephrine, 10µg/ml transferrin, 5µg/ml insulin, 1.5µg/ml bovine serum albumin fraction V, 6.5ng/ml triiodothyronine, 50µg/ml gentamicin and 50ng/ml amphotericin-B. The media was also complemented with freshly prepared 50nM all-trans retinoic acid, which induces differentiation. 1.8–2.0*10⁵ cells were seeded on semi-permeable support membranes (24-well; Costar Transwell-clear culture insert, polyester, 0.4µm pore, Corning Costar), which were coated with human placenta collagen, type IV (15µg/cm²). After 2 days the media on top of the membrane was removed and cells were grown and differentiated at an air-liquid interphase (ALI) for up to 21 days.

**FIGURE 7:** Air-liquid system (3D). Primary lung epithelial cells are grown at the air-liquid interphase and differentiate into several cell types as indicated. Adapted from Dr. Mandy Lehmann (The Scripps Research Institute).

2.3.4 Treatments
5-Aza-2’-deoxycytidine (Aza): Lung cancer cell lines were cultured in appropriate medium supplemented with 5% FCS in the absence or presence of 1µM Aza. Due to its instability at warmer temperatures (Lin et al., 1981), the media was changed daily and replenished with fresh Aza.

Trichostatin A (TSA): Lung cancer cells were grown in 5% medium and 5ng/ml TSA was added for 48 hours. Control cells were grown in the presence of vehicle alone.

Succinylacetone (SA): NCI-H661 cells were grown for 4 days in RPMI, containing Heps and 1% NuSerum V in the presence or absence of 10µg/ml SA.

2.3.5 Human and rodent tissue samples
**Human Samples:** A total of eleven paired specimen of primary lung carcinomas and adjacent non-cancerous tissue were obtained from the Cooperative Human Tissue Network (Western Division, Vanderbilt University Medical Center, Nashville, TN), which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects. The tissue samples were collected from surgical resections and quick-frozen in liquid nitrogen immediately after excision and stored at -80°C until further use. The histological type of the cancer and the
corresponding differentiation state is listed in (Figure 8A). Those specimens were used for promoter methylation studies and immunohistochemistry. In addition, 39 primary adenocarcinomas from the Lovelace Respiratory Research Institute (Albuquerque, NM) were analyzed for promoter methylation. Tissue sections from normal human upper lung and diseased lung (cystic fibrosis) were obtained from Pantomics, Inc. (San Francisco).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>INM Mapping Stage Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Large Cell Carcinoma</td>
<td>T3N1 IIA</td>
</tr>
<tr>
<td>2</td>
<td>Squamous Cell Carcinoma</td>
<td>T1NO IA</td>
</tr>
<tr>
<td>3</td>
<td>Squamous Cell Carcinoma</td>
<td>T2NO IB</td>
</tr>
<tr>
<td>4</td>
<td>Squamous Cell Carcinoma</td>
<td>T2NO IB</td>
</tr>
<tr>
<td>5</td>
<td>Squamous Cell Carcinoma</td>
<td>T2NO IB</td>
</tr>
<tr>
<td>6</td>
<td>Squamous Cell Carcinoma</td>
<td>T2N2 IIIA</td>
</tr>
<tr>
<td>7</td>
<td>Adenocarcinoma</td>
<td>T2NO IB</td>
</tr>
<tr>
<td>8</td>
<td>Adenocarcinoma</td>
<td>T2NO IB</td>
</tr>
<tr>
<td>9</td>
<td>Adenocarcinoma</td>
<td>T2NO IB</td>
</tr>
<tr>
<td>10</td>
<td>Adenocarcinoma</td>
<td>T1NO IA</td>
</tr>
<tr>
<td>11</td>
<td>Adenocarcinoma</td>
<td>T2NO IB</td>
</tr>
</tbody>
</table>

Rodent samples: Mice (strains: C57BL/6 and BALB/c) and rats (strain: Sprague Dawley) were obtained from the core rodent breeding facility at The Scripps Research Institute (TSRI) and Harlan Sprague Dawley, Inc. (San Diego, CA), respectively and were housed in a pathogen-free facility. Animals were sacrificed by CO₂ inhalation and tissues were excised and immediately processed. All animal procedures were reviewed by the Institutional Animal Care & Use Committee (IACUC) of TSRI and approved by the Animal Welfare Act.

2.4 Molecular Biology

2.4.1 Nucleic Acid Extraction

2.4.1.1 Total RNA and genomic DNA extraction from cell culture

Total RNA from primary cells and cell lines was isolated using an RNA extraction kit (RNaseasy Mini Kit, Qiagen). Cells were lysed in RLT buffer (provided in the kit) containing β-mercaptoethanol. The cell lysate was applied to a Qiashredder column (Qiagen) and homogenized via microcentrifugation. Total RNA was eluted with 30µl DEPC-treated RNase-free water. DEPC reacts with reactive residues (such as amines, thiol- and hydroxy-groups) of proteins, thus inactivating their biological activity. Genomic DNA (gDNA) was extracted from 2*10⁶ cells using a DNA extraction kit (DNeasy Tissue Kit, Qiagen). Integrity of gDNA was determined by Alu-PCR.
2.4.1.2 Total RNA and genomic DNA extraction from tissue specimen

Total RNA from tissue specimen was isolated with a combination of phenol/chloroform and spin-column purification. 10-20mg of frozen lung tissue was homogenized in 1ml Trizol reagent, using a tissue homogenizer. The aqueous phase was mixed with 1 volume of 75% ethanol after phase separation and transferred to an RNeasy column. RNA was bound to the column and eluted in 50µl RNase-free water. Genomic DNA from tissue specimen was isolated at the same time as total RNA. After complete removal of remaining aqueous phase, gDNA was precipitated from the interphase and underlying organic phase by addition of 300µl 100% ethanol. Samples were inverted and centrifuged at 2,000g for 5 minutes at room temperature. The DNA pellet was washed twice with 1ml 10% ethanol in 0.1M trisodium citrate for 30 minutes at room temperature. Subsequent to each wash, DNA was spun down for 5 minutes at 2,000g at 4°C. After the second wash, the pellet was suspended in 1ml 75% ethanol in order to remove the pinkish color from the DNA. The pellet was briefly air-dried after ethanol removal and resuspended in 50µl 8mM sodium hydroxide by pipetting up and down. Insoluble material was removed by centrifugation at 12,000g for 10 minutes.

2.4.2 Determination of the concentration of RNA or DNA

Concentration of RNA or DNA was determined by measuring the absorbance at 260nm on a Nanodrop spectrophotometer. The following equation was used to calculate the concentration.

\[c[\mu g/\mu l] = \frac{A_{260} \cdot dilution factor \cdot X}{1000}\]

The value for X depends on the type of nucleic acid analyzed: 50 (dsDNA), 40 (RNA), 30 (ssDNA).

2.4.3 cDNA Synthesis

Complementary DNA (cDNA) was synthesized from total RNA using two different approaches, depending on the downstream application. Total RNA (2µg) from cultivated cells was reverse transcribed using 200 units SuperScript II (SSII) and oligo(dT) (both Invitrogen). Reverse transcription was carried out for 50 minutes at 42°C in a 20µl reaction volume, containing First-strand buffer, 0.01M DTT (both supplied with SSII) and RNase inhibitor (40 units, Roche Diagnostics, Indianapolis, IN). Remaining RNA was removed through subsequent incubation with 2 units RNase H (Invitrogen). cDNA generated this way was used for semi-quantitative PCR. Total RNA (1µg) from tissue specimen was reverse transcribed using the High Capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The cDNA (in a final volume of 20µl) was subject to quantitative (real-time) PCR.

2.4.4 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was used to amplify a single or few copies of a DNA fragment. Reactions were performed in a Thermocycler for generally 35 cycles. Particular variations are mentioned in the text.

2.4.4.1 Qualitative (semi-quantitative) PCR

One µl of cDNA from cultivated cells was used for qualitative PCR amplification. For all PCR reactions a master mix was prepared that contained a multiple of the following (25µl total reaction volume):
2.4.4.2 Quantitative (real-time) PCR

cDNA from tissue specimen and SAEC (differentiated for a time course of 21 days) was subject to quantitative real-time PCR analysis utilizing TaqMan™ technology. The cDNA was diluted in water to a final concentration of 10ng/µl, assuming 100% reverse-transcription efficiency during cDNA synthesis. 5µl of the diluted cDNA were mixed with 12.5µl 2xTaqMan® Universal PCR Master Mix (no AmpErase® UNG), 1.25µl 20x gene expression assay probe and 6.25µl sterile water. Each sample (in a 25µl reaction volume) was pipetted in triplicate into a 96-well reaction plate and 3 samples per specimen or time point were analyzed. Gene-specific PCR products were continually measured with an ABI PRISM 7700 or 7900 Sequence Detection System (Applied Biosystems). The probes for Duox1 (Hs00213694_m1, Applied Biosystems), DuoxA1 regulation, Duox2 (Hs00204187_m1) and human β-actin (Hs99999903_m1) were labeled with 6FAM™ dye - MGB and were designed over an exon-exon boundary to eliminate the possibility of genomic DNA amplification. Relative DUOX1, DUOX2 and DUOXA1 mRNA amounts in the different samples were standardized against the amount of actin mRNA and expressed as ∆CT = (CTβ-actin - CTGOI). The mRNA copy ratio of GOI to actin was calculated as 2^ACT and normalized to the highest (tissue specimen) or lowest (SAEC) expression of the respective GOI.

2.4.5 Agarose Gel Electrophoresis

DNA fragments were separated according to their size in 1% (w/v) agarose in 1x TAE buffer, containing 0.5 mg/ml ethidium bromide. 8-10µl PCR product was electrophoresed at 100V. Integrity of total RNA (0.5-2.0µg) was also assessed by electrophoresis, as non-degraded RNA shows 2 distinct bands, corresponding to ribosomal subunits 28S and 18S.

2.4.6 Bisulfite conversion of gDNA and methylation specific PCR

Isolated gDNA (2µg) from cell lines and tissue specimen was subject to bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Orange), converting unmethylated cytosines into uracil (FIGURE 9A). CpG islands in the promoter regions of DUOX1, DUOX2, DUOXA1 and DUOXA2 were identified by utilizing CpG island searcher (Takai and Jones, 2003). A CpG island was defined by the following criteria: GC >55%, observed CpG/expected CpG >0.65, and length >500bp. In the first step of the nested methylation specific PCR, universal primers, which are unbiased for potential CpG conversion sites, were used to amplify a fragment of the DUOX1 and DUOX2 promoter, respectively,
spanning the region of interest. One µl of the universal PCR product was used as template for subsequent methylation (MSP) and unmethylation (USP) specific PCR (FIGURE 9B). Primer sequences are shown in (TABLE 4).

**FIGURE 9:** Bisulfite treatment and methylation-specific primer design. (A), bisulfite converts unmethylated cytosines stoichiometrically to uracil via deamination. Methylated cytosines (5′-methylcytosine) are resistant to bisulfite conversion. (B), isolated genomic DNA is treated with bisulfite, which leads to conversion of all unmethylated cytosines into uracil (U), while methylated cytosines (CH₃) remain as cytosine in the sequence. Two primer sets, which are used in two individual PCR reactions, are designed for the same DNA region, based on the assumption that cytosines of given CpG dinucleotides are unmethylated (USP) or methylated (MSP).

### 2.4.7 TOPO-cloning and Bisulfite sequencing

The region between -894 to +1411 of the DUOX1 and -755 to -52 of the DUOX2 promoter was amplified from the bisulfite modified gDNA, using BSM primers. These sequences comprise 72 (DUOX1) and 39 CpG sites (DUOX2), respectively. As PCR purification is not sufficient to selectively purify the correct sized product, the desired PCR products were gel purified with the Pure Link Quick Gel Extraction Kit and subsequently cloned into the pCR4-TOPO vector (Invitrogen), according to the manufacturer’s instructions. The plasmid vector is supplied linearized with single 3′-thymidine (T) overhangs, thus allowing for easy ligation with the PCR amplicon, as Taq polymerases always add a single deoxyadenosine (A) to the 3′ ends of PCR products, due to their non-template-dependent terminal transferase activity. Upon ligation of the insert with the vector, the construct was chemically transformed into One Shot Mach1™-T1R Competent E. coli cells. Positive selection was achieved through 50 µg/ml ampicillin. Colonies were picked for plasmid purification and at least 8 positive clones per condition were sequenced using M13 primers (supplied with the kit).

### 2.4.8 Lentivirus production and cell transduction

Human Duox1, DuoxA1-2, Duox2 and DuoxA2 were cloned into CGW lentiviral expression vector. Virus particles were produced by transient transfection of HEK293T cells. Approximately 24 hours
prior to transfection, cells were seeded in 5% DMEM high glucose media (2*10^6 cells/10 cm dish, pre-coated with 20µg/ml poly-L-lysine). The following mix was prepared in a 5ml FACS tube:

<table>
<thead>
<tr>
<th>Per 10 cm dish:</th>
<th>10µg</th>
<th>transfer vector plasmid</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>6.5µg</td>
<td>GAG/pol plasmid</td>
</tr>
<tr>
<td></td>
<td>3.5µg</td>
<td>VSV.G plasmid</td>
</tr>
<tr>
<td></td>
<td>2.5µg</td>
<td>pRSV-rev plasmid</td>
</tr>
<tr>
<td></td>
<td>Ad 450µl</td>
<td>with 0.1x TE</td>
</tr>
</tbody>
</table>

While vortexing, 500µl warm 2xHBS and 50µl 2.5M CaCl2 were added dropwise and the mixture was immediately added to the cells. Fresh media was added to the cells after approximately 16 hours. The supernatant was collected 24 and 48 hours post-transfection, sterile filtered through a 0.22µm filter and centrifuged at 19400 rpm for 2.2 hours at 4°C. The liquid was discarded and the virus-pellet resuspended in 5% BSA/PBS, aliquotted and frozen at -80°C. Lung cancer cell lines and primary lung cells were incubated with viral supernatant for 48-72 hours in the presence of 4µg/ml polybrene (Rizzi et al., 2007; Swan et al., 2006).

2.5 Immunodetection of Proteins

2.5.1 Protein isolation and quantification

For isolation of total cell lysate (TCL) cells were frozen at -80°C for a minimum of 30 minutes to disrupt the cell structure. Ice-cold RIPA buffer (containing 10µM Aprotinin, 10µMLeupeptin, 1mM PMSF) was added to the cells and the lysate was scraped off into a microcentrifuge tube, vortexed and incubated on ice for 10 minutes. Cell debris was parted from the solubilized protein by centrifugation at 14,000rpm for 10 minutes at 4°C. Protein concentrations were determined using a BCA assay (Smith et al., 1985). It is critically important that the cell lysates are kept ice-cold at all times, to prevent aggregation of Duox-protein. For isolation of cytosolic and membrane fractions cells were lysed in relaxation buffer (containing 1 mM PMSF, 100 µM leupeptin, 10 µM aprotinin, 1 mM Na3VO4, 25 mM NaF) and briefly sonicated. Cell debris was removed after centrifugation at 2150rpm for 10minutes at 4°C. The supernatant was transferred into microfuge tubes and lysates were ultracentrifuged at 49,000rpm (100,000g) for 30 minutes. The remaining pellet, which corresponds to membrane-associated proteins, was separated from the cytosolic proteins in the supernatant, washed and finally resuspended in Laemmli-buffer.

2.5.2 Immunoblotting (IB)

Frozen protein extracts were thawed on ice. TCL (30µg) was mixed with ice-cold 4x Laemmli buffer and immediately loaded onto 8% (v/v) discontinuous denaturing polyacrylamide gel (Laemmli, 1970). Heating of the lysates must be avoided, as Duox proteins aggregate and will be only detectable as high molecular weight smear. The proteins were transferred to nitrocellulose membranes (2 hours, 0.3A), blocked for 1 hour with 5% non-fat dry milk/TBS-T and subsequently probed with primary antibodies (generally 1:1,000 in 1% BSA/TBS-T) for 1 hour at RT or over night at 4°C. After washing for 3 x 5 minutes with TBS-T, appropriate secondary antibodies (HRP-labeled; 1:10,000) were applied for 1 hour. Membranes were washed again 3 x 5 minutes with TBS-T, followed by short incubation with SuperSignal West Dura Extended Duration Substrate and exposed to autoradiography film.
2.5.3 Immunoprecipitation (IP)
Cells were washed with PBS and proteins were crosslinked by incubation with 2mM DSP in PBS (prepared freshly) at 37°C for 20 minutes. All following steps were carried out at 4°C or on ice. The cells were washed with ice-cold PBS and their proteins were alkylated for 15 minutes with 20mM NEM in PBS. The cells were scraped into a microcentrifuge tube and incubated for 15 minutes with an equal volume of 2x IP buffer (containing 20μM Aprotinin, 20μM Leupeptin, 2mM PMSF). Cell debris was separated from the lysate via centrifugation for 10 minutes at 14000rpm and the supernatant was transferred to a new microcentrifuge tube. An aliquot of the lysate was stored at -80°C and the remainder was cleared by incubation with 1:1 slurry of BSA-coated protein G beads, rotating for 1 hour. The cleared lysate was incubated with primary antibody (monoclonal anti-myc or anti-GFP) and control antibody (monoclonal anti-GST) rotating for 1.5 hours. Subsequently, BSA-coated protein G beads were added and incubated while rotating for 45-60 minutes. Bound antibody was precipitated through centrifugation for 10 minutes at 3000rpm and the beads were washed 3 times with IP buffer. Immunoprecipitated proteins were separated from the beads by addition of 4x Laemmlli buffer and vigorous vortexing.

2.5.4 Immunofluorescence (IF)
For localization studies of exogenous and endogenous protein, cells were seeded on collagenized coverslips, if needed transfected or transduced, and fixed with 4%PFA/PBS for 10 minutes at RT. After permeabilization with 0.5% Triton-X cells were blocked with blocking buffer for 1 hour RT or at 4°C over night. Coverslips were transferred to a light-impermeable humidified chamber and primary antibodies were applied directly onto the coverslip for 1 hour RT. Generally, primary antibodies were diluted 1:200 in antibody diluent. Before and after the addition of the Alexa-conjugated fluorescent antibodies for 1 hour RT (1:500 in antibody diluent), the coverslips were washed with PBS. The nucleus was stained for 10 minutes with 1μg/ml DAPI and the coverslips were mounted on glass slides with Fluoromount G. Samples were dried at RT over night and images were acquired with a 2100 Rainbow laser scanning confocal microscope (Nikon 60x oil, Plan-Apo-1.4 NA; Nikon 100x oil Plan-Apo-1.4 NA). 3D-differentiated cells were processed identically, except that fixation and permeabilization were conducted for 20 minutes. Images were analyzed for colocalization with Image J and LSM Analyzer software. Orthogonal planes were illustrated by Metamorph software.

2.5.5 Immunohistochemistry (IHC)
50 to 200mg of frozen tissue from patient specimen or whole organs (trachea, upper lung, lower lung) were fixed in zinc formalin for 12 hours minimum and subsequently embedded in paraffin wax. For histological assessment, 3μm thick sections were H&E and PAS stained according to standard procedures. Sections containing airway cross cuts were deparaffinized in xylene, rehydrated in graded alcohol (two minutes each in ethanol 100%, 95%, 70%, 50%, 30%; followed by two minutes in PBS) and autoclaved (10 minutes, 120 psi) in 0.01M sodium citrate buffer (pH 6.0) for antigen retrieval. Use of citrate buffer older than four weeks can result in decreased antigen retrieval. Endogenous peroxidase activity was inhibited through 30 minute incubation with 3% hydrogen peroxide in methanol. After blocking with 2% non-fat dry milk in water the sections were incubated with serial dilutions of polyclonal rabbit anti-Duox serum in blocking solution for 16 hours at 4°C (1:1000 for human sections, 1:200 for rodent sections). The antibody was raised in rabbits to amino acid residues 775 to 1,026 of hDuox1. The slides were washed in PBS containing 0.1% Tween-20 (PBS-T) and incubated with biotinylated anti-rabbit IgG for 60 minutes at room temperature (1:200 in blocking solution). After three short washes with PBS-T, strepavidin conjugated HRP was applied for
60 minutes at room temperature (1:200 in ddH2O) and subsequently the labeled antigen was visualized with 3-amino-9-ethy carbazole (AEC) as chromogen with a brownish color indicating the area of Duox expression. The slides were counterstained with Mayer’s Hematoxylin and washed with Scott’s water for 7 minutes before mounting. As negative controls, slides were incubated with preimmune serum, but otherwise were treated identically. Images were taken with a Nikon Eclipse E800 microscope (Nikon 10x air, 0.3 NA; Nikon 40x air, 0.75 NA).

2.6 Cell Biology Assays

2.6.1 Homovanillic acid (HVA) assay
Release of H2O2 into the extracellular milieu was measured by H2O2-dependent horseradish peroxidase-mediated oxidation of HVA (3-methoxy-4-hydroxyphenylacetic acid) into a highly fluorescent dimer (2,2'-dihydroxy-3,3'-dimethoxydiphenyl-5,5'-diacetic acid). Cells were grown in 6 well plates and incubated for 30 minutes with 1ml HVA-solution in the presence or absence of 2µM ionomycin or 1µM thapsigargin. 500µl supernatant were transferred into a microcentrifuge tube and mixed with 75µl Stop buffer. Fluorescence was read on a Perkin Elmer Luminescence Spectrometer LS50B (320nm excitation, 420nm emission, slid width = 3). A preparation of a standard curve allowed for the accurate determination of H2O2 produced by the cells. For this, H2O2 concentrations ranging from 0µM to 5µM, were incubated in HVA solution for the duration of the assay and fluorescence of the standards were read at the same time as the samples.

2.6.2 Wound closure assay
For the quantitative analysis of wound closure cell lines transduced with Duox1/DuoxA1-2 or EV-control cell lines were grown on Fibronectin-coated photoetched coverslips until 100% confluency was reached. The cells were starved over night in their respective media, containing 0.5% FCS and the monolayer was wounded with a 10µl pipette tip in a linear fashion. Floating cells were removed through subsequent washes with sterile PBS and the cells on the coverslips were incubated in 10% media for 2-4 hours. For better statistics, at least 9 fields were imaged per condition and pictures of the same areas were taken hourly using a phase-contrast microscope. Wound areas were measured using Metamorph software and were expressed as percent closed wound of the average wound area of Duox1+DuoxA1-2. For IF studies, cells were grown on standard collagenized coverslips. The cells were fixed after 1 hour upon wounding and stained using the standard IF protocol.

2.6.3 Transwell Migration Assay
Migration experiments were performed in semi-permeable support membranes (24well; Costar Transwell-clear culture insert, polycarbonate, 8.0 µm pore, Corning Costar), which were coated with 20µg/µl bovine fibronectin or 0.15µg/µl human placenta collagen, type IV. Transduced lung cancer cell lines expressing control vector, DuoxA1 or Duox1/DuoxA1 were washed twice in plain media and resuspended at 5x10^5 cells/ml in media containing 0.5% FCS. 1x10^5 cells were seeded on top of the insert and were allowed to attach for 30 min. Directional migration was induced by addition of 0.8ml 10% FBS-containing medium into the bottom chamber. Cells were cultured in a humidified incubator at 37°C and 5% CO₂ for up to 3.5 hours. The remaining cells on top of the insert were removed with a cotton swab and cells adherent to the bottom membrane were fixed and stained with Quik-Dip Stain according to the manufacturer’s instructions and quantified with an Axiovert 100 microscope.
2.6.4 Cell Viability Assay
Lung cancer cell lines were seeded in triplicate and grown for up to 5 days in low-serum conditions. Proliferation was measured by means of mitochondrial activity, which reduces yellow water-soluble MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) into water insoluble purple formazan. The MTT stock solution (5 mg/ml in water) was diluted 10-fold in pre-warmed culture medium and added to the cells. After incubation for 30 minutes at 37°C, the solution was aspirated and the cells were carefully washed with PBS. The violet formazan precipitate was solubilized in DMSO and the absorbance (540nm) of the solution was determined spectrophotometrically (Synergy HT).

2.7 Statistical analysis
Data are presented as mean ± SEM and were graphed using GraphPad Prism 4.0 software. Differences in hydrogen peroxide production per milligram of protein per hour were analyzed by using a two-tailed Student’s t test. Differences in mRNA expression between cancerous and non-cancerous matched human lung specimen and in 3D cultures were also assessed using the Student’s t-test and the equality of variances was determined by the F-test. $P < 0.05$ was considered as statistically significant. Significance levels were *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
3 Results

3.1 Regulation and expression of Duox1 and Duox2
Dual oxidases were first identified in human and porcine glands, where they supply H$_2$O$_2$ for thyroperoxidase-mediated iodination and cross-linking of thyroglobulin tyrosine residues (De Deken et al., 2000; Geiszt and Leto, 2004). However, Duox enzymes seem to be distributed in a differential manner, as Duox2 is predominantly expressed in the thyroid and the highest levels of Duox1 were found in the lung (Caillou et al., 2001; Geiszt et al., 2003). Yet, not much is known about their cellular localization in lung epithelial cells and their dependence on other factors needed for correct processing and transfer of full enzymatic activity.

3.1.1 Expression analysis of Duox1 and Duox2 in primary lung epithelial cells
In order to get qualitative information about Duox1 and Duox2 levels in lung epithelial cells, their expression was characterized in human primary small airway epithelial cells (SAEC) and normal human bronchial epithelial (NHBE) cells. RT-PCR revealed expression of DUOX1 and DUOX2 in both cell types, with DUOX2 being less abundantly expressed (FIGURE 10A). Total cell lysates (TCL) were probed with a Duox-specific and a distinct band of approximately 180 kDa was detected (FIGURE 10B), which corresponds to the glycosylated and mature form of Duox (Caillou et al., 2001; De Deken et al., 2002; Morand et al., 2003). As only fully matured Duox is functional and localizes to the plasma membrane, endogenous Duox was analyzed in SAEC via confocal microscopy (FIGURE 10C). Interestingly, a clear stain was visible at particular regions of the cell, reminiscent of protrusions of the leading edge.

![Figure 10](image-url)
Functional analysis of Duox enzymatic activity demonstrated Ca\(^{2+}\)-inducible H\(_2\)O\(_2\) production in primary lung epithelial cells (Geiszt et al., 2003). However, based on their structural homology with gp91phox/Nox2, it is also conceivable that Duox enzymes are able to produce superoxide (O\(_2^-\)), which then rapidly dismutates into hydrogen peroxide (see **Figure 3**). To test for both types of reactive oxygen species (ROS), SAEC and NHBE cells were stimulated with ionomycin, which leads to an amplification of intracellular Ca\(^{2+}\). Duox enzymes contain two EF-hand motifs, a structural domain for calcium-binding, and consequently an increase of Ca\(^{2+}\) should lead to their activation. Both primary human lung epithelial cell types produced H\(_2\)O\(_2\) upon stimulation with ionomycin, which was determined by oxidation of homovanillic acid (HVA assay; **Figure 11**), thus suggesting an involvement of EF hand-containing Duox enzymes. Treatment of these cells with DPI, a flavoprotein inhibitor, led to complete inhibition of ROS production despite stimulation with ionomycin. While this inhibitor is not specific for NADPH oxidases, it still indicates the involvement of a flavoenzyme in ROS generation. The addition of catalase diminished detectable H\(_2\)O\(_2\) upon stimulation with ionomycin. Because this enzyme promotes the conversion of hydrogen peroxide to water and molecular oxygen, this result suggested that the produced ROS was indeed H\(_2\)O\(_2\).

**Figure 11**: Production of H\(_2\)O\(_2\) in primary lung epithelial cells. SAEC and NHBE were stimulated with ionomycin (3\(\mu\)M) for one hour. Catalase was added to determine the nature of the generated ROS and DPI (10 \(\mu\)M) was used to inhibit NADPH oxidase activity. This experiment was repeated several times and one representative experiment is shown.

In summary, Duox1 and possibly Duox2 are expressed in primary human lung epithelial cells and are localized to the plasma membrane. The inducibility with Ca\(^{2+}\) and utilization of FAD as cofactor suggest that Duox is the likely source of H\(_2\)O\(_2\) in lung airway epithelial cells.

### 3.1.2 Duox proteins require DuoxA maturation factors for function

Attempts have been made to reconstitute Duox enzymes in several different cell types; yet, functional enzyme was only recovered if Duox proteins were re-introduced into thyroid cells (De Deken et al., 2002; Morand et al., 2004). A recent study however, described a maturation factor for Duox2, termed DuoxA2, which enabled the translocation of the oxidase from the ER to the plasma membrane, thus allowing the functional reconstitution of Duox2 in HeLa cells (Grasberger and Refetoff, 2006). The authors also identified a putative maturation factor for Duox1, termed DuoxA1. Since no functional studies on Duox1/DuoxA1 were conducted, a model system for lung epithelial cells was established. Lung cancer cells NCI-H661, which are deficient of Duox1, Duox2, DuoxA1 and...
DuoxA2 (see Figure 27), were transiently transfected with either Duox1 and DuoxA1 (343aa) or Duox2 and DuoxA2. Since both Duox enzymes possess intracellular EF-hands (see Figure 4), the cells were stimulated with either ionomycin or thapsigargin. Both compounds raise cytosolic calcium concentration via Ca\textsuperscript{2+} influx from intracellular calcium stores. A significant amount of H\textsubscript{2}O\textsubscript{2} was released into the extracellular milieu upon stimulation, in both Duox1/DuoxA1 and Duox2/DuoxA2 reconstituted cells (Figure 12). These results correlate well with the already published data for Duox2/DuoxA2 (Grasberger and Refetoff, 2006) and confirm a similar dependence of Duox1 on DuoxA1.

![Figure 12](39.png)

**Figure 12:** Duox-dependent H\textsubscript{2}O\textsubscript{2} production. Duox1 and DuoxA1 (343aa) or Duox2 and DuoxA2 were transiently expressed in Duox-deficient lung cancer cells NCI-H661. Release of H\textsubscript{2}O\textsubscript{2} was measured in response to thapsigargin (1\(\mu\)M) or ionomycin (2\(\mu\)M). Immunoblots from TCL were probed for Duox and myc-DuoxA expression. Tubulin served as loading control. Graphs show mean±SEM.

### 3.1.3 Duox and DuoxA co-localize at the same sub-cellular compartments

Duox proteins have been identified in the apical membrane of follicular cells in the thyroid (Caillou et al., 2001) and within the epithelium of the airways (Geiszt et al., 2003). However, the detailed localization of both oxidases and their maturation factors at the single cell level has not been determined yet. Since Duox1/DuoxA1 and Duox2/DuoxA2 reconstituted NCI-H661 cells produced similar amounts of H\textsubscript{2}O\textsubscript{2}, it is conceivable that both oxidases localize to same cellular locations. Confocal immunofluorescence revealed that Duox1 was expressed at the plasma membrane, where it co-localized with DuoxA1 (343aa) (Figure 13, left panel). As shown by staining for calnexin, a marker for the ER, no co-localization was detected with either Duox1 or DuoxA1. Surprisingly, Duox2 mostly co-localized with DuoxA2 in vesicular structures and the ER, while some Duox2/DuoxA2 was also targeted to the plasma membrane (Figure 13, right panel). These findings are in contrast to previous reports in HeLa cells (Grasberger et al., 2007; Grasberger and Refetoff, 2006; Zamproni et al., 2008), where DuoxA proteins did not co-localize with Duox at plasma membrane-associated compartments, but were rather completely retained in the ER. Lung cells might possess other yet unknown factors which allow DuoxA proteins to exit the ER. Hence, it is possible that HeLa cells lack those tissue specific factors, as they were derived from a carcinoma of the cervix, a tissue not expressing endogenous Duox.
3 RESULTS

3.1.4 DuoxA maturation factors form complexes with Duox proteins

The co-localization of Duox with DuoxA indicates a close spatial proximity of those two proteins. Since Duox1 and Duox2 need the presence of DuoxA1 and DuoxA2, respectively, to be fully mature and active, it is likely that they form a functional complex. In order to test for heterodimer formation, NCI-H661 cells were first stably transduced with lentiviruses encoding for Duox1 and DuoxA1 (343aa) or Duox2 and DuoxA2, thus allowing integration and stable, low-level expression. The cells produced similar amounts of H$_2$O$_2$ upon stimulation with ionomycin or thapsigargin compared to transiently transfected cells, when reconstituted with functional Duox1 or Duox2 (FIGURE 14A). Expression of empty vector (EV), DuoxA1 or DuoxA2 alone resulted only in background levels of H$_2$O$_2$, as maturation factors alone cannot generate ROS. Next, DuoxA proteins were immunoprecipitated using anti-myc monoclonal antibody, since both maturation factors were C-terminally fused to a myc-tag (FIGURE 14B). Both Duox1 and Duox2 co-immunoprecipitated with their respective DuoxA maturation factor, as indicated by positive Duox-detection in the myc-immunoprecipitates. No Duox protein was detectable in the control lanes, suggesting specific co-immunoprecipitation of Duox with DuoxA. Positive detection of myc (DuoxA) in the immuno-precipitates confirmed the presence of DuoxA proteins, thus ruling out that Duox protein was bound unspecifically to the beads in the pull down process.
Figure 14: Stable expression of functional Duox1 and Duox2. Lung cancer cells NCI-H661 cells were transduced with lentiviruses encoding for Duox1 and DuoxA1 (343aa) or Duox2 and DuoxA2. (A), Duox-dependent $H_2O_2$ production was determined in response to ionomycin (2µM) or thapsigargin (1µM). The immunoblots depict Duox1 or Duox2 (Duox), DuoxA1 or DuoxA2 (myc) and tubulin expression in cell lysates. (B), after anti-myc immunoprecipitation (IP) immunoblotting for Duox and DuoxA (myc) was performed. Control IP (ctrl) was done with anti-GST. Immunoblotting (IB) on TCL was performed to confirm expression of Duox and DuoxA (myc).

The formation of heterodimers has been identified for other members of the Nox family, particularly Nox2/p22 (DeLeo et al., 2000; Yu et al., 1997). The incorporation of heme into Nox2 was shown to be essential for the interaction of both proteins, a phenomenon, which was also crucial for homodimer formation of inducible Nitric oxide synthases (Espinosa and Alfano) (Espinosa and Alfano, ; Panda et al., 2005). To analyze if the acquisition of heme by Duox is also necessary for association with DuoxA, the stably transduced cells were cultured in the presence or absence of succinyl acetone (SA), a specific inhibitor of heme biosynthesis. Indeed, co-immunoprecipitation of Duox with DuoxA was diminished in SA-treated cells (Figure 15A). Also, the enzymatic activities of both Duox1 and Duox2 were significantly reduced in cells treated with SA compared to untreated cells and only background levels of $H_2O_2$ were detectable (Figure 15B).

These results demonstrate that DuoxA1 and DuoxA2 are not only maturation factors for Duox1 and Duox2, but they are rather an integral part of the functional oxidase complex at the plasma membrane or other particular Duox1- or Duox2-containing membrane-associated compartments. Moreover, the insertion of heme into Duox1 and Duox2 is essential for the formation of the heterodimer and its disruption leads to the abrogation of $H_2O_2$ production.
RESULTS

3.1.5 Characterization of DuoxA isoforms
DuoxA1 and DuoxA2 are predicted to comprise five membrane-integral regions and harbor three NX(S/T) consensus sites for N-glycosylation within an extended extracellular loop between transmembrane (TM) domains two and three (FIGURE 16A). A search for both proteins in the Entrez Protein Database yields one DuoxA2 protein comprised of 320aa (NM_207581.2), while three isoforms of DuoxA1 were found: DuoxA1-1 (298aa; BC020841.1), DuoxA1-2 (343aa, DQ489735.1) and DuoxA1-3 (483aa, BC029819.1) (FIGURE 16B). All three DuoxA1 isoforms share the putative 5 transmembrane structure. DuoxA1-1 lacks two of the three N-glycosylation sites and DuoxA1-3 features an extended intracellular C-terminus.

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FIGURE 15: Inhibition of heme-insertion into Duox1 and Duox2. NCI-H661 cells stably expressing Duox1/DuoxA1 (343aa) or Duox2/DuoxA2 were cultured for 4 days in low serum media in the presence or absence of the heme synthesis inhibitor succinyl acetone (SA; 10µg/ml). (A), Immunoblotting (IB) for Duox and DuoxA (myc) was performed after immunoprecipitation of DuoxA (myc IP). Control IP was done with anti-GST. TCL were probed for expression of Duox and DuoxA (myc). (B), H2O2-production was measured upon stimulation with ionomycin (2µM). Graphs show mean ± SEM.

FIGURE 16: Putative structure of DuoxA1 and DuoxA2. (A), suggested topology model for DuoxA proteins features 5 transmembrane (TM) domains. The longer extracellular loop between TM2 and TM3 contains 3 putative N-glycosylation sites (Grasberger and Refetoff, 2006). (B), schematic representation of the three identified DuoxA1-variants, which all share the presumed five TM structure. DuoxA1-1 (298aa) lacks two of three putative N-glycosylation sites. DuoxA1-3 possesses an extended C-terminus. Green, extracellular regions; yellow, TM domains; blue, intracellular regions; •, N-glycosylation sites.
To date, no extensive functional analysis of DuoxA1 was published. Expression of all three DuoxA1 isoforms, as well as expression of DUOXA2, DUOX2 and DUOX1, was analyzed in primary lung epithelial cells NHBE and SAEC (FIGURE 17). Cells were either grown in normal tissue culture conditions (2D) or in air-liquid interphase condition for 14 days (3D), as polarized epithelia are known to differentiate and upregulate certain genes (de Jong et al., 1994; Gray et al., 1996; Wu et al., 1997). Semi-quantitative PCR of DUOXA1-1 revealed constant expression levels, suggesting a possible housekeeping role. DUOXA1-2 expression was slightly decreased in 3D. The transcript of DUOXA1-3 was not detected in any of the samples. Primer specificity and PCR-conditions were confirmed via transfection with the full length construct (data not shown). Since this isoform was initially identified from pooled libraries of brain, lung and testis, it is possible that the longest of the three DuoxA1 isoforms is not expressed in the lung. Also, the polarized epithelium lacks alveolar type II cells, which could be the source for this protein (Fischer et al., 2007; Nagai et al., 2008). Expression of DUOX2 and DUOX2A was greatly enhanced in differentiated cells.

To investigate if all three DuoxA1 proteins can contribute to Duox1 function, Duox1 was co-expressed with each isoform in NCI-H661 cells (FIGURE 18A). Reconstitution of DuoxA1-3 induced only modest Duox1-dependent H₂O₂ production compared to DuoxA1-2 (see also FIGURE 12). Surprisingly, reconstituted DuoxA1-1 did not support Duox1 function, suggesting a potentially different role for this protein than DuoxA1-2 or DuoxA1-3. Confocal analysis revealed different cellular localization of all three isoforms and of the heterodimer (FIGURE 18B). DuoxA1-1 remained exclusively in the ER, where it co-localized with Duox1 and Calnexin (data not shown). It is possible that the lack of two of the three potential N-glycosylation sites in the putative extracellular loop hinders maturation of this isoform, resulting in ER retention. While DuoxA1-3 resided mostly in the ER, it also localized partially to the plasma membrane. This correlates well with the functional assay, as most Duox1 seemed to remain in the ER rather than being transported to the plasma membrane and consequently H₂O₂-production was only moderate in Duox1/DuoxA1-3 reconstituted cells. Only co-transfection of DuoxA1-2 allowed Duox1 to exit the ER completely. The notion of a functional interaction of Duox1 with DuoxA1-2 and DuoxA1-3, respectively was further substantiated as Duox1 did only co-immunoprecipitate with those two proteins, but not with DuoxA1-1 (FIGURE 18C).
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**Figure 18:** Reconstitution of Duox1 and DuoxA1-isoforms. NCI-H661 cells were transiently transfected with Duox1 and DuoxA1-1, DuoxA1-2 and DuoxA1-3, respectively. All three DuoxA1 isoforms were fused to eGFP. (A), Duox1-dependent H₂O₂ production was determined in response to stimulation with ionomycin (2µM) or thapsigargin (1µM). Data are presented as mean values ±SEM. Expression of Duox1 and DuoxA1 (GFP) was confirmed by immunoblotting. Tubulin served as loading control. (B), Detection of Duox1 and the three DuoxA1 isoforms by immunofluorescence. Cells were stained with Duox antibody. DuoxA1-1(1-3) detection was based on the eGFP fusion protein. Co-localization is shown in white. Bar, 10µm. (C), Immunoblotting for Duox1 and DuoxA1 (GFP) was conducted upon anti-GFP immunoprecipitation. Control IP was done with anti-GST. TCL were probed for Duox1 and DuoxA1-1, DuoxA1-2 and DuoxA1-3 (GFP) expression.

Taken together, these findings demonstrate that the localization and consequentially the function of Duox1 are dependent on the DuoxA1-isoform. While both DuoxA1-3 and DuoxA1-2 form a heterodimer with Duox1, only the latter enables complete translocation of the heterodimer to the plasma membrane, which is essential for Duox1/DuoxA1 activity.

### 3.1.6 Mismatch pairing of Duox and DuoxA

The aforementioned experiments showed that reconstitution of Duox1 and Duox2 with their respective maturation factor, Duox A1-2 and DuoxA2, resulted in the highest ROS production (Figure 12, 14, 18). Those two maturation factors share the predicted 5 transmembrane structure (see Figure 5, 16) and are 52% identical in their primary structure. To test if both could support function of the paralog of their respective oxidase, DuoxA1-2 and Duox2 were co-expressed with Duox1 and DuoxA2, respectively. Surprisingly, DuoxA1-2 could sustain Duox2-dependent H₂O₂ production, with levels similar to those of the combination Duox2/DuoxA2 (Figure 19A). However, pairing of Duox1 with DuoxA2 did not result in any substantial H₂O₂ release. In contrast, a recent report showed a minimal rescue of Duox2 function when expressed together with DuoxA1-2 (Zamproni et al., 2008). Therefore, this experiment was repeated in Duox-deficient HeLa cells, the cell type used by Zamproni and coworkers (Figure 19B). Only minimal levels of basal and ionomycin-stimulated H₂O₂ were
produced by the mismatch, indicating cell-type specific differences in functional dimer formation. It is possible that HeLa cells lack additional lung-cell-specific factors, thus preventing functional recovery of the mismatched Duox2/DuoxA1-2 pair.

**FIGURE 19:** Analysis of Duox-dependent H$_2$O$_2$ production by matched and mismatched pairs of Duox and DuoxA. Duox1 and Duox2, respectively, was transiently co-expressed with DuoxA1-2 or DuoxA2 in NCI-H661 (A) or HeLa (B) cells. The release of H$_2$O$_2$ was measured upon stimulation with ionomycin (2µM). Shown are mean values ±SEM. Levels of Duox and DuoxA expression were determined by immunoblotting. Tubulin served as loading control.

Immunostaining of NCI-H661 cells expressing Duox2 in the presence of DuoxA1-2 showed distinct localization of both proteins at the plasma membrane, whereas Duox2 localized mainly in vesicular structures when co-expressed with DuoxA2 (**FIGURE 20**, left panel, see also **FIGURE 13**).

**FIGURE 20:** Duox2 localization is contingent on co-expression of DuoxA1-2 or DuoxA2. Duox2 localization was analyzed via confocal microscopy in NCI-H661 and HeLa cells, which were transiently transfected with Duox2/DuoxA1-2 or Duox2/DuoxA2 and stained with Duox and myc antibodies. Co-localization is shown in white. Bar, 10µm.

In HeLa cells Duox2 was not targeted to the plasma membrane when co-expressed with DuoxA1-2 and remained almost exclusively in the ER (**FIGURE 20**, right panel). The formation of a mismatched Duox2/DuoxA1-2 heterodimer in lung cells was further confirmed via co-immunoprecipitation. Duox2 was detected after pull down of both DuoxA2 and DuoxA1-2, confirming a close interaction of Duox2 with either maturation factor (**FIGURE 21**).
3 RESULTS

These results suggest that lung cells can utilize not only DuoxA2 but also DuoxA1-2 to support Duox2-dependent H₂O₂ production. Also, the particular interaction of Duox2 with either DuoxA subunit seems to define the subcellular localization of the oxidase, which could possibly impact the spatiotemporal generation of ROS. In addition, these findings demonstrate that results can vary depending on the model cells used. Hence, this emphasizes the use of cells, which are derived from the same tissue as the protein of interest.

3.1.7 Generation of isoform-specific Duox1, Duox2 and DuoxA1 antibodies

To investigate endogenous expression of Duox and DuoxA, antibodies specific for human Duox1, Duox2 and DuoxA1 were generated in the lab, finally allowing for differential expression analysis of those proteins (FIGURE 22). Isoform specificity was confirmed by detection of total Duox (using anti-Duox1,2) and DuoxA (using anti-myc). The anti-DuoxA1 antibody does not discriminate between any of the three DuoxA1 isoforms.

3.1.8 Analysis of endogenous Duox and DuoxA expression

Quantitative PCR of SAEC, which were cultured and differentiated for a time course of 21 days, showed no change in DUOX1 transcript levels, whereas DUOX2 mRNA was significantly increased over time (FIGURE 23A). Western blot analysis revealed an increase of Duox1 protein, which might be a result of increased protein stability in differentiated cells (FIGURE 23B). Despite the fact that DUOX2 mRNA was markedly upregulated during differentiation (FIGURE 17, FIGURE 23A) Duox2 protein could not be detected in the lysates (data not shown). It is possible that the protein levels were still below

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**FIGURE 21**: Heterodimer formation of Duox2 with DuoxA1-2. Immunoprecipitation of DuoxA1-2 and DuoxA2 was conducted on lysates from NCI-H661 cells, which were transiently transfected with Duox2 and DuoxA1-2 or DuoxA2. Immunoprecipitates and TCL were probed for Duox2 and DuoxA (myc). Control IP was done with anti-GST.

**FIGURE 22**: Validation of isoform-specific antibodies. TCL from cells expressing Duox1/DuoxA1-2 or Duox2/DuoxA2 were probed with isoform-specific antibodies for Duox1, Duox2 and DuoxA1. The latter does not discriminate between any of the DuoxA1 isoforms. Detections of total Duox using the isoform-unspecific antibody (Duox1,2) and of total DuoxA (myc) served as expression control. Tubulin-probing confirmed equal loading.
the detection limit of the antibody, at least for immunoblotting applications, especially because overexpressed Duox2 was readily detectable.

**Figure 23:** Analysis of endogenous Duox expression in primary lung epithelial cells. (A), real-time PCR specific for Duox1 or Duox2 was performed on SAEC, which were either undifferentiated (day 0) or differentiated in ALI-culture for 7, 14 and 21 days. Per sample, two inserts were pooled for extraction of total RNA. Data points consisted of three samples each and were individually normalized to actin, which served as internal control. Data were expressed in relation to transcript levels at day 0 and are presented as mean±SEM. (B), detection of endogenous Duox1 protein in lysates from SAEC, which were either undifferentiated (2D) or ALI-differentiated (3D) for 14 and 23 days (d), by probing with the specific anti-Duox1 antibody. Lysates from cells transiently transfected with Duox1/DuoxA1-2 were used as positive expression control (+). Tubulin served as loading control.

Previous results have shown distinct localization patterns for both Duox1 and Duox2, when co-expressed with DuoxA maturation factors (Figure 13, Figure 20). Since these localization experiments were conducted in transformed lung cancer cells, these findings were further validated in the context of normal lung epithelial cells. Primary SAEC were transduced with lentivirus, encoding for Duox1, Duox2, DuoxA1-2 and DuoxA2 or empty vector (EV) as control. Cells were stained with anti-Duox1,2 and anti-myc antibodies, which detect both Duox isoforms and the myc epitope tag of DuoxA, respectively. Confocal analysis revealed the same heterodimer co-localization in SAEC compared to the previous observations in NCI-H661, with Duox1/DuoxA1-2 and Duox2/DuoxA1-2 being expressed at the plasma membrane and Duox2/DuoxA2 being localized to vesicular structures and the ER (Figure 24). When antibodies specific for Duox1, Duox2 and DuoxA1 were used, the same pattern was detected. Endogenous Duox1 and DuoxA1 were detected at the plasma membrane in cells expressing either EV or DuoxA1 alone. Duox2 could not be visualized, as it is not expressed in undifferentiated SAEC (Figure 17).
RESULTS

**FIGURE 24:** Analysis of exogenous and endogenous Duox and DuoxA localization in primary human lung epithelial cells SAEC. Cells were lentivirally transduced with Duox1/DuoxA1-2, Duox2/DuoxA2, Duox2/DuoxA1-2, DuoxA1-2/empty vector (EV) or EV/EV and stained with specific antibodies as indicated. Co-localization is illustrated in white. Bar, 10µm.

Thus far, not much is known about the intracellular distribution of Duox1 and Duox2 in *in vitro* polarized cells. The generation of the specific antibodies in our lab allowed for the detection of not only the presence of Duox1, Duox2 and DuoxA1, but also their localization in differentiated cells. As a first step SAEC were grown and differentiated for 2 weeks in ALI culture and stained with markers for mucus (MUC5A) and ciliated (β-tubulin IV) cells, as well as for Duox1, Duox2 and DuoxA1 (**FIGURE 25**). A Z-series of confocal images revealed expression of Duox1, Duox2 and DuoxA1 on the apical side of the multiple cell layer. Surprisingly, Duox2 was also expressed in ciliated cells, where it colocalized with β-tubulin in the cilia. Some DuoxA1 and little Duox1 was also detectable in the cilia but to a much lesser extent than Duox2. Duox1 was not found in mucus-expressing cells.
FIGURE 25: Localization of endogenous Duox1, DuoxA1 and Duox2 in in vitro differentiated primary lung epithelial cells. SAEC were differentiated for 14 days in ALI-culture and stained for mucus-containing cells (MUC5A, red, R), ciliated cells (β-tubulin, red, R) and Duox1, DuoxA1 and Duox2 (all green, G) as indicated. Nuclei were stained with DAPI (blue, B). Z-sections at 0.3µm intervals were taken throughout the entire depth of the multiple cell layer at several positions of the insert. Orthogonal views correspond to crosslines depicted in the rectangular overview and show two color overlays of green and blue (GB), red and blue (RB) and three color merge (red, green, blue; RGB), with co-localization indicated in yellow. Bar, 10µm.
3.1.9 in vivo expression of Duox in lung epithelium

In order to get information about Duox expression in vivo, sections from human normal upper lung were stained for Duox1,2 expression. The red stain indicative for Duox protein revealed localization within the pseudostratified epithelium of the airways, oriented towards the lumen (Figure 26A).

Figure 26: Detection of endogenous Duox in human lung tissues. Immunohistochemical staining for Duox1,2 was performed on human tissue sections from a normal donor (A) and a patient with cystic fibrosis (B). Duox protein is indicated by a red stain. (A), Duox protein was predominantly expressed in the ciliated airway epithelial cells, which are directed towards the lumen of the airways. (B), strong staining for Duox1,2 was detected in serous acinar cells, depicted in the inset. They are localized in enlarged submucosal glands, in close proximity to the airways. Bar, 10µm.

Duox was also detected in airway epithelial cells of lung tissue sections from a donor with cystic fibrosis (CF) (Figure 26B). Additionally, a very strong red stain was visible in serous acinar cells. These cells are located in submucosal glands, which were enlarged in this patient, a typical phenotype of CF (Kamio et al., 2005; Yang et al., 1988).

As this data show Duox-specific staining in serous aciner cells, they suggest an additional site for Duox expression in vivo, in addition to the already established localization to airway epithelial cells (Geiszt et al., 2003).
3.2 Duox-silencing in human lung cancer

ROS are known to damage biomolecules, such as proteins, DNA or lipids, and this in turn can potentially lead to abnormalities of cellular function and regulation (Lambeth, 2004). Since Duox enzymes produce ROS and are the predominant NADPH oxidases in the lung, it is possible that an upregulation of those proteins could lead to oxidative stress and cellular damage, potentially promoting lung cancer development and progression.

3.2.1 Expression analysis of Duox and DuoxA in lung cancer cell lines

To investigate potential differences between normal and cancerous lung epithelial cells, expression of DUOX was analyzed in primary (SAEC, NHBE) and immortalized primary (BEAS-2B, SALE) lung cells as well as lung cancer cell lines, which were derived from primary small cell lung carcinomas (NCI-H69, NCI-H82, SHP-77), and non-small cell lung carcinomas (Adenocarcinoma: NCI-H292, NCI-H727, Calu-3, NCI-H441, UCLA-P3, A549; Squamous cell carcinoma: NCI-H460, NCI-H661; Large cell carcinoma: NCI-H157) (Figure 27).

(Figure 27: Expression analysis of DUOX1, DUOX2, DUOXA1, DUOXA2 in lung epithelial cells and lung cancer cell lines. RT-PCR was performed on primary human lung epithelial cells (SAEC, NHBE), immortalized normal lung epithelial cells (BEAS-2B, SALE), three small cell lung cancer cell lines (H69, H82, SHP-77) and 9 non-small cell lung cancer cell lines (Adenocarcinoma: H292, H727, Calu-3, H441, UCLA-P3, A549; Squamous cell carcinoma: H460, H661; Large cell carcinoma: H157). DuoxA1 mRNA was detected with primers detecting DuoxA1-2 and DuoxA1-3. Actin was used as internal control.

Surprisingly, DUOX1 message was reduced or absent in 9 out of 12 lung cancer cell lines, while it was expressed in primary and immortalized primary lung epithelial cells. Because DUOX2, DUOXA1 and DUOXA2 are localized at the same locus as DUOX1 on chromosome 15q15 (see Figure 5A), their expression was analyzed as well. DUOX2 was clearly detectable in normal lung cells, although the levels were lower than DUOX1. This correlates well with previous data that also showed lower DUOX2 transcript levels in cells, which were grown in normal 2D tissue culture conditions (see Figure 17). The endogenous levels of DUOXA1 demonstrated a similar expression pattern as its corresponding oxidase, while DUOXA2 was not present in primary lung cells and most cell lines.
3.2.2 Restoration of Duox/DuoxA expression through global demethylation

Since reduced DUOX message was detected in most lung cancer cell lines, we hypothesized aberrant promoter hypermethylation being involvement in DUOX silencing. Promoter hypermethylation has been identified as a frequent key event in both early- and late-stage lung carcinoma pathogenesis (Bowman et al., 2006; Machida et al., 2006; Palmisano et al., 2000; Tsou et al., 2002; Widschwendter et al., 2004). Since this epigenetic mechanism almost exclusively occurs in CpG-islands, areas with a high density of CpG dinucleotides, the regions -1000bp upstream of the transcriptional start site (TSS) of DUOX1, DUOX2 and -2000bp upstream of DUOXA1 and DUOXA2 were analyzed (FIGURE 28).

![Figure 28](image)

**FIGURE 28:** Analysis of the promoter regions upstream of DUOX1, DUOX2, DUOXA1 and DUOXA2. Contingent on the stringent criteria described by (Takai and Jones, 2003) CpG islands were detected within the analyzed 1000bp upstream of the TSS of DUOX1 and DUOX2. Similarly, CpG islands were also detected in the 2000bp upstream of the TSS of DUOXA1 and DUOXA2.

The regions of all four genes are CpG-rich and contain CpG islands. Hence, it is likely that their promoters are subject to non-random hypermethylation. It has been shown that inhibition of the cellular DNA methyltransferase (DNA (cytosine-5)-methyltransferase 1; Dnmt1) recovered expression of genes that were silenced by promoter hypermethylation. Therefore, in order to analyze if DUOX genes are also affected by the epigenetic phenomenon, selected DUOX-deficient cell lines were treated with 5’-aza-2’-deoxycytidine (Aza) (FIGURE 29).

![Figure 29](image)

**FIGURE 29:** Recovery of DUOX1, DUOX2, DUOXA1 and DUOXA2 expression after treatment with 5-aza-2’-deoxycytidine (Aza). DUOX-deficient lung cancer cell lines were grown for 5 days in the presence (+) or absence (-) of Aza (1µM). Gene expression was analyzed by RT-PCR. Actin served as internal control.
Indeed, expression of DUOX1 was restored in all tested lung cancer cell lines upon inhibiton of Dnmt1. Aza-treatment also induced expression of DUOX2, DUOXA1 and DUOXA2 in four out of five cell lines. It is possible that in NCI-H441 cells other epigenetic mechanisms, such as histone acetylation, are responsible for the transcriptional repression of the investigated genes. TCL of cell lines A549 and NCI-H661 cultured in the presence or absence of Aza were probed for total Duox expression and a band of 180kDa was detected (FIGURE 30A). However, even upon Ca^{2+} mobilization via stimulation with ionomycin, no increase of H_{2}O_{2} was detectable (FIGURE 30B). It is possible that Duox recovery through Aza is too modest to support function, a notion that was supported by comparison of exogenous levels of Duox versus endogenous achived by Aza treatment (FIGURE 30C). While Duox protein was easily observed after one second of enhanced chemiluminescence (ECL) exposure in NCI-H661 transiently transfected with Duox1/DuoxA1-2, Aza-recovered Duox was barely detectable after 20 seconds. It is possible that several concomitant events are taking place in cancer cells, besides inhibition of Duox protein expression, which prevent the re-establishment of the functional oxidase.

![Figure 30: Recovery of Duox protein in lung cancer cells. Duox-deficient lung cancer cell lines A549 and NCI-H661 were grown in the presence or absence of Aza (1µM) for 5 days. (A), 150µg total lysate were loaded on each lane and membranes were probed for total Duox expression. (B), H_{2}O_{2} production was analyzed in NCI-H661 upon stimulation with ionomycin (2µM). Shown are mean values ±SEM. n=3. (C), NCI-H661 cells were either transfected with Duox1/DuoxA1-2 for 48h or grown in the presence of 1µM Aza for 5 days. 50 µg of total cell lysate for each treatment were loaded per lane. Immunoblots were probed with anti-Duox and anti-tubulin antibodies and proteins were visualized by ECL with exposure times ranging from 1 to 20 seconds.](image)

### 3.2.3 Detailed methylation analysis of the DUOX1 and DUOX2 promoter

The promoter of Duox1 encompasses three binding sites for Sp1. This transcription factor is an activator of RNA polymerase II-driven transcription (Yeh et al., 2006). Its consensus binding sequence (CCGCC) does enclose one CpG site, which could be methylated. In turn, this could lead to sterical inhibition of the RNA-Polymerase complex during transcription. Since Aza causes global demethylation throughout the entire cell, so far no conclusions could be drawn regarding the definite methylation status of the promoter regions of DUOX. In order to analyze specific CpG islands, genomic DNA (gDNA) was treated with sodium bisulfite, resulting in deamination of cytosines into uracil. Methylated cytosines, however, are not affected and remain in the DNA as cytosines (see methods for details). A larger region of the promoter was amplified with universal primers, which are unbiased for CpG sites. The product of this PCR reaction was subsequently probed with primers specific for either methylated (MSP) or unmethylated (USP) sequences, revealing the methylation status of few CpG sites close to the TSS of both DUOX1 and DUOX2 (FIGURE 31). Methylated
sequences were identified in 4 out of 5 Duox-negative cell lines (A549, NCI-H157, NCI-H441, NCI-H661). As expected, Duox-expressing cells (NHBE, SAEC, NCI-H292) were negative for methylation in this region and were positive for unmethylated sequences. Cell line NCI-H727 was negative for MSP, however, DUOX1 message was restored upon Aza-treatment (see Figure 29). This could indicate that methylation occurred at a different CpG-rich region of the promoter, which was not covered by the MSP primers used in this assay, or that other epigenetic modifications, such as histone acetylation, are responsible for the inactivation of DUOX1 in these cells. Cancer cell line NCI-H157 was positive for both methylated and unmethylated sequences, indicative for partial methylation throughout the cell population. MSP of the DUOX2 promoter was negative in primary and immortalized primary cells and all but one investigated cell line (NCI-H441) were partially methylated.

Figure 31: Methylation specific PCR. The methylation status of lung cancer cell lines and normal lung epithelial cells was characterized using primers specific for methylated (MSP) and unmethylated sequences. The promoter regions of DUOX1 and DUOX2 were amplified with Universal-a primers, followed by PCR with MSP-a and USP-a primers. Primer locations: Duox1-Universal-a: -263 and +58; Duox1-MSP-a: -96 and +19; Duox1-USP-a: -186 and +21; Duox2-Universal-a: -566 and +65; Duox2-MSP-a: -199 and -18; Duox2-USP-a: -517 and -402.

MSP only allows for the analysis of a few CpG sites, which are determined by the chosen primer sequences. In order to get information about the methylation status of all existent CpG-dinucleotides in a given sequence, the regions 1000bp upstream of the TSS of DUOX1 and DUOX2 were sequenced following bisulfite modification. The sequencing of at least eight individual clones disclosed very dense hypermethylation of the 72 CpG dinucleotides within the DUOX1 promoter in cell lines NCI-H661 and A549 (Figure 32, left panel). Only 30-35% of the CpG sites were methylated in NCI-H157 cells, correlating very well with the MSP results (Figure 31). DUOX1-expressing primary cells NHBE showed virtually no methylated CpG sites. The bisulfite sequencing of the three investigated cell lines revealed less overall methylation within the 39 CpG sites of the DUOX2 promoter, especially in NCI-H157 and NCI-H661 (Figure 32, right panel). Since both cell lines showed an explicit recovery of DUOX2 upon Aza-treatment, it is possible that not all alleles present in the whole cell population of those cell lines were characterized by the bisulfite sequencing. It is also conceivable that other cis- or trans-acting stretches of DNA outside the investigated sequence, are methylated in this cells, leading to a signaling-inhibition upstream of DUOX2 transcription.
3 RESULTS

Figure 32: Bisulfite sequencing. Genomic DNA of three DUOX-deficient lung cancer cell lines and primary lung epithelial cells NHBE were treated with sodium bisulfite. BSP primers located outside the investigated sequences were used to amplify the regions of interest. PCR products containing the 72 and 39 CpG sites of the DUOX1 and DUOX2 promoter, respectively, were cloned and eight individual clones per sample were sequenced. Each row represents one sequenced clone and each circle represents one CpG dinucleotide. The promoter regions upstream of DUOX1 and DUOX2 are shown at the top, with each vertical bar representing one CpG dinucleotide. O, unmethylated CpG site; ●, methylated CpG site. Arrows indicate the location of MSP-a and USP-a primers, used in Figure 31.

3.2.4 Expression of Duox in lung cancer tissues

Particularly squamous and small cell lung cancer cell lines preserve resemblance with their equivalent tumor (Virtanen et al., 2002). Nevertheless, molecular changes can arise in the establishment and maintenance of cell culture lines. In fact, cell lines developed from adenocarcinomas have been shown to alter their phenotype in vitro (Virtanen et al., 2002; Wistuba et al., 1999). Moreover, established cancer cell lines show significantly higher levels of CpG methylation compared to their corresponding tumor tissue (Flatau et al., 1983). Therefore, to rule out artefacts which can only be observed in vitro, mRNA levels of DUOX1, DUOX2 and DUOXA1 were analyzed in 11 primary non-small cell lung carcinomas and paired adjacent normal tissues. Real-time PCR was performed on three different regions of each specimen to average the results per tissue sample. When probed for DUOX1 expression, 9 out of 11 patients showed a statistically significant downregulation in tumor tissue compared to the corresponding adjacent tissue (Figure 33A). One patient showed lower DUOX1 levels in tumorigenic tissue, but failed to be statistically significant (patient 2), while one patient had elevated DUOX1 levels in the cancer tissue compared to adjacent tissue (patient 6). Interestingly, the pattern of DUOXA1 is highly similar to that of DUOX1 (Figure 33B). Analysis of DUOX2 revealed statistically significant lower transcript levels in 7 out of 11 patients (Figure 33C). Two patients (patient 5 and 7) retained expression of DUOX2, while DUOX1/DUOXA1 were reduced in the cancer tissue. Since DUOX and DUOXA genes are arranged head-to-head on chromosome 15q15 (see Figure 5A), a linked deregulation of these genes is possible.
Levels of mRNA do not always directly correlate to total protein, which can arise through differences in mRNA/protein stability or post-transcriptional/-translational modifications (Yoshiko et al., 2002). Therefore, in order to evaluate the relationship between DUOX message and protein, total Duox protein was detected via immunohistochemistry in cancerous and adjacent healthy tissues. Duox was detected predominantly in the ciliated airway epithelial cells in the matched normal tissues of patient 11 and 7, where it appears as a red stain (FIGURE 34A). A substantially weaker staining was seen in the corresponding tumor-samples of both patients. Limited amounts of tissue samples impeded the analysis of every patient. Moreover, samples without airway crosscuts were removed from further analysis, due to the predominant localization of Duox in airway epithelial cells. No Duox-specific stain was detectable when the sections were stained with Duox preimmune serum, confirming the specificity of the antibody. These results substantiate the real-time data, supporting the hypothesis of Duox being downregulated or even completely absent in lung cancer in vivo.
Based on the in vitro results showing DUOX1 and DUOX2 promoter hypermethylation (see Figure 31, 32), MSP was used to determine if these promoters are also hypermethylated in vivo. Five patients, whose tumors displayed significant downregulation of DUOX1 in real-time PCR, were further analyzed with MSP and USP specific primers (patient 1, 3, 7, 9 and 11). Methylated sequences were detected in all cancer tissues, indicating that these tumors have methylated alleles of the DUOX1 promoter (Figure 34B). The absence of a PCR product in the non-cancerous/healthy samples suggests that these tissues have unmethylated alleles only. Only patient 9 was also MSP positive in the healthy tissue, albeit to a much lesser extend than in the matched cancerous tissue. It is possible that the healthy tissue of this patient has been infiltrated with cancerous cells or that the healthy tissue was in close proximity to the tumor and had already undergone some changes towards an abnormal or even pre-cancerous phenotype (Spira and Ettinger, 2004), as evident by the hyperplasia in the healthy tissue of patient 11 (Figure 34A; compare to histology in Figure 8B). USP yielded PCR
products in all patient samples, whether of cancerous or healthy origin (data not shown). This most likely arises from contamination of normal cells within the tumor sample (Chang et al., 2007; Krassenstein et al., 2004). Although it is tempting to hypothesize that promoter hypermethylation of DUOX1 and DUOX2 is a common event in lung cancer, the sample size of 11 patients is too small for any conclusions. Therefore, this theory was further substantiated through a larger sample size. In collaboration with Steven S. Belinsky at the Lovelace Respiratory Research Institute, the methylation status of the DUOX1 and DUOX2 promoter was analyzed via MSP analysis in 39 patients with primary adenocarcinomas (FIGURE 34C). A hypermethylated promoter of DUOX1 and DUOX2 was detected in 28% and 39% of the tumors, respectively. And while 50% of the tissues contained at least one methylated gene, 18% displayed promoter hypermethylation for both DUOX genes.

In conclusion, these results indicate that downregulation of Duox and DuoxA proteins is a prevalent occurrence in lung cancer cells in vitro and in vivo and is contingent on non-random aberrant promoter hypermethylation. As the promoter regions of all genes feature CpG islands, it is possible that the whole chromosomical region is prone to this epigenetic event, causing loss or deregulation of these transcriptionally linked enzymes.

3.2.5 Reconstitution of Duox leads to enhanced migration

It has been previously shown that Duox1 is implicated in airway homeostasis and cell migration of lung epithelial cells (Koff et al., 2006; Wesley et al., 2007). As Duox1 is the predominant Duox-isoform in the lung, the impact of Duox1 on lung epithelial cells was investigated. First, two Duox-deficient lung cancer cell lines (A549 and H157) were stably reconstituted with functional Duox1. Control cells were transduced either with empty vector (EV) or DuoxA1-2 alone. As expected, the cells expressing the functional oxidase regained the ability to produce H₂O₂ upon Ca²⁺ mobilization through thapsigargin or ionomycin (FIGURE 35).

![Figure 35](image)

In order to investigate if functional Duox1 influences directional cell migration, cells were seeded in serum-free media into transwell migration chambers and were allowed to migrate to the bottom of the chamber, containing serum-rich medium. In both cell lines, control cells expressing either empty vector (EV) or DuoxA1-2 alone did migrate towards the bottom of the insert, but to a much lesser
extent than cell expressing Duox1/DuoxA1-2 (Figure 36A). Directional migration was further analyzed under more physiological conditions by wounding a confluent monolayer of transduced A549 and NCI-H157 cells (Figure 36B, top). This in vitro wound closure assay mimics in vivo cell migration during wound closure to a certain degree. Upon wounding, cells expressing Duox1/DuoxA1-2 were able to migrate and close the wound faster than Duox1-deficient cells (Figure 36B, bottom). Wound closure is a process that requires not only cell migration but also cell proliferation, in order to repopulate the injured areas. As cell proliferation has been linked to increased ROS-generation by a variety of sources within the cell, the influence of Duox1 on cellular growth rates was investigated (Figure 33C). Proliferation was not altered in cells expressing Duox1/DuoxA1-2 compared to control cells, which expressed EV or DuoxA1-2 alone, suggesting that functional Duox1 does not influence proliferation in selected lung cancer cell lines.

Figure 36: Duox1 increases directional migration and wound closure but not proliferation. (A), Cells were seeded in serum-free media on top of the membrane and directional migration was initiated by the addition of FBS to the bottom of the transwell migration chamber. Cells on bottom of the filter were counted after 2 hours and 3.5 hours for A549 and H157 cells, respectively. Values are presented as percent of EV control (±SEM) from triplicates of one representative experiment. (B), a confluent monolayer of cells expressing EV or functional Duox1 was injured. Top, pictures were taken from the same positions over a time course of several hours. Bottom, cell migration into the wound area was analyzed for 9 and 15 h for transduced A549 and NCI-H157 cells. Data were collected from at least 9 random fields and are expressed as percent closed wound of the average wound area of Duox1+EV (±SEM). (C), cells were cultured in low-serum conditions for up to 5 days. Every 24 hours proliferation was spectrophotometrically determined at 540nm based on mitochondria-based reduction of MTT. Shown is a triplicate of one representative experiment.

It has been shown that H₂O₂ production is increased upon wounding of confluent monolayers of aortic endothelial and airway epithelial cells. In addition, the detected ROS was localized to the cells lining the wound edge (Moldovan et al., 1999; Wesley et al., 2007). Treatment with NADPH oxidase inhibitor DPI or siRNA-mediated silencing of Duox1 diminished ROS production in airway epithelial cells (Wesley et al., 2007). Since functional Duox localizes to the plasma membrane of transfected cells (Figure 13), seems to concentrate on the leading edge of randomly migrating cells (Figure 24 EV/EV).
and is beneficial for wound closure (Figure 36), it is conceivable that Duox proteins are targeted to the leading edge during cellular migration. This hypothesis was analyzed in NCI-H661, which stably express both functional oxidases, Duox1/DuoxA1-2 and Duox2/DuoxA2, respectively. Control cells expressed EV, DuoxA1-2 or DuoxA2 alone (see Figure 14A). In a first step, these cells were analyzed in transwell migration and quantitative wound closure assays to see, if they also feature the same Duox-based migrational advantage as lung cancer cells A549 and NCI-H157. Indeed, cells that expressed functional Duox1 migrated faster towards the bottom of the transwell chamber or into the wound area (Figure 37 A and B). Surprisingly, Duox2-based transwell migration was greatly increased compared to either control cells as well as Duox1-expressing cells, while wound closure was slightly faster compared to cells expressing functional Duox1. This suggests that the presence of functional Duox1 or Duox2 leads to increased motility of cells and that Duox2 has an even greater impact on cellular movement compared to Duox1. Transwell migration rates in DuoxA2-expressing cells were also improved. This could indicate that the maturation factor alone can already influence migration via yet undefined pathways.

![Graph A and B](image)

**Figure 37:** Duox1 and Duox2 increase migration in NCI-H661. Cells were stably transduced with either Duox1/DuoxA1-2 or Duox2/DuoxA2. Control cells expressed EV, DuoxA1-2 or DuoxA2. (A), Wounding assay. A confluent monolayer of cells expressing EV, functional Duox1 or functional Duox2 was injured. Cell migration into the wound area was analyzed after 6 h. Data were collected from at least 9 random fields and are expressed as percent closed wound of the average wound area of Duox1+A1 (±SEM). (B), Transwell migration assay. Cells were seeded on top of the membrane in serum-free media. Directional migration was initiated by the addition of serum-rich media to the bottom of the chamber. Cells on bottom of the filter were counted after 5.5 hours. Values are presented as percent of EV control (±SEM) from triplicates of one representative experiment.

Since both oxidases increased migration rates, their localization was analyzed along wound edges. One hour after generation of a linear injury to a confluent cell layer, both Duox1 and Duox2 localized at the leading edge of cells migrating into the wound, where they co-localized with their respective maturation factor (Figure 38).
In conclusion, these results suggest an important role for functional Duox enzymes, as they not only increase directional migration but also accelerate wound repair. It is possible that functional Duox allows the airway epithelium to heal faster in response to injury, an ability that is constricted in tissues that have lost Duox as a result of non-random promoter hypermethylation.
3.3 Characterization of Duox expression in Rodents

In human lung cancer, little is known about the cellular origins of the disease or the process of tumor initiation, because early-stage tumors are hardly detectable. Yet, animal models of human lung cancer have helped to improve the understanding of the cellular and molecular changes within lung cells during development and progression of the disease (Kim et al., 2005). Particularly, pulmonary tumor models in rodents are being used, as they are easily inducible and resemble lung carcinomas found in humans.

3.3.1 Duox expression in murine airways

Several models for induction of murine lung carcinomas have been described, which are mostly derived through carcinogen exposure, conditional deletion of tumor suppressors and/or Cre-recombinase-induced activation of proto-oncogenes (Jackson et al., 2001; Meuwissen et al., 2003; Meuwissen et al., 2001; Wang et al., 2006). The selective activation of oncogenic mutations in a subset of cells permits the study of tumor initiation and progression. Moreover, this model allows for the investigation of the cell of origin and the role of cancer stem cells in the preservation of those tumors. An Entrez Protein Database search for murine DUOX isoforms confirmed that Duox1, Duox2 and both maturation factors DuoxA1 and DuoxA2 are encoded in the mouse genome, encoding for proteins with respective sizes of 1551 aa (NM_001099297), 1517 aa (NM_177610), 341 aa (NM_145395) and 320 aa (BC031111). Since DUOX genes are silenced in human lung cancer, it is reasonable to assume that mouse DUOX might also be affected during lung tumori-genesis. In a first step, Duox expression was analyzed in the airways of C57BL/6 mice. Duox protein was immunohistochemically detected in the epithelial cell lining trachea, bronchi and airways of the lungs (Figure 39).

Figure 39: Immunohistochemical detection of mouse Duox in C57BL/6 mice. Sections from mouse trachea, bronchus and lung lobe were stained with anti-human Duox1,2 antibody. Duox protein was stained in red and was primarily expressed in the epithelial cells lining the airways. The inserts depict enlarged areas with specific Duox staining. Adjacent sections served as negative controls and were treated with preimmune serum in order to ensure the specificity of the antibody. Bar, 10µm.
Since the antibody used for the immunohistochemical detection of endogenous mouse Duox was directed against human Duox1, but also recognizes Duox2, the identity of the Duox isoform in mouse tissues was still unknown. Therefore, specific primers for mouse DUOX1, DUOX2, DUOXA1 and DUOXA2 were designed and expression of these genes was probed in whole tissue lysates from trachea, upper lung and lower lung. The tissues were derived from two different mouse strains, namely C57BL/6 and BALB/c. Both strains are inbred and are commonly used for lung cancer studies; however they are unequally susceptible for lung tumor development. While BALB/c mice are considered to be intermediate sensitive to spontaneous lung tumor incidence and chemically induced lung tumors (through exposure to cigarette smoke, tar or chemical carcinogens), C57BL/6 are almost completely resistant (Meuwissen and Berns, 2005). Surprisingly, DUOX1 was only expressed in the trachea but not in the lungs of C57BL/6 mice (FIGURE 40). In BALB/c mice DUOX1 was also greatly expressed in the trachea, while some DUOX1 was also detectable in the lung. In both strains the expression of DUOXA1 was reduced or completely absent, except for high levels in the trachea of BALB/c mice, suggesting that Duox1 can only be functional in the trachea of those animals, while C57BL/6 mice do not possess a functional Duox1 in their airways per se. DUOX2 and DUOXA2 were detectable in all tissues of both mice strains, indicating that Duox2 could be the main oxidase in murine airways.

![FIGURE 40: Endogenous expression of mouse DUOX1, DUOX2, DUOXA1 and DUOXA2. RT-PCR with mouse-specific primers was performed in C57BL/6 and BALB/c mice. Whole tissue lysates were obtained from trachea (T), upper lung (UL) and lower lung (LL). Actin was used as internal control.](image)

### 3.3.2 Duox expression in rat airways

As Duox1 might not to be functional in the murine lung, a mouse model of human lung cancer with regard to DUOX1 expression is not useful. Fortunately, lung cancer in rats features many cytogenetic and molecular mechanisms involved in tumor evolution, which are similar to the human disease (Dano et al., 2000). As rat lung cancer can easily be induced by exposure to X rays, cigarette smoke, beryllium metal, plutonium-239 oxide, radon or tumor xenografts, it appears to be an excellent model for de novo gene methylation analysis (Dano et al., 2000; March et al., 2001; Swafford et al., 1997). A database search in the rat genome revealed the existence of genes encoding for DUOX1, DUOX2, DUOXA1 and DUOXA2, translating to proteins with respective sizes of 1551aa (NM_153739.1), 1517aa (NM_024141.1), 340aa (NM_001107767.1) and 320aa (XM_575222.1). Expression of both DUOX and DUOXA isoforms was analyzed in whole tissue lysates from trachea, upper lung and lower lung of Sprague Dawley rats, a strain commonly used in lung cancer studies (FIGURE 41A).
In contrast to the results obtained in murine tissues, both \textit{DUOX1} and \textit{DUOXA1} were expressed in all analyzed tissues, while \textit{DUOX2} and \textit{DUOXA2} were only detectable in the trachea.

\textbf{FIGURE 41}: Endogenous expression of Duox in rat airways. (A) Whole tissue lysates from trachea (T), upper lung (UL) and lower lung (LL) of Sprague Dawley rats were analyzed for expression of \textit{DUOX1}, \textit{DUOX2}, \textit{DUOXA1} and \textit{DUOXA2} via RT-PCR. Actin served as internal control. (B) Sections of upper lung and lower lung from Sprague Dawley rats were stained anti-human Duox1,2 antibody. Duox protein was stained in red and was primarily expressed in the epithelial cells lining the airways. The inserts depict enlarged areas with specific Duox staining. Adjacent sections served as negative controls and were treated with preimmune serum in order to ensure the specificity of the antibody. Bar, 10\textmu m.

Staining with Duox-specific antibody demonstrated a distinct red stain in the epithelial cells of the airways of upper and lower lung (\textbf{FIGURE 41B}). As no \textit{DUOX2} transcript was detected in the lungs of Sprague Dawley rats, the immunohistochemical staining indicates that Duox1 is the main oxidase of the lung.

In conclusion, these results show that \textit{DUOX1} is not expressed in the murine lung, and therefore this species cannot be used for studies of \textit{DUOX1} silencing in the context of lung cancer onset and progression. As rat lungs express \textit{DUOX1}, this species might make a candidate model for the investigation of \textit{DUOX1}-silencing in lung cancer.
4 Discussion

The family of NADPH oxidases (Nox) has been linked to host defense, cell differentiation, transformation and inflammation. Two particular members, termed Duox1 and Duox2, are abundantly expressed in the epithelial cells lining the airways of the lung. To date, their functions are still largely unknown, but involvement in innate immunity and airway homeostasis has been suggested. Duox enzymes were also linked to oxidant-mediated signaling and chronic airway pathologies, based on their ability to produce reactive oxygen species (ROS).

The key findings of my work can be grouped in two categories. With respect to the regulation of the enzymes I discovered that Duox1 and Duox2 are differentially localized and expressed in lung epithelial cells, that they form paired functional heterodimers with their respective maturation factors DuoxA1 and DuoxA2, and that Duox2 functionally interacts with the paralog DuoxA1. In the context of lung cancer, my studies demonstrated that expression of both Duox enzymes as well as their maturation factors is markedly decreased in lung cancer and that their silencing is based on aberrant promoter hypermethylation.

4.1 Regulation of Duox1 and Duox2

The regulation as well as the specific functions of Duox enzymes are largely unknown. Harper and coworkers demonstrated that expression of both Duox enzymes was regulated differently in lung epithelial cells (Harper et al., 2005). While Duox1 mRNA was modestly upregulated through the Th2 cytokines interleukin (IL)-4 and IL-13, the Th1 cytokine interferon (IFN)-γ greatly enhanced expression of Duox2 mRNA. This suggests that Duox enzymes might have different functions in the airways in response to diverse stimuli or insults. It was not determined how much Duox1 or Duox2 protein was expressed or where the proteins were localized in primary or air-liquid differentiated epithelial cells.

Confocal analysis of lung epithelial cells demonstrated different cellular localizations for Duox1 and Duox2, which may indicate distinct cellular function. Duox1 has been linked to increased cellular migration rates in earlier studies (Koff et al., 2006; Wesley et al., 2007). As Duox1 is localized along the plasma membrane, and may generate H2O2 on-site, thus facilitating cellular migration via an ERK-activation pathway. For the most part Duox2 remained in the ER; yet, some Duox2 was also localized to the plasma membrane and to intracellular vesicles. The nature of these vesicles is still unresolved. However, they are reminiscent of trafficking vesicles, which can be latent and remain in the cytosol until an activating signal induces their transport to the membrane. These intracellular membrane reservoirs fuse with the plasma membrane and the contents of the vesicle will be released into the extracellular space, while enclosed integral membrane proteins will be exposed on the cell surface. H2O2 can actively control exocytosis through inhibition of a protein that regulates membrane fusion events necessary for exocytosis, termed N-ethylmaleimide sensitive factor (NSF) (Matsushita et al., 2005). Thus, Duox2-derived H2O2 could actively control the release of proteins and compounds stored in transport vesicles, including Duox2 itself.

Peroxisomes are small cellular respiratory organelles located in the cytoplasm that participate in the metabolism of fatty acid and amino acid degradation. They contain a wide variety of enzymes, including catalase, an enzyme that facilitates the decomposition of hydrogen peroxide to water and oxygen (del Rio et al., 1992). It is not surprising that peroxisomes are also a source of reactive oxygen species (Singh, 1997). H2O2 is generated in the peroxisomal respiratory pathway by different flavin oxidases, such as D-amino acid oxidase or uric acid oxidase (Corpas et al., 2001; del
Rio et al., 1992; Gootjes et al., 2004). Interestingly, the intracellular staining pattern of catalase and consequently of peroxisomes (Gootjes et al., 2004) is highly similar to the one observed for Duox2. It seems possible that Duox2 is one of the several flavin enzymes enclosed in lung epithelial peroxisomes and that Duox2-mediated H$_2$O$_2$ could participate in beta-oxidation, a process that breaks down fatty acids.

The expression of Duox2 was greatly induced upon differentiation in 3D culture, which could indicate that the enzyme is involved in polarization and maturation of airway epithelial cells, or that it performs a function characteristic of differentiated cells. In fact, it has been demonstrated for several other genes (such as mucin MUC3, MUC5AC, MUC5B, and MUC6) that their expression is upregulated through differentiation (Bernacki et al., 1999; Yoshisue et al., 2004). Duox1 has been linked to MUC5AC expression in cultured human airway epithelial cells via a Duox1-ROS-TACE-pro-ligand-EGF receptor cascade (Shao and Nadel, 2005), and it is conceivable that Duox2 could have a similar function in inducing epithelial mucin production.

The development of isoform-specific Duox and DuoxA antibodies allowed localization analysis of Duox1 and Duox2. Interestingly, although both isoforms were localized apically in the in vitro polarized airway system, Duox2 was selectively enriched in ciliated cells, where it mainly localized to the cilia. A recent report demonstrated that hyaluronan (HA), a high molecular weight polymer, is synthesized at the apical pole of airway epithelial cell. HA is broken down by ROS, resulting in several low molecular weight fragments that stimulate the ciliary beat frequency (Manzanares et al., 2007). As Duox2 is localized in the cilia of differentiated cells and is also expressed in vesicular structures, it is tempting to speculate that Duox2 will be transported via an intraflagellar mechanism along the microtubules into the cilia (Ainsworth, 2007). Duox2-mediated ROS could induce HA de-polymerization, thus contributing to the beating of cilia during mucociliary clearance. This hypothesis could be tested, for instance, by selectively knocking down either Duox or DuoxA isoforms utilizing lentivirus-mediated shRNA and studying the effect on cilia motility.

The diverse expression and localization patterns suggest different functions for Duox1 and Duox2. As directional migration was strikingly enhanced in cells expressing functional Duox2 compared to functional Duox1, it seems possible that upon insult or injury of the epithelium Duox2 enhances migration and/or wound closure to a larger extent than Duox1. Duox1 may perform a homeostatic function, whereas Duox2 is only expressed and transported to the plasma membrane when its function is needed by the cell. In fact, the promoter of Duox2 contains a putative ATF/CRE-binding site, a stress-responsive element. ROS have also been shown to mediate phosphorylation of cAMP-responsive element-binding protein (CREB) (Bedogni et al., 2003). Expression of Duox2 could be upregulated through activation of stress-pathways and its expression might be sustained via a positive feedback loop.

It has become evident that Duox function is not solely dependent on transcriptional regulation. In fact, Duox function is critically dependent on the presence of the maturation factors DuoxA1 and DuoxA2, allowing the oxidase to exit the ER and translocate to the plasma membrane (Grasberger and Refetoff, 2006). Surprisingly, contrary to the hypothesis that DuoxA1 and DuoxA2 are ER-resident proteins, DuoxA1 and DuoxA2 form heterodimeric complexes with their respective Duox partner. This phenomenon of complex formation had already been described for other members of the Nox family. In particular, the phagocytic oxidase Nox2 forms a heterodimer with p22$^{Prox}$ (DeLeo et al., 2000; Yu et al., 1997). Consistent with the concept of the Nox2/p22$^{Prox}$
heterodimer system, my studies unraveled that incorporation of heme into Duox was required for the assembly of the complex with DuoxA. These results suggest that Duox1 as well as Duox2 follow the paradigm of heterodimer formation with an associated subunit in a heme incorporation-dependent manner.

Based on the fact that several DuoxA isoforms exist, one might be tempted to speculate, how this could impact the function of Duox1. Unexpectedly, although Duox1 could form a heterodimeric complex with DuoxA1-3, this DuoxA1 isoform was not expressed in undifferentiated or differentiated airway epithelial cells. DuoxA1-3 expression might be age-dependent and is only expressed in embryonic tissues, as known e.g. for splice variants of G-protein coupled receptors (Pilzer and Gozes, 2006). Alternatively, it is possible that this isoform is not expressed in the lung per se, as it was originally identified from pooled libraries of brain, lung and testis. In addition, the polarized 3D system lacks alveolar type II cells, which could be the source for DuoxA1-3. Those cells are only expressed in the terminal alveoli, where they secrete lung surfactant and serve as progenitor cells for the terminally differentiated alveolar type I cells (Blaskaran et al., 2005). The primary lung epithelial cells used for 3D differentiation were isolated from the bronchi (NHBEC) or the smaller airways (SAEC), which are both localized afar from the terminal bronchioles (Fischer and Widdicombe, 2006). Therefore, these cells can differentiate into goblet, basal or ciliated cells, but not into alveolar type II cells. The identification of two simultaneously expressed splice variants, DuoxA1-1 and DuoxA1-2, could indicate that Duox1 is regulated by both isoforms in a competitive manner. The differential expression of DuoxA1-1 and DuoxA1-2 may modulate Duox1 activity by competing and therefore affecting functional Duox1/DuoxA1-2 heterodimer formation, a scenario identified for p73, a homolog of tumor suppressor p53 (De Laurenzi et al, 1998). In addition, as alternative splicing of mRNA transcripts generates additional genomic complexity from the low number of genes present in the human genome, the different DuoxA1-isoforms may have different roles in various Duox-expressing tissues (such as testis, thyroid, tongue and cerebellum).

The finding that Duox2 is localized differentially depending on its associated subunit (DuoxA2 or DuoxA1-2) might be indicative of a dual assignment for Duox2. It is plausible that by heterodimerization with DuoxA1-2, Duox2 could compensate for Duox1 in case of genetic or mutational inactivation. Based on their high degree of sequence homology, it might also be possible that DuoxA1-2 could substitute for DuoxA2, thus providing another fallback mechanism to maintain Duox2 function when mutational inactivation of DuoxA2 occurs. However, a recent report demonstrated that mutations in DuoxA2 resulted in hypothyroidism (Zampron et al., 2008), a condition generally attributed to genetic abnormalities in the sodium-iodide symporter (NIS), thyroperoxidase (TPO) or Duox2 (Ris-Stalpers, 2006). This finding contradicts the theory that DuoxA1-2 could generally substitute DuoxA2. Nevertheless, since the study demonstrated that DuoxA2 was still expressed as a truncated version, it is conceivable that the mutant DuoxA2 may not be able to support function anymore, but could still bind to Duox2. It would act as a dominant-negative suppressor of Duox2 function by binding to Duox2 and preventing the formation of a functional mismatch heterodimer with DuoxA1-2. However, the inability to form a mismatch might by confined to the thyroid, while still being applicable to the lung. Functional analysis in thyroid cells transiently reconstituted with the Duox2/DuoxA1-2 heterodimer would shed some light on such mechanism.

Nox5 function is tightly regulated based on its localization within the cell (Kawahara and Lambeth, 2008). This spatial regulation is achieved by phosphorylated phosphatidylinositol (PI) lipids (PI(4,5)P2 and PI(3,4,5)P3), which target proteins with polybasic clusters, such as Nox5, to the plasma
membrane (Heo et al., 2006; McLaughlin et al., 2002). In analogy to this observation, Duox enzymes could be regulated in a similar manner. Since all DuoxA-isofoms contain polybasic motifs, it is possible that directed association with a given maturation factor could determine the subcellular location of the oxidase, constituting another form of spatial targeting and therefore regulation. In conclusion, the presence of distinct Duox and DuoxA genes might result in a larger variety of function and in addition, might also secure preservation of Duox function in case of genetic alteration/mutation.

4.2 Duox in lung cancer

A deregulation of Duox enzymes has already been indirectly linked to several lung pathologies, including cystic fibrosis (Wright et al., 2006), chronic obstructive pulmonary disease (COPD) (Nagai et al., 2008) and asthma (van der Vliet, 2008). ROS had been connected to carcinogenesis, as they are important for the initiation and promotion of cells to neoplastic growth (Chung-man Ho et al., 2001). Because Duox enzymes have the ability to produce potentially harmful ROS, which may induce recurring DNA damage, inhibition of apoptosis and activation of proto-oncogenes (Azad et al., 2008), increased Duox expression or function could contribute to the pathogenesis of lung malignancy. However, in contrast to our expectations, this study showed that the expression of both Duox enzymes as well as their DuoxA maturation factors was clearly reduced in lung cancer cell lines as well as non-small cell lung cancer, thus ruling out the hypothesis of Duox being overexpressed or constitutively active.

The functions of many critical genes are lost in lung cancer, which can result from genomic amplification, homozygous deletion, frame shift or other coding-region mutations (Belinsky, 2004; Motiwala et al., 2004). The most common mechanism of gene silencing in lung cancer constitutes aberrant methylation of the 5' promoter region (Zochbauer-Muller et al., 2002). Genomic analysis of the promoters of both DUOX as well as DUOXA genes disclosed high density of CpG dinucleotides. Aberrant hypermethylation was confirmed for DUOX1 and DUOX2 and suggested for DUOXA1 and DUOXA2.

Promoter hypermethylation is frequent in both early-stage and late-stage lung tumor pathogenesis (Bowman et al., 2006; Machida et al., 2006; Palmisano et al., 2000; Tsou et al., 2002; Widschwendter et al., 2004). So far, more than 40 genes have been identified as being inactivated by promoter hypermethylation, many of which are involved in cell cycle regulation (CDKN2A, RARβ, PAX5α, β), DNA repair (MGMT), apoptosis (Caspase-8, Fas, DAPK, TRAILR1), Ras signalling (RASSF1A, NRE1A), and invasion (h-cadherin, E-cadherin, TIMP3, laminin family) (Belinsky, 2004; Bowman et al., 2006; Krunkosky et al., 2003; Tang et al., 2004; Zhou et al., 2005). In fact, it has been shown that aberrant CpG island methylation is not random (Costello et al., 2000). Bren et al. reported that loci at 15q15.1 and 15q22.2, which are located close to the Duox gene cluster at 15q15-21, are aberrantly methylated in lung cancer (Brena et al., 2007). In this regard, it is likely that methylation-based silencing of DUOX/DUOXA is not accidental, but rather a lung cancer-associated incident.

In general, promoters of tumor suppressor genes, such as RB1, p53, CDKN2A, BRCA1, BRCA2 and APC, are affected by this epigenetic event. Their gene products have pivotal roles in normal cells, as they control cell cycle repression, DNA repair, apoptosis or suppress metastasis (Baylin and Herman, 2000; Belinsky, 2004; Jones and Laird, 1999). Overall, their essential function is to inhibit the process of tumorigenesis. For instance, the gene CDKN2A, which encodes for INK4A (also known as
p16), is frequently silenced by aberrant methylation in many cancers. As INK4A inhibits cell-cycle progression, the inactivation of CDKN2A was proposed to be a first step towards immortalization by allowing cells to escape the immortality checkpoint M0 (Belinsky, 2004). Given that the promoters of both DUOX genes are frequently hypermethylated in vitro as well as in vivo, we hypothesized that Duox enzymes may be involved in a tumor suppressing network. They might transduce growth-inhibiting signals through regulated ROS production, which in turn act as secondary messengers. In fact, accumulation of high levels of intracellular ROS have recently been linked to senescence (Ramsey and Sharpless, 2006). In addition, the peroxidase-homology domain of Duox could be utilized to selectively oxidize target proteins of the tumor suppressing network, resulting in their inhibition or activation.

Reconstitution of functional Duox1 however resulted in improved migration rates. This phenotype is generally attributed to increased malignancy, as cell migration is the first step in metastasis, allowing a cell to spread from the initial site of tumor growth and invade the surrounding tissue. Another hallmark of malignant cells is uncontrolled proliferation (Tfelt-Hansen et al., 2004), which correlates with tumorigenesis and metastasis (Tagliaferri et al., 2000). Other studies have shown that reconstitution of tumor suppressors, such as PTEN (Zhao et al., 2004) or p53 (Marchetti et al., 2004) resulted in decreased cell growth. However, functional Duox1 did not influence cellular growth rates. Furthermore, anchorage-independent growth in soft agar or on poly-HEMA was not altered by reintroduction of Duox1 (data not shown), a function generally attributed to tumor suppressing proteins (Georgescu et al., 1999; Ha et al., 2007). In conclusion, as functional Duox1 did not have any repressive effects on cell migration or cell cycle control, a role for Duox1 as tumor suppressor is not likely.

The fact that functional Duox1 as well as Duox2 lead to increased wound healing, might suggest that these enzymes have a crucial role in facilitating fast and efficient closure of a wounded epithelium. Interestingly, clinical studies and experimental data demonstrated that smokers heal poorly and are more susceptible to incisional wound infection than non-smokers (Wong and Martins-Green, 2004; Yang and Longaker, 2003). Additionally, cigarette smoke has been shown to inhibit repair processes of airway epithelial cells (Kode et al., 2006; Luppi et al., 2005; Wang et al., 2001). It thus seems likely that smoking inhibits Duox function, resulting in sustained tissue injury and inflammation.

In fact, Nagai and coworkers have shown that chronic exposure to cigarette smoke was connected to down-regulation of DUOX1 (Nagai et al., 2008). Moreover, cigarette smoke has already been associated with promoter hypermethylation in various organs (Enokida et al., 2006; Marsit et al., 2007), especially in the lung (Belinsky et al., 1998; Belinsky et al., 2002; Eguchi et al., 1997; Kim et al., 2003; Kim et al., 2001). Therefore, cigarette smoking might be responsible for hypermethylation of the DUOX promoter, potentially leading to silencing of the whole locus harboring the DUOX/DUOXA genes. My in vivo expression analysis showed a concerted down-regulation of DUOX1/DUOXA1 in 82% of the patients, while only 64% demonstrated diminished DUOX2 levels. This could be due to the fact that the DUOX2 promoter contains less CpG dinucleotides than the DUOX1 promoter. This increases the probability of aberrant methylation, resulting in more efficient silencing of DUOX1 than DUOX2. Therefore, my findings might provide a first link between smoking-induced promoter hypermethylation and smoking-induced silencing of DUOX1.
In summary, Duox enzymes might contribute to tissue repair and thus constitute a prerequisite of the healthy lung epithelium. Upon such injury, a repair process is initiated that includes cell migration, proliferation, and differentiation (Aarbiou et al., 2004). Duox enzymes have already been linked to mucociliary cell differentiation through induction of mucin expression (Shao and Nadel, 2005) as well as to acidification of the airway surface liquid ASL (Schwarzer et al., 2004). My results further substantiate a homeostatic role of Duox enzymes as they enhance cell migration, thereby ensuring epithelial integrity and consequently maintenance of lung tissue functions such as ion transport and ciliary beating. Based on the prevalence of CpG islands within the promoter region of the DUOX/DUOXA genes, their genomic location might constitute a hot spot for methylation events that occur in the earliest stages of premalignancy.

As Duox enzymes seem to orchestrate several homeostatic functions, their silencing may lead to an altered lung epithelium without enough mucus, a basic pH change of the ASL and a reduced ability to regenerate from damage.
4.3 Outlook

Future studies should further elucidate the role of epigenetic Duox silencing in the development and progression of lung cancer. As the in vitro as well as in vivo studies demonstrated an overall high occurrence of aberrant DUOX promoter hypermethylation – despite a relatively small sample size – we propose that this mechanism is an early event in the development of lung cancer. Therefore, DUOX methylation could serve as a biomarker for individual risk assessment of genetic predisposition to lung cancer.

Epigenetically-mediated gene silencing by promoter hypermethylation has been shown to be the most common mechanism for gene silencing in lung cancer and these abnormalities arise in the early stages of lung pathogenesis, even before any pathological changes become apparent (Belinsky, 2004). For instance, research identified the genes for INK4A (p16) and MGMT as being 100% hypermethylated in patients with squamous cell lung carcinoma three years prior to clinical diagnosis. Several studies have shown that during the gradual change of the normal tissue to hyper-, meta-, dysplasia, and finally to carcinoma, the prevalence for promoter hypermethylation increases as time to lung cancer diagnosis decreases (Belinsky, 2004; Belinsky et al., 2006; Zochbauer-Muller et al., 2002).

![Figure 42: Aberrant promoter hypermethylation as biomarker for lung cancer prediction.](image)

If our theory is correct, case-control studies should demonstrate that Duox hypermethylation is indeed an early event, possibly present in preneoplastic lesions. Therefore, Duox would be a biomarker of genetic predisposition to cancer rather than an indicator of current malignancy. As such, Duox could be utilized for a highly sensitive, PCR-based sputum screening analysis for examination of high risk individuals, for example heavy smokers or workers exposed to airborne carcinogens (e.g. miners, textile or railroad workers). The probing of exfoliate material constitutes a non-invasive and side effect-free alternative to other more conservative examination methods, such as spiral CT or bronchoscopy.

However, if DUOX silencing does not occur during premalignancy, further studies might be able to link DUOX silencing directly to a certain tumor stage. Duox expression analysis of extensive tissue panels, which include detailed information regarding pathology and disease history, might reveal a connection between DUOX methylation and a certain lung cancer subtype and/or stage, thus
allowing the use of Duox as a tumor stage marker for clinical lung cancer. Particularly, linking of Duox silencing to either K-ras or EGFR mutations, which are present in up to 50-60% and 60% of non-small cell lung cancer (Duarte and Pascoal, 2006; Lynch et al., 2004) may provide an additional marker for tumor classification. As research is currently exploring the efficacy of demethylating agents (Belinsky et al., 2005), hypermethylated DUOX could prove to be a key therapeutic target to prevent or reverse malignant transformation in the airways.

Additionally, studies are important which investigate the association of Duox with other lung pathologies that are characterized by high oxidative stress (e.g. asthma, cystic fibrosis, COPD). It is possible that – in contrast to DUOX silencing in lung cancer – Duox enzymes are over-expressed or constantly activated in these diseases. To that end, the generation of animal models would allow for a more detailed correlation between Duox function and disease progression.

However, as my preliminary data suggest, both mouse and rat are not appropriate models for analysis of Duox1 and Duox2 in lung disease, as they lack co-expression of both oxidases throughout their pulmonary tract. In addition, transgenic mouse models of cystic fibrosis (CF) have been shown to fail to recapitulate the natural progression of the human disease, as they manifest subtle to no CF lung disease (Guilbault et al., 2007). Ferret and pig have emerged as new models for cystic fibrosis (Li and Engelhardt, 2003; Li et al., 2006; Rogers et al., 2008). Particularly pigs may constitute a better model for human genetic diseases based on the similarity to humans in regard to anatomy, biochemistry, physiology, size and genetics. However, ferrets might be the more appropriate lab model. Nevertheless, the expression patterns of Duox1 and Duox2 as well their maturation factors remain to be identified in ferrets as well as in pigs in order to use these models for Duox-focused research.

Our knowledge of the complex Duox biology will increase through histological assessment of clinical samples for Duox1 and Duox2 expression, utilizing the newly generated specific antibodies. This might identify abnormalities in specific lung diseases, possibly allowing the identification of specific isoform de-regulation. Answers to these questions could move the field towards a better understanding of onset and progression of lung diseases and might provide opportunities to develop novel therapeutic strategies.
5 Summary

Duox NADPH oxidases are the ROS-generating enzymes in the respiratory epithelium and the thyroid. Duox1 and Duox2 generate H₂O₂ at the air-liquid interphase of lung epithelial cells and at apical membranes of thyroid follicular cells. Duox enzymes have been linked to host defense and thyroid hormone biosynthesis. Duox1 was further connected to acidification of the airway surface liquid as well as wound healing. Besides dependence on co-expression of DuoxA maturation factors, not much is known about the spatial expression and regulation of both Duox enzymes in airway epithelial cells.

Here, I demonstrate that Duox enzymes form functional heterodimers with the corresponding DuoxA subunits, in close analogy to the phagocyte NADPH oxidase Nox2. Several DuoxA1-isoforms (DuoxA1-1, DuoxA1-2 and DuoxA1-3) were identified, which do not contribute equally to Duox1 function. Duox1 and Duox2 localize to different cellular compartments in lung epithelial cells, depending on divergent DuoxA co-expression. It is possible that this localization defines the signaling specificity of Duox isoforms. In fact, Duox2 but not Duox1 is expressed in ciliated cells in an ex vivo differentiated lung epithelial model.

In addition, based on their ability to produce potentially harmful ROS in the airways, Duox expression was investigated. The expression of DUOX1, DUOX2 and their maturation factors was markedly reduced or absent in lung cancer cell lines as well as in lung cancer tissues. Analysis of the CpG-rich promoter regions of DUOX1 and DUOX2 revealed frequent aberrant hypermethylation. Reconstitution of Duox increased cellular migration and wound healing, but did not affect growth rates.

In conclusion, the results suggest that Duox1 and Duox2 have distinct functions in the lung epithelium and that aberrant DUOX promoter hypermethylation is a common silencing mechanism of DUOX1 and DUOX2 in lung cancer. Therefore, epigenetic modifications of the genomic locus harboring the DUOX genes might be a potential biomarker for developing or progressing lung cancer. Further, analysis of isoform expression in other lung diseases might present an initial step towards early screening approaches and drug development.
6 Zusammenfassung


Zusammenfassend deuten die Ergebnisse darauf hin, dass Duox1 und Duox2 unterschiedliche Aufgaben im Lungenepithel besitzen, und dass beide Enzyme häufig im klinischen Lungenkrebs nicht mehr exprimiert werden. Der die DUOX Gene umgebende Lokus stellt ein mögliches Ziel für häufige epigenetische Modifikationen im Lungenkrebs dar. Veränderungen in diesem chromosomalen Bereich könnten als potenzielle Biomarker für die Kanzerogenese genutzt und eventuell sogar einer der drei Stufen (Initiation, Promotion, Progression) zugeordnet werden. Darüberhinaus könnte die Analyse der Expressionsmuster von Duox1 und Duox2 Hinweise für neue Entwicklungsansätze von spezifischen Medikamenten liefern, und so die Behandlung verschiedener Lungenkrankheiten ermöglichen.
7 References


References


Wong, J.L., R. Creton, and G.M. Wessel. 2004. The oxidative burst at fertilization is dependent upon activation of the dual oxidase Udx1. Dev Cell. 7:801-14.


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9 Erklärung


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