British Journal of Pharmacology

British Journal of Pharmacology (2018) **175** 1401–1418 1401

REVIEW ARTICLE

Dual oxidase: a novel therapeutic target in allergic disease

Correspondence Albert van der Vliet, PhD, Department of Pathology and Laboratory Medicine, The Robert Larner, M.D. College of Medicine, University of Vermont, HSRF Room 216, 149 Beaumont Avenue, Burlington, VT 05405-0068, USA. E-mail: albert.van-der-vliet@uvm.edu

Received 14 November 2017; **Revised** 17 January 2018; **Accepted** 22 January 2018

Albert van der Vliet[1,2], Karamatullah Danyal[1,2] and David E Heppner[3,4]

[1]Department of Pathology and Laboratory Medicine, The Robert Larner, M.D. College of Medicine, University of Vermont, Burlington, VT, USA, [2]Vermont Lung Center, University of Vermont, Burlington, VT, USA, [3]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA, USA, and [4]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

NADPH oxidases (NOXs) represent a family of enzymes that mediate regulated cellular production of reactive oxygen species (ROS) and play various functional roles in physiology. Among the NOX family, the dual oxidases DUOX1 and DUOX2 are prominently expressed in epithelial cell types at mucosal surfaces and have therefore been considered to have important roles in innate host defence pathways. Recent studies have revealed important insights into the host defence mechanisms of DUOX enzymes, which control innate immune response pathways in response to either microbial or allergic triggers. In this review, we discuss the current level of understanding with respect to the biological role(s) of DUOX enzymes and the unique role of DUOX1 in mediating innate immune responses to epithelial injury and allergens and in the development of allergic disease. These novel findings highlight DUOX1 as an attractive therapeutic target, and opportunities for the development of selective inhibitor strategies will be discussed.

Abbreviations

CPZ, chlorpromazine; DPI, diphenylene iodonium; DUOX, dual oxidase; DUOXA, dual oxidase maturation factor; EGFR, EGF receptor; ER, endoplasmic reticulum; HDM, house dust mite; ILC2, type 2 innate lymphoid cell; LPO, lactoperoxidase; MPO, myeloperoxidase; NOX, NADPH oxidase; NOXA, NOX activator; PDB, protein database; PHD, peroxidase homology domain; ROS, reactive oxygen species; TK, tyrosine kinase; TLR, toll-like receptor; TPO, thyroperoxidase; TRX, thioredoxin

DOI:10.1111/bph.14158

Introduction

The concept of oxidative stress has been widely embraced as a major factor in disease pathology and has fuelled a billion dollar industry based on antioxidant dietary supplements. However, clinical studies using antioxidant supplementation strategies have generally been unsuccessful in attenuating disease risk or progression, and antioxidant supplementation was in some cases found to even worsen pathological outcomes (Ghezzi *et al.*, 2017). This lack of success likely relates to the flawed concept of oxidative stress as a pharmacological target. First, oxidative stress can be brought about by a range of ROS (the commonly used acronym to define this group of reactive metabolites), which can originate from external sources (e.g. environmental pollution and metabolism of xenobiotics) or be generated endogenously by various enzymatic systems, in some cases in a regulated and deliberate fashion to serve important biological functions [e.g. by activation of NADPH oxidases (NOXs)]. Therefore, generic non-selective approaches that indiscriminately suppress ROS may not have the desired outcome. A second flaw with such generic antioxidant approaches is the fact that ROS represent a diverse group of reactive metabolites that each have unique (bio)chemical properties, and it is often unclear to what extent antioxidant supplements counter the action of individual ROS species. To clarify this, it is helpful to distinguish *primary* from *secondary* ROS, with primary ROS representing the initial products of (enzymatic) O_2 reduction [i.e. superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide ($\mathbf{H_2O_2}$)] and secondary ROS comprising reactive metabolites formed by subsequent reactions of these primary ROS [e.g. with $\mathbf{NO^{\cdot}}$ to form peroxynitrite ($ONOO^-$), with transition metals or metalloenzymes to form hydroxyl radical ($OH^{\cdot}$) or hypohalous acids, or with other biomolecules to form, for example, lipid peroxides]. The biological literature on oxidative stress has tended to focus primarily on these secondary ROS as being responsible for pathology associated with oxidative stress, whereas dysregulated production of primary ROS ($O_2^{\cdot-}$, H_2O_2) might be equally or even more important.

With respect to the production of primary ROS in biological systems, their main cellular source include the family of NOXs, and ROS production is often considered the sole function of NOX enzymes to mediate their biological functions (Lambeth, 2004; Bedard and Krause, 2007). These functions rely on the production of cytotoxic secondary ROS (as a host defence mechanism against infection) and also on primary ROS (mostly H_2O_2) that can control protein function *via* reversible oxidation of susceptible cysteine or methionine residues in a process known as redox signalling (Janssen-Heininger *et al.*, 2008; Holmstrom and Finkel, 2014). Since the discovery of various NOX homologues about two decades ago, it has become apparent that several disease conditions are characterized by inappropriate expression or activation of NOXs. From this perspective, rather than attempting to prevent the adverse biochemistry of secondary ROS using generic approaches of antioxidants or radical scavengers, pharmacological targeting of specific NOX enzymes or other cellular sources of ROS might be more fruitful. The biology and pathology of NOX enzymes have been summarized in several excellent recent reviews (Lambeth, 2004; Bedard and Krause, 2007; Lassegue *et al.*, 2012; Bernard *et al.*, 2014), and a number of efforts over the past decade have attempted to develop isoform-selective inhibitors of NOX family enzymes. Among these, the dual oxidases (DUOX1 and DUOX2) have been relatively ignored in such efforts, although emerging findings indicate their potential involvement in disease pathology. Therefore, this review will focus on recent advances with respect to the biology and pathology of DUOX enzymes and will discuss molecular features that may inform approaches for their selective pharmacological targeting.

Discovery and tissue expression of DUOX enzymes

The dual oxidases DUOX1 and DUOX2 are isoforms of the long-known phagocyte (now known as NOX2), but do not require any protein cofactors for activation, which is primarily dictated by the presence of calcium-regulated EF-hand domains (a feature also shared with NOX5). In addition, the DUOX enzymes also contain a unique extracellular NH_2-terminal domain with high homology to haem peroxidases, which has earned them the name 'dual oxidase', even though the function of this peroxidase homology domain (PHD) is still somewhat unclear and may be unrelated to catalytic peroxidase activity (Meitzler and Ortiz de Montellano, 2009). Both DUOX genes share a bidirectional promoter with their corresponding DUOX maturation factors (DUOXAs) and are located in a head-to-head fashion in tandem on the long arm of chromosome 15 (De Deken *et al.*, 2014). The DUOXAs are critical for full maturation of the DUOX proteins and coordinate their location at the plasma membrane, although immature DUOX proteins within the endoplasmic reticulum (ER) also possess catalytic activity (Morand *et al.*, 2004; Ameziane-El-Hassani *et al.*, 2005; Grasberger and Refetoff, 2006). Transcriptional modes of regulation of DUOX1/DUOX2 and their corresponding maturation factors are still incompletely characterized (Christophe-Hobertus and Christophe, 2010; Xu *et al.*, 2012).

The DUOX enzymes were first identified in human and porcine thyroid tissues and were originally described as $p138^{Tox}$ or ThOX1 and ThOX2 (Dupuy *et al.*, 1999; De Deken *et al.*, 2000) and were later renamed as DUOX1 and DUOX2 based on the presence of a PHD domain (Edens *et al.*, 2001). DUOX1 and DUOX2 are also expressed in various other tissues, with significant expression of DUOX1 mRNA in lung, placenta, testis, prostate, pancreas and heart and expression of DUOX2 mRNA primarily in the colon and also in lung, kidney, liver, pancreas, prostate and testis (Edens *et al.*, 2001). A more detailed tissue distribution of human DUOX1 and DUOX2 is illustrated in Figure 1, based on extracted RNAseq data from the genotype-tissue expression project from the Broad Institute of MIT and Harvard (www.gtexportal.org). In the thyroid, DUOX is critical for H_2O_2 production to support oxidative iodination chemistry by thyroperoxidase (TPO) to generate thyroid hormone. This function appears to be restricted primarily to DUOX2 based on identification of a number of missense mutations

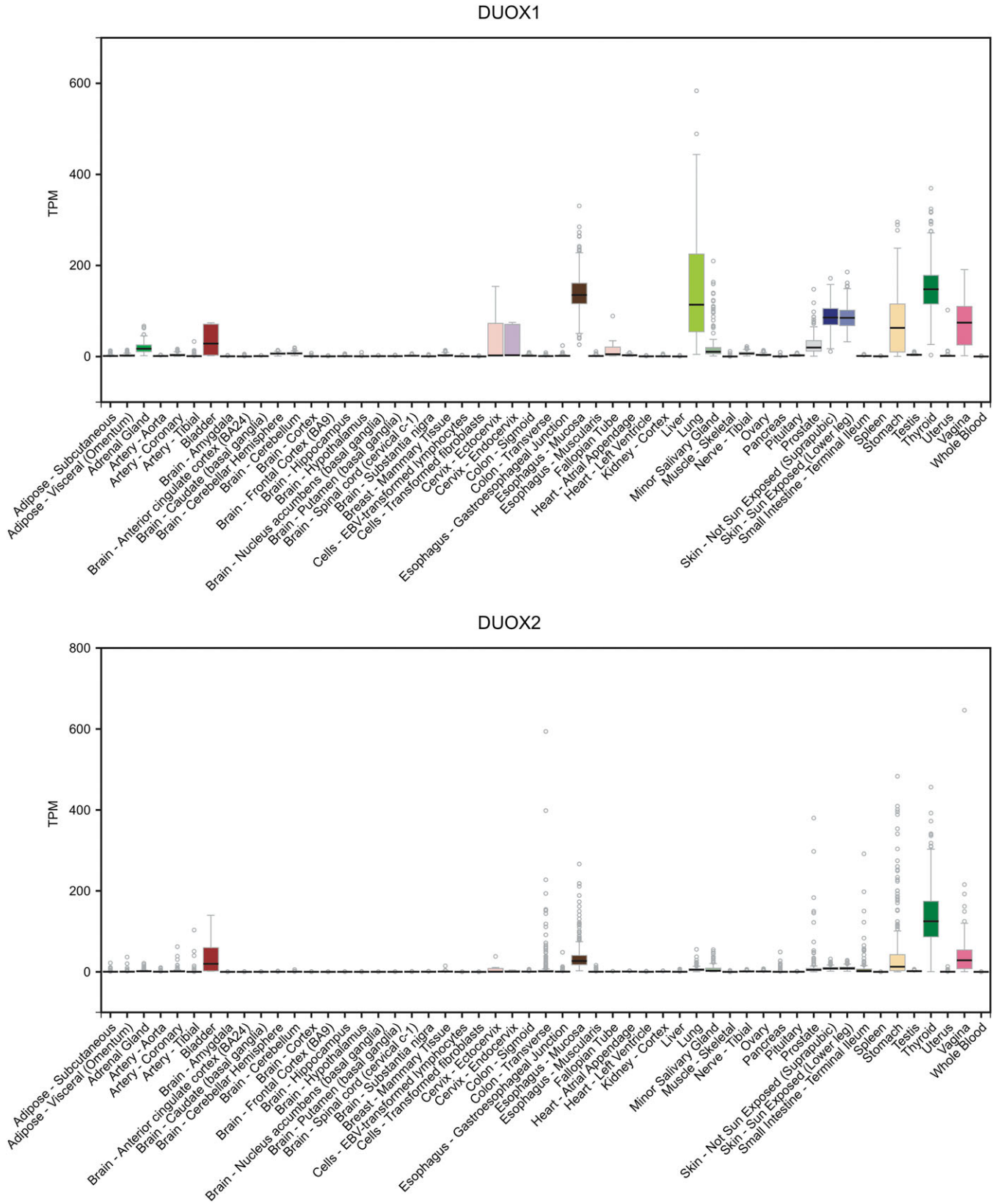

Figure 1

Tissue distribution of mammalian DUOX enzymes. RNAseq data were extracted from public data deposited by the Broad Institute for the Gene Tissue Expression (GTEx v.7) project (www.gtexportal.org). EBV, Epstein–Barr virus; TPM, Transcripts Per Kilobase Million mapped reads.

in DUOX2 that result in diminished function and hypothyroidism (De Deken *et al.*, 2014). DUOX2 is also the main isoform within the gastrointestinal (GI) tract and is expressed most prominently within the colon epithelium (El Hassani *et al.*, 2005) at the tip of intestinal villi (Sommer and Backhed, 2015) and within rectal glands (Geiszt *et al.*, 2003). *In situ* hybridization analysis of respiratory tissues has shown the presence of both DUOX1 and DUOX2 mRNA, with DUOX1 mostly expressed in the tracheal and bronchial epithelium and DUOX2 within salivary glands (Geiszt *et al.*, 2003). Subsequent studies indicate that DUOX protein is primarily present at the apical epithelial surface in major airways (Forteza *et al.*, 2005) and also in the alveolar epithelium, primarily in type II cells (Fischer *et al.*, 2007). Cell-specific expression of DUOX1 in other tissues is less well characterized, but several reports have highlighted the presence of DUOX1 in epidermal keratinocytes (Hirakawa *et al.*, 2011), urothelial cells (Donko *et al.*, 2010) and in non-epithelial cell types such as T-cells (Kwon *et al.*, 2010), alveolar macrophages (Rada *et al.*, 2014b) and innate lymphoid cells (Habibovic *et al.*, 2016).

Host defence properties of DUOX in non-mammalian organisms

Homologues of DUOX are found in almost all multicellular organisms, and DUOX enzymes therefore likely have evolved to serve fundamental functional roles, such as host defence, cell differentiation and development (Kawahara *et al.*, 2007). Early studies in *Caenorhabditis elegans* indicated the presence of Ce-Duox1 (also known as BLI-3) in the hypodermis, which was found to support oxidative cross-linking of tyrosine residues to promote stabilization of the cuticular extracellular matrix (Edens *et al.*, 2001). Similar functions for Duox in extracellular tyrosine cross-linking were also described in *Drosophila melanogaster* or in other arthropods, to stabilize wing cuticle structures or enhance defence against invading pathogens (Anh *et al.*, 2011; Yang *et al.*, 2014). Although such tyrosine cross-linking was originally attributed to its PHD domain, it also requires the involvement of a distinct haem peroxidase (Kumar *et al.*, 2010; Yang *et al.*, 2014; Hurd *et al.*, 2015). More recent studies also indicate alternative functions for Ce-Duox1/BLI-3 (the single functional NOX in *C. elegans*) in intestinal host defence, which are related to its ability to activate intracellular signalling pathways involving p38 MAPK signalling and the Nrf (NF-E2 related factor) orthologue SKN-1, thereby augmenting resistance to invading pathogens (Hoeven *et al.*, 2011). Similar intestinal host defence functions were also described for dDUOX, one of the two NOX homologues in *D. melanogaster* (Ha *et al.*, 2005; Ha *et al.*, 2009; Xiao *et al.*, 2017), and Duox in zebrafish (Flores *et al.*, 2010). Furthermore, studies in *D. melanogaster* have revealed both positive and negative regulatory mechanisms to control Duox expression or activation, which likely serve to assure its adequate response to pathogenic bacteria while tolerating commensal bacteria (Kim and Lee, 2014; Xiao *et al.*, 2017).

Mammalian DUOX in the gastrointestinal tract

Similar host defence functions of DUOX were also proposed in mammalian systems (Geiszt *et al.*, 2003), although the situation is more complex because of the presence of two distinct functional DUOX isoforms. While the two DUOX genes are highly homologous (>85%), they possess distinct differences with respect to their gene regulation (Harper *et al.*, 2005; Linderholm *et al.*, 2010; Raad *et al.*, 2013) and activation mechanisms (Rigutto *et al.*, 2009). DUOX2 expression within the GI tract is sustained by the intestinal biota, although it is not affected by commensal bacteria (Sommer and Backhed, 2015). The precise mechanisms of DUOX2 activation by pathogenic bacteria are not known but are thought to involve **toll-like receptors (TLRs)**, intracellular **NOD2** receptors and MAPK/NF-κB pathways (Lipinski *et al.*, 2009; Sommer and Backhed, 2015). Intestinal DUOX2 is enhanced in patients with Crohn's disease and ulcerative colitis (Aviello and Knaus, 2017), and a functional role for DUOX2 in intestinal homeostasis is implicated by several reports linking inactivating DUOX2 mutations with Crohn's disease (Levine *et al.*, 2016) or very early onset inflammatory bowel disease (Hayes *et al.*, 2015; Parlato *et al.*, 2017). Experimental studies in mice with functional DUOX2 deficiency showed increased gastric colonization after infection with *Helicobacter felis* and intestinal alterations indicative of mucosal dysbiosis (Grasberger *et al.*, 2015). In contrast to DUOX2, DUOX1 is minimally expressed in the GI tract, although DUOX1 has been detected in the stomach lining and in some gastric and colorectal epithelial cell lines (Aviello and Knaus, 2017), and virtually nothing is known regarding its potential functional role in GI biology or pathology. Although current evidence supports a functional role for DUOX2 in antimicrobial responses and in the maintenance of intestinal microbiobial homeostasis, no direct evidence exists to date to suggest that DUOX2 contributes to the pathology of inflammatory bowel disease.

Host defence functions of DUOX in the respiratory tract

Antimicrobial/antiviral host defence

Both DUOX1 and DUOX2 are expressed in the respiratory epithelium, DUOX1 being the main isoform responsible for extracellular H_2O_2 production in response to exogenous stimuli such as **ATP** (Forteza *et al.*, 2005), **histamine** (Rada *et al.*, 2014a) and bacterial stimuli such as **LPS** or **flagellin** (Koff *et al.*, 2008; Boots *et al.*, 2009; Rada and Leto, 2010). In apparent contrast, studies with differentiated airway epithelial cells indicate a prominent role for DUOX2 in basal H_2O_2 production (Gattas *et al.*, 2009; Linderholm *et al.*, 2010). Moskwa *et al.* (2007) were the first to report a role for DUOX in mammalian airway epithelial antimicrobial activity, which was attributed to DUOX2-dependent extracellular H_2O_2 production. In agreement with these findings, epithelial exposure to bacterial flagellin was found to promote interactions between **TLR5** and DUOX2 to stimulate ROS production and innate immune responses (such as **IL-8** and MUC5AC production) (Joo *et al.*,

2012). Although bacterial triggers can also activate DUOX1 (see above), studies in mice have so far failed to identify a role for DUOX1 in airway production of inflammatory cytokines or neutrophil recruitment in response to bacterial LPS (Chang *et al.*, 2015; Hristova *et al.*, 2016).

The potential importance of DUOX2 in airway antiviral responses was initially suggested by findings that DUOX2 and its maturation factor DUOXA2 are both strongly up-regulated by viral infection and by **types I (α and β) and II (γ) IFNs** and Th1 (type 1) cytokines (Harper *et al.*, 2005; Fink *et al.*, 2013; Strengert *et al.*, 2014), although viral infection can in some cases also induce DUOX1 (Grandvaux *et al.*, 2015). DUOX2-mediated antiviral responses were found to be mediated by the induction of retinoic acid-inducible gene 1 and melanoma differentiation-associated protein 5 and activation of type 1 IFN responses (Fink *et al.*, 2013; Kim *et al.*, 2015), which in conjunction with type 1 cytokines (**TNF-α** and **IL-1β**) induce DUOX2 to subsequently participate in antiviral pathways *via* IFN-β and IFN-γ (Fink *et al.*, 2013; Grandvaux *et al.*, 2015). The functional importance of DUOX2 in antiviral responses was demonstrated in studies with airway epithelial cells *in vitro* (Fink *et al.*, 2013; Strengert *et al.*, 2014) and in studies of influenza A infection in mice, which was augmented by intranasal inoculation with DUOX2-targeted short hairpin RNA and attenuated after administration of DUOX2 DNA (Hong *et al.*, 2016; Kim *et al.*, 2017). Collectively, even though bacterial and viral stimuli can activate both DUOX1 and DUOX2, evidence so far primarily implicates DUOX2 in antibacterial or antiviral host defence, and no reports exist to date to suggest a role for DUOX1.

Role in epithelial wound responses

In addition to being endowed with elaborate defence mechanisms against rapidly replicating microorganisms (bacteria, viruses, etc.), vertebrates have also evolved defence mechanisms against physical trauma or epithelial injury caused by invasion of helminths or other metazoan parasites. Parasites do not complete their life cycle within the host, and activation of cytotoxic (type 1) defence mechanisms to kill these organisms would be ineffective and too dangerous to the host. Instead, host defence against these pathogens involves type 2 immune response pathways that have evolved to promote expulsion by, for example, mucus production as well as activation of regenerative pathways to repair breaches in epithelial barrier integrity, thereby enhancing tolerance to parasite infection (Gause *et al.*, 2013). Among the first reports addressing a host defence function of airway epithelial DUOX1 are findings highlighting its role in controlling the expression of the mucin gene MUC5AC in response to various triggers (Shao and Nadel, 2005; Li *et al.*, 2013; Habibovic *et al.*, 2016), thus indicating a role for DUOX1 in promoting mucociliary clearance. Moreover, studies with cultured airway epithelial cells also demonstrated a contribution of DUOX1 to epithelial wound responses to physical or chemical injury, by promoting intrinsic cell dynamics (Wesley *et al.*, 2007; Hristova *et al.*, 2014) and expression of various wound response genes such as **MMP-9** and IL-8 (CXCL8) (Koff *et al.*, 2008; Sham *et al.*, 2013). Similar DUOX1-mediated wound responses were also observed in an *in vivo* model of lung epithelial injury in mice (Gorissen *et al.*, 2013), and analogous DUOX-mediated epidermal wound healing was also demonstrated in zebrafish-based models of tail fin injury (Niethammer *et al.*, 2009; Razzell *et al.*, 2013), thus highlighting a highly conserved role for DUOX1 in epithelial wound responses (van der Vliet and Janssen-Heininger, 2014). The mechanisms by which DUOX1 is activated in these contexts are not fully understood but likely involve the participation of damage-associated molecular patterns such as the purine metabolite ATP, which activates epithelial DUOX1 through stimulation of **purine nucleotide P2Y receptors** at the epithelial surface (Wesley *et al.*, 2007; Hristova *et al.*, 2016).

Production of epithelial alarmins

Cytokines of the IL-1 family are central mediators of innate immunity, and some of these (e.g. the interleukins IL-1α and **IL-33**) are expressed constitutively within epithelial cells or other structural cell types within their nuclei or cytoplasm, allowing them to be released rapidly upon cell stimulation or injury, to serve as 'alarmins' by acting as extracellular cytokines that alert the immune system to induce appropriate responses (Cayrol and Girard, 2014). With respect to IL-33, a major epithelial alarmin, its release causes the induction of type 2 cytokines (e.g. **IL-5** and **IL-13**) and growth factors (e.g. **amphiregulin** or **TGFβ**) within various effector cells, including locally resident type 2 innate lymphoid cells (ILC2s), and such IL-33-mediated activation of ILC2-mediated type 2 responses is critical in host defence against parasitic infection (Molofsky *et al.*, 2015). Recent studies highlighted the importance of Ca^{2+}-dependent signalling pathways induced by mechanical triggers or danger signals such as ATP in epithelial secretion of IL-33, and our group recently demonstrated a critical role for activation of DUOX1 (but not DUOX2) in IL-33 secretion by non-classical pathways (Hristova *et al.*, 2016). These findings would imply a specific host defence role for DUOX1 against infections by hookworms or other parasites, since lung epithelial and vascular injury due to worm penetration represent important features of hookworm infection (Bouchery *et al.*, 2015), but such a role for DUOX1 has not yet been demonstrated. Consistent with such a role for DUOX1 in type 2 immunity are findings that airway epithelial DUOX1 expression is enhanced in the presence of type 2 cytokines such as IL-13 (Harper *et al.*, 2005; Hristova *et al.*, 2016), thereby enhancing its functional role in the context of prolonged or repeated insults. Curiously, studies in thyrocytes and intestinal epithelial cells indicate that type 2 cytokines induce DUOX2 rather than DUOX1 (Raad *et al.*, 2013) and may, therefore, suggest a similar role for DUOX2 in intestinal defence against hookworm infection (Monticelli *et al.*, 2015).

DUOX in other epithelia and non-epithelial cell types

DUOX1 represents the predominant NOX isoform in human keratinocytes, the major epidermal cell type (>90%) and has been linked with Ca^{2+}-dependent keratinocyte differentiation (Choi *et al.*, 2014). DUOX1 may also be involved in innate cutaneous host defence to infectious triggers or in epidermal wound responses, based on findings in non-mammalian organisms (Niethammer *et al.*, 2009; Juarez

et al., 2011; van der Hoeven *et al.*, 2015), although DUOX1 in oral and skin epithelia does not appear to be essential for induction of defensins in response to *Acinetobacter baumannii*, a Gram-negative pathogen that threatens healthcare delivery systems (Feng *et al.*, 2014). DUOX1 is also prominently expressed in urothelial cells, with a potentially similar host defence function in the bladder (Donko *et al.*, 2010). Finally, evidence is emerging for the presence of DUOX1 in non-epithelial lineages, such as T and B lymphocytes (Singh *et al.*, 2005; Kwon *et al.*, 2010), macrophages (Rada *et al.*, 2014b) and innate lymphoid cells (Habibovic *et al.*, 2016), with it being involved in T-cell or B-cell receptor signalling and having potential roles in macrophage or ILC polarization, thus indicating broader host defence functions of DUOX1.

Specific roles for DUOX1 and DUOX2 in distinct innate immune response pathways?

Epithelial barriers in vertebrates are critical in providing adequate defence against common external challenges and form a critical sentinel function to alert the immune system to coordinate appropriate immune responses to these diverse challenges (Gallo and Hooper, 2012; Holtzman *et al.*, 2014; Lambrecht and Hammad, 2017). The presence of both DUOX1 and DUOX2 within epithelial lineages, particularly at epithelial surfaces, ideally positions them to serve critical roles in mucosal host defence and regulating appropriate immune responses. In contrast to non-vertebrates, which rely solely on innate immune responses that involve recognition of microorganisms by non-rearranging receptors and rapid response mechanisms in which their single functional DUOX gene may participate, vertebrate species also evolved with an adaptive immune system to coexist with innate immune pathways, allowing for greater variability in antigen recognition and development of memory, but also enhancing the potential for autoimmunity, allergy or allograft rejection (Janeway and Medzhitov, 2002; Hoffmann, 2003). Since vertebrate species typically contain two highly homologous DUOX genes and DUOXAs, likely as a result of gene duplication events during evolution of vertebrates from early deuterostome ancestors (Kawahara *et al.*, 2007), we propose that DUOX1 and DUOX2 may have evolved to acquire unique roles in these specific arms of the innate immune response. Indeed, while DUOX2 appears to participate primarily in antimicrobial/antiviral responses associated with TLR activation and production of interferons and type 1 cytokines, DUOX1 is primarily involved in the activation of type 2 immune responses induced by epithelia-damaging events, such as parasite infection, protease allergens and toxins. (Figure 2). Observations of isoform-specific modes of regulation with respect to gene regulation and activation of DUOX1 and DUOX2 (Harper *et al.*, 2005; Rigutto *et al.*, 2009) are consistent with such unique roles in specific aspects of epithelial-derived host defence initiated by distinct receptor-mediated pathways (e.g. TLRs vs. P2Y receptors and **proteinase-activated receptors**). Coordinated activation of either response pathway typically serves to restore tissue homeostasis, but inappropriate activation may result in autoimmunity or metabolic disease or in allergic disease and fibrosis respectively (Figure 2). Because of the considerable cross-talk and communication between individual cell types involved in these immune responses (Rivera *et al.*, 2016), it is likely that DUOX1 and DUOX2 are both engaged in most cases and their relative contributions may differ depending on the type of pathogen or injury. In some cases, such DUOX-specific events may be antagonistic, as illustrated by the potential suppression of DUOX1 by viral infection or IFN (Grandvaux *et al.*, 2015), but may also be cooperative as highlighted by a recent study indicating that DUOX2 can mediate pannexin-mediated ATP release in response to hypotonic stress (Krick *et al.*, 2016), thereby also resulting in activation of DUOX1.

Mechanisms of DUOX-mediated host defence: from oxidant-mediated killing to redox-dependent cell signalling

Analogous to the proposed antimicrobial functions of phagocyte NOX (NOX2) in conjunction with haem peroxidases such as **myeloperoxidase (MPO)** in neutrophils (Winterbourn *et al.*, 2016), DUOX1/DUOX2 are thought to exert their host defence function by generating H_2O_2 and inducing lactoperoxidase (LPO)-catalysed oxidation of thiocyanate (SCN^-) or iodide (I^-) within the airway lumen, to form secondary oxidants such as hypothiocyanous acid (HOSCH) and hypoidous acid (HOI), which serve to kill or repel bacteria (De Deken *et al.*, 2014) and minimize virus infectivity (Grandvaux *et al.*, 2015). In addition, such oxidative host defence mechanisms may also be augmented by NOX-dependent proton secretion, which compensates for charge transfer and local pH changes due to NOX activation and leads to NOX2-dependent acidification of phagosomes in phagocytic cells or DUOX1-dependent acidification of the airway lumen (Fischer, 2009; Segal, 2016), in both cases enhancing the activity of locally secreted peroxidases to generate antimicrobial oxidants (Winterbourn *et al.*, 2016).

In addition to these extracellular oxidative host defence mechanisms, DUOX-derived H_2O_2 is also capable of inducing cellular responses by autocrine or paracrine mechanisms *via* redox-dependent regulation of cell signalling pathways, by reversible oxidation of functional cysteine residues. A proteomic screen revealed that DUOX1 activation induces cysteine oxidation within a number of cellular targets, including cytoskeletal proteins, oxidoreductase enzymes and proteins involved in cell metabolism (Hristova *et al.*, 2014). Reversible cysteine oxidation also has a major impact on tyrosine kinase (TK) signalling, either by inactivation of protein tyrosine phosphatases or by activation of protein TKs (Heppner *et al.*, 2016). Indeed, DUOX1-dependent airway epithelial wound responses as well as IL-33 secretion were found to be mediated by activation of the non-receptor TK **Src** and the **EGF receptor (EGFR)**, in part by direct oxidation of cysteine residues within these kinases (Sham *et al.*, 2013; Heppner *et al.*, 2016; Hristova *et al.*, 2016). Such DUOX1-dependent protein cysteine oxidation may involve a direct interaction of

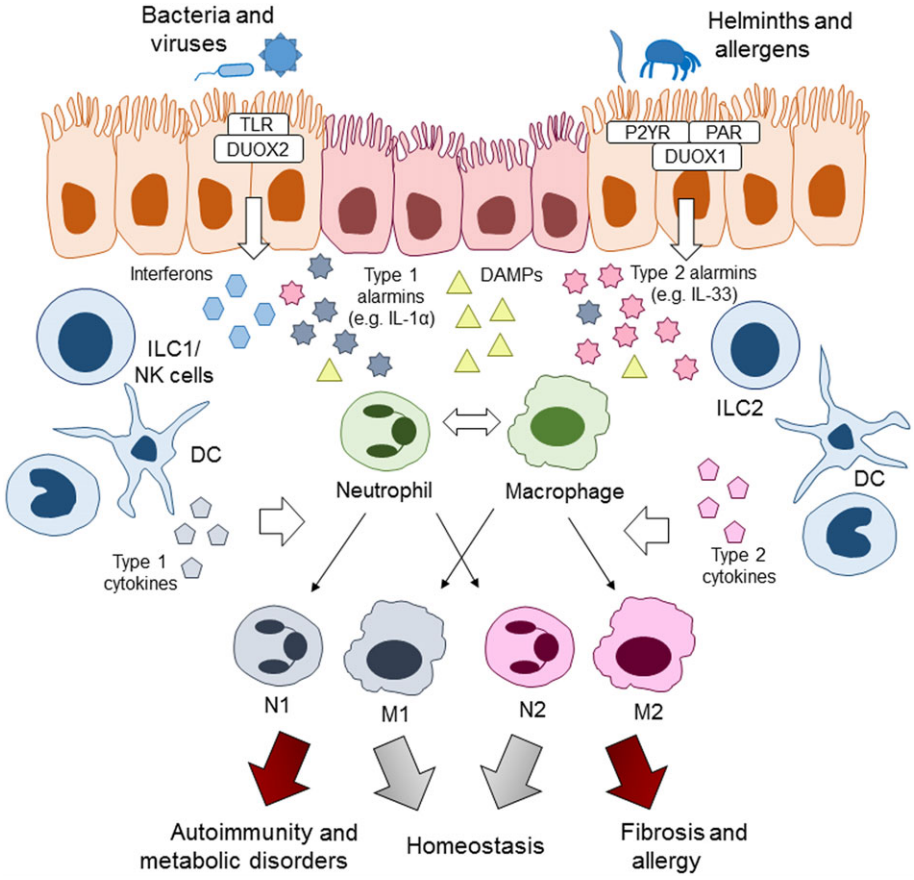

Figure 2

Schematic illustration of proposed roles of DUOX1 and DUOX2 in shaping innate type 2 and type 1 immune responses. DUOX2 is activated primarily by bacterial and viral triggers through TLR-mediated signalling and is associated with increased production of interferons and type 1 cytokines. In contrast, DUOX1 is activated primarily by non-microbial stimuli that induce tissue injury, involving activation of type 2 purine receptors (P2YR) or protease-activated receptors (PAR), leading to activation of type 2 alarmins and type 2 cytokine production. Effector cells such as neutrophils and macrophages are activated differently (e.g. N1 vs. N2 and M1 vs. M2 polarization), leading to a tailored response for efficient defence against these pathogens and restoration of homeostasis (grey arrows). Harmful inflammation due to overstimulation of either DUOX2- or DUOX1-mediated innate immune pathways may result in autoimmune disease or allergic disorders respectively (red arrows). DAMPs, damage-associated molecular patterns; DC, dendritic cell; ILC1/2, type 1/2 innate lymphoid cell; NK cells, natural killer cells.

DUOX1 with its target protein, in the case of, for example, Src (Sham *et al.*, 2013), but may also involve more indirect mechanisms, for example, secondary activation of other NOX enzymes (Heppner *et al.*, 2016). DUOX2-dependent antiviral responses have been linked with activation of NF-κB (Joo *et al.*, 2012) and **ADAM17** sheddases (Yu *et al.*, 2011), factors that are both subject to redox regulation (Janssen-Heininger *et al.*, 2008; Sham *et al.*, 2013), although it is unclear whether these enzymes are direct targets of DUOX2-derived H_2O_2.

One important area of controversy with respect to DUOX-mediated redox signalling and host defence relates to its subcellular localization. For example, while DUOX1 is thought to be present primarily at the airway epithelial surface, DUOX-dependent extracellular H_2O_2 production is marginal in murine airway epithelial cells compared with, for example, human epithelia (Moskwa *et al.*, 2007; Hristova *et al.*, 2014), which would suggest that DUOX does not contribute significantly to mucosal LPO-dependent antimicrobial killing mechanisms in mice. However, comparative analysis of redox signalling in human and murine epithelial cells showed qualitatively and quantitatively similar protein cysteine oxidation in response to activation of DUOX1, which was largely insensitive to extracellular catalase (Wesley *et al.*, 2007; Heppner *et al.*, 2016). This suggests that DUOX1-dependent redox signalling originates primarily from activation of intracellularly localized DUOX1 (e.g. at ER membranes) or occurs after DUOX1 internalization in redox signalling complexes (redoxosomes). Another issue with respect to the proposed involvement of DUOX or other NOX isozymes in cellular redox signalling is the fact that NOX-derived ROS are generated extracellularly or within phagosomes/endosomes and are thus segregated from their putative cytoplasmic redox-sensitive targets by a lipid membrane. The recent discovery of **aquaporins** as selective channels for H_2O_2 resolves this issue, and aquaporin-dependent transmembrane H_2O_2 transfer likely helps confine the oxidative potential of H_2O_2 to assure specificity in redox signalling (Bertolotti *et al.*, 2016; Thiagarajah *et al.*, 2017).

DUOX in disease pathology

Up until recently, a function for DUOX1/DUOX2 in pathology has largely remained elusive, with the exception of somatic mutations within DUOX2 that have been associated with hypothyroidism (van der Vliet, 2011; Bernard *et al.*, 2014). Recent studies over the past few years have, however, highlighted important contributions of DUOX1/DUOX2 to chronic diseases such as cancer and allergic disease and provide a strong rationale for development of selective therapeutic strategies to inhibit these enzymes. The next sections will summarize the current evidence implicating DUOX1 and DUOX2 in disease pathology.

Acute inflammation/infection

Based on the proposed roles of DUOX1/DUOX2 in mucosal host defence by providing extracellular H_2O_2 to support LPO-mediated production of HOSCN, it has been speculated that genetic diseases associated with increased lung injury and infection, such as cystic fibrosis, may be related to defects in this host defence system. Indeed, defects in the cystic fibrosis transmembrane conductance regulator gene that cause cystic fibrosis have been associated with impaired transepithelial SCN^- transport, thus resulting in reduced DUOX–LPO-dependent mucosal host defence (Conner *et al.*, 2007; Moskwa *et al.*, 2007), although they do not appear to significantly affect DUOX1/DUOX2 expression (Pongnimitprasert *et al.*, 2012; van der Vliet *et al.*, unpubl. data). Conversely, minimal evidence exists to date to support a contribution of DUOX1/DUOX2 to acute lung injury or pneumonia, and studies in mouse model systems have not demonstrated a role for DUOX1/DUOX2 in LPS-induced innate cytokine responses or neutrophilia (Chang *et al.*, 2015; Hristova *et al.*, 2016). However, one recent study indicated a role for DUOX2 in alveolar type II cells in mediating acute lung injury in response to hyperoxia (Kim *et al.*, 2014).

Cancer

NOX enzymes are often dysregulated in various cancers (Little *et al.*, 2017). Overexpression of DUOX2/DUOXA2 during ulcerative colitis is thought to be responsible for oxidative DNA damage as a predisposing factor for development of colon cancer (MacFie *et al.*, 2014). Indeed, DUOX2 is often overexpressed in cancers of the alimentary tract, including colorectal cancers, and may contribute to cancer progression (Wu *et al.*, 2013; Qi *et al.*, 2016), suggesting that pharmacological inhibitors with selectivity towards DUOX2 may have clinical utility in anticancer treatment (Lu *et al.*, 2017). Recent studies also indicated a potential contribution of DUOX1 in promoting oxidative DNA damage and genomic instability in the thyroid in response to ionizing radiation, as a potentially important contributing feature to thyroid cancer (Ameziane-El-Hassani *et al.*, 2015). In apparent contrast, several other reports indicate that DUOX1 is frequently suppressed in epithelial cancers by epigenetic mechanisms and may be associated with poor prognosis, as was reviewed recently (Little *et al.*, 2016; 2017).

Allergic airway disease

Based on findings linking DUOX1 with type 2 immune responses and the ability of type 2 cytokines to induce DUOX1, it has been speculated that DUOX1 is commonly elevated in allergic disease (Figure 2). Indeed, enhanced expression of DUOX1 and, to a lesser extent, DUOX2 has been observed in the nasal mucosa of patients with chronic sinusitis (Cho *et al.*, 2013), and increases in DUOX1 mRNA and protein were also observed in cultured nasal or bronchial epithelial cells from subjects with allergic asthma (Hristova *et al.*, 2016; Wan *et al.*, 2016) and in animal models of allergic airway inflammation (Habibovic *et al.*, 2016). Other studies also report increases in DUOX2 in fresh bronchial epithelial cells from patients with severe asthma (Voraphani *et al.*, 2014) and in mice during allergic inflammation induced by cockroach allergens (Nadeem *et al.*, 2015). The functional importance of DUOX2 was suggested in a model of house dust mite (HDM)-induced allergic rhinitis and allergic asthma, which indicated translocation of TLR2/TLR4 to the cell surface in response to HDM-derived β-glucans and LPS and TLR-dependent activation of DUOX2 as critical mediators for innate immune responses to HDM activation (Ryu *et al.*, 2013), although the significance of these pathways for mucus hyperplasia and airway hyperresponsiveness, two clinically relevant features of allergic asthma, was not assessed. Studies using a genetic mouse model of DUOX deficiency, induced by deletion of both *Duoxa* genes, demonstrated the importance of DUOX in mucus metaplasia, airway hyperresponsiveness and neutrophilic inflammation in an ovalbumin-induced model of allergic inflammation (Chang *et al.*, 2013), although these latter studies did not address the relative contribution of DUOX1 and DUOX2. Our group recently demonstrated a role for DUOX1 in HDM-induced allergic inflammation, using DUOX1-deficient mice and siRNA targeting approaches, which revealed the critical importance of DUOX1 in HDM-induced type 2 inflammatory responses, as well as mucus metaplasia, subepithelial remodelling and airway hyperresponsiveness to methacholine (Habibovic *et al.*, 2016). Moreover, DUOX1 was found to mediate neutrophilic rather than eosinophilic inflammation in these studies, which may be particularly relevant for severe asthma endotypes that are typically dominated by neutrophilic inflammation and are resistant to current therapies (Svenningsen and Nair, 2017). More importantly, these clinically relevant aspects of allergic inflammation could be reversed by siRNA-mediated inhibition of DUOX1 after establishing allergic disease in this model (Habibovic *et al.*, 2016), indicating the therapeutic potential of inhibiting DUOX1 in the context of allergic asthma. The involvement of DUOX1 in allergic airway inflammation and mucus hyperplasia was linked to increased activation of Src and EGFR within the airway (Habibovic *et al.*, 2016). Both TKs have been recognized as important mediators of asthma pathology (Vallath *et al.*, 2014), but currently available inhibitors of these kinases have not been used in the treatment of asthma due to undesirable side effects. Selective inhibition of DUOX1 might thus be an attractive alternative strategy for treating severe allergic asthma. It should be noted that other NOX isoforms expressed in airway epithelial cells (e.g. NOX4) may also contribute to asthma pathology (van der Vliet, 2011; Wan *et al.*, 2016).

Skin pathologies

Exaggerated immune responses to allergic stimuli are typically due to atopy, characterized by high IgE that can impact on allergic disease at different anatomical sites. The natural history of atopic diseases often involves initial manifestation of atopic dermatitis, which is then followed by food allergy, allergic rhinitis and/or allergic asthma, in a process referred to as the atopic march (Han *et al.*, 2017). Since DUOX1 is present in epidermal keratinocytes and induced by type 2 cytokines (Hirakawa *et al.*, 2011), it may also play a role in the development of atopic dermatitis with similar involvement of type 2 inflammation by epithelial-derived IL-33 and ILC2s (Savinko *et al.*, 2012; Salimi *et al.*, 2013), although this has not yet been tested directly. Interestingly, some pro-inflammatory responses to HDM-mediated stimulation of keratinocytes, as a model of atopic dermatitis, were linked to DUOX2 rather than DUOX1 (Ko *et al.*, 2015). DUOX1 has been reported to be enhanced in dermal tissues from patients with psoriasis and lichen planus, autoimmune-type disorders associated with abnormal growth in the epidermal layer of the skin (Candel *et al.*, 2014). These skin disorders are typically associated with enhanced Th1 and Th17 responses and with pro-inflammatory cytokines such as TNF-α. Paradoxically, several studies have shown that anti-TNF treatment can also lead to new-onset psoriasis or lichen planus, and studies in zebrafish keratinocytes demonstrated that silencing of TNF-α or its receptor **TNFR2** resulted in induction of DUOX and DUOX-dependent pro-inflammatory cytokine production, suggesting a role for DUOX in psoriasis development (Candel *et al.*, 2014).

Remaining questions

While our understanding of the biology of DUOX enzymes has grown dramatically, controversies still remain with respect to the individual contributions of DUOX1 and DUOX2. One issue with findings implicating DUOX2 is that they are largely based on mouse models with genetic DUOX2 deficiency, which have severe congenital hypothyroidism and require continuous L-thyroxine treatment for normal development. Such approaches might alter the metabolome or microbiome in these animals and thus affect outcomes in models of complex disease. The involvement of specific DUOX isoforms has also been difficult to assess due to the lack of isoform-specific antibodies and the fact that both isoforms are often expressed simultaneously. Although DUOX1 and DUOX2 may be associated with distinct immune pathways (Figure 2), both isoforms may participate in complex chronic diseases such as allergic asthma. Asthma is not a singular disease but is composed of different endotypes that may be related to diversity in genetic background, exposure history and the presence of bacterial or viral infections, which could have variable impacts on DUOX2. Moreover, intricate communications exist between different cells of the immune system, including negative regulatory mechanisms to ensure appropriate immune responses (Rivera *et al.*, 2016). Disruptions in such regulatory mechanisms in association with Western lifestyles are thought to favour pathologies characterized by exaggerated type 2 immune responses (de Kouchkovsky *et al.*, 2017; Lambrecht and Hammad, 2017). Emerging evidence indicating the presence of DUOX in various immune cells types would further highlight complex roles for DUOX in pathologies associated with chronic inflammation or dysregulated immune processes. Nevertheless, the growing evidence implicating DUOX in disease pathology, particularly in the context of allergic diseases that are rapidly increasing in Westernized societies, provides a strong rationale for the development of DUOX-selective inhibitors that could be used in their therapeutic management.

The search for NOX-selective inhibitors

Current status of development of NOX inhibitors

The growing appreciation of specific role(s) of individual NOX enzymes in various biological and pathological contexts has spurred various efforts to develop isoform-specific inhibitors of NOX enzymes (Drummond *et al.*, 2011; Gatto *et al.*, 2013; Altenhofer *et al.*, 2015; Cifuentes-Pagano *et al.*, 2015; Teixeira *et al.*, 2017). Unfortunately, the relative lack of high-resolution structural information of NOX enzymes (either in active or inactive states) has hampered rational structure-based design of isoform-selective inhibitors, and the development of NOX-selective inhibitors has relied mostly on high-throughput screens of molecular libraries. While such approaches have had some success, the molecular mechanisms of actions are often unknown. Many putative NOX inhibitors appear to act by interfering with the binding of NADPH or flavin adenine dinucleotide (FAD) to the enzyme or their electron transfer properties, but given the widespread use of these co-factors in many flavoenzymes, it is difficult to achieve selectivity for NOX. Indeed, diphenylene iodonium (DPI), one of the earliest described NOX inhibitors, was demonstrated to interact with flavins by forming covalent adducts (O'Donnell *et al.*, 1993) and thus inhibits all NOX isoforms with similar potency (Altenhofer *et al.*, 2015). A recent evaluation of 36 analogues of DPI identified some nitro-substituted analogues with some apparent selectivity towards NOX5 and DUOX2 compared with other NOX isoforms (Lu *et al.*, 2017), suggesting that it may be feasible to develop isoform-selective inhibitors based on flavin-targeted compounds such as DPI. Continued structural insights based on new crystallographic data (Magnani *et al.*, 2017) may help guide such efforts. Several other NOX inhibitors with some selectivity have been identified from high-throughput screens of molecular libraries on cell models in which different NOX enzymes were ectopically expressed (Gianni *et al.*, 2010; Altenhofer *et al.*, 2015;Hirano *et al.*, 2015; Joo *et al.*, 2016), and two recently developed NOX2-selective inhibitors were found to act by competitive inhibition of NADPH binding based on detailed mechanistic studies and molecular docking studies (Hirano *et al.*, 2015; Joo *et al.*, 2016). However, inhibitory actions of putative NOX inhibitors may also involve indirect mechanisms rather than selective targeting of the NOX protein, and technical issues with the assay methodology to assess NOX activity have in some cases led to false positives (Maghzal *et al.*, 2012; Seredenina *et al.*, 2015). Therefore, reliable identification of NOX-selective inhibitors requires the use of multiple assay methodologies to assess

NOX activity (e.g. O_2 consumption, NADPH utilization and analysis of ROS products using several probes). Among the various efforts to date to develop NOX-selective inhibitors, one such effort launched by Genkyotex based on screening 136 000 compounds, including various pyrazolopyridine diones, has yielded GKT831 as an orally bioavailable NOX1/NOX4-selective inhibitor which represents the only NOX-selective inhibitor to date that has progressed into clinical trials. Although a recently completed phase 2 study in patients with type 2 diabetes with diabetic nephropathy unfortunately did not reach its primary clinical endpoint (Labiotech.eu. 2015-09-10), other clinical studies have been initiated to address efficacy in primary biliary cholangitis (Labiotech.eu. 2017-05-03).

Do current NOX inhibitors also inhibit DUOX?

Common to almost all of the drug screening efforts so far is that they did typically not address efficacy against DUOX1 or DUOX2 (Lu *et al.*, 2017). This is likely due to the absence (until recently) of experimental data demonstrating a role for DUOX1/DUOX2 in disease and perhaps also related to concerns that the development of DUOX2-selective inhibitors would be undesirable because of their negative impact on thyroid hormone synthesis. However, most small molecule NOX inhibitors developed to date have structural similarities with NADPH or FAD and would thus be expected to also inhibit DUOX enzymes. Indeed, the classic NOX inhibitor DPI was also found to potently inhibit DUOX (Wesley *et al.*, 2007; Lu *et al.*, 2017), and the recently developed NOX1/NOX4-selective inhibitor GKT831 was also reported to inhibit DUOX (Strengert *et al.*, 2014). We recently surveyed several putative NOX inhibitors for their ability to inhibit DUOX1, and found that apocynin and VAS2870, two putative NOX inhibitors with poorly defined inhibitory mechanisms (Altenhofer *et al.*, 2015), were rather ineffective and displayed low potency compared with DPI. In contrast, the phenothiazine compound ML171 (2-acetyl-phenothiazine), originally identified in a drug screen as a NOX inhibitor with relative selectivity against NOX1 (Gianni *et al.*, 2010), displayed strong inhibition against ATP-mediated H_2O_2 production as well as DUOX1-dependent IL-33 secretion (Habibovic *et al.*, 2016). Phenothiazines represent a family of heterocyclic compounds that are recognized as a class of 'privileged structures' with versatile binding properties that exhibit a number of desirable drug-like characteristics and have had a long history of drug development with various indications, as antimalarials (late 19th century), anthelminthics and antibiotics (mid-20th century), antihistaminics (1940s), and sedatives and antipsychotics (1950s) (Ohlow and Moosmann, 2011). Their mechanisms of action are highly diverse and include antihistaminergic, antidopaminergic and antiserotonergic effects, as well as more recently identified effects on dynamin and clathrin-endocytotic pathways (Daniel *et al.*, 2015). Recent studies also indicated that phenothiazine-based compounds can exert direct inhibitory actions against NOX2 and other NOX isoforms, with some isoform specificity (Seredenina *et al.*, 2015). One important consideration is that phenothiazines in which their B ring nitrogen atom is unsubstituted, such as the putative NOX1 inhibitor ML171 (Figure 3), also possess strong radical-trapping properties and thus have more generic antioxidant properties, and *N*-unsubstituted phenothiazines such as ML171 were found to be inactive as direct inhibitors of NOX1–NOX5 (Seredenina *et al.*, 2015). In contrast, *N*-substituted phenothiazine compounds such as the antipsychotic drug **chlorpromazine** (CPZ) lack such radical-scavenging properties (Figure 3) and may act as direct NOX inhibitors. Indeed, our studies indicate that CPZ and other *N*-unsubstituted phenothiazines can effectively inhibit DUOX1-dependent H_2O_2 production, with potency comparable with ML171 (van der Vliet *et al.*, unpubl. data). Together with observations of NOX-selective actions of other phenothiazines (Gianni *et al.*, 2010; Seredenina *et al.*, 2015), these findings suggest opportunities for development of DUOX1-selective inhibitors based on phenothiazine scaffolds. In fact, previous uses of phenothiazine-based compounds such as CPZ or structurally similar compounds for the treatment of allergic disease (Baum *et al.*, 1957) might have been related in part to their ability to inhibit DUOX1.

Towards development of DUOX-selective inhibitors

Since the DUOX enzymes contain several structural features that are distinct from other NOX isozymes, the successful development of DUOX1-selective inhibitors most likely relies on targeting unique structural determinants of DUOX1 that are absent or variant in the other isoforms. Unfortunately, minimal structural information of NOX enzymes is available from crystallographic data, which only exist for the NADPH-binding domain of human NOX2 [protein database (PDB) ID: 3A1F] and the transmembrane domain (PDB ID: 5O0T) and dehydrogenase domain (PDB ID: 5O0X) of NOX5 from *Cylindrospermum stagnale* (Magnani *et al.*, 2017), and can be used to gain structural insights into other NOX isoforms by homology modelling. No structural data exist for their more unique PDH domains and their EF-hand-binding domain regions. However, several recent studies have highlighted potentially unique molecular features within these and other domains within DUOX that could be

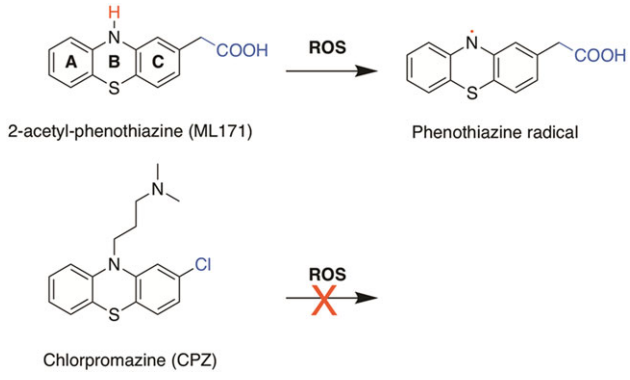

Figure 3

Structures of 2-acetyl-phenothiazine (ML171) and CPZ and their reactivity with ROS by radical-trapping mechanisms.

exploited for rationalized development of targeted inhibitors (Figure 4), which will be discussed in the next sections.

PHD domains

The *N*-terminal extracellular PHDs within the DUOX enzymes are so named because of their homology with mammalian haem peroxidases, but the mammalian DUOXs lack various residues within these PHD domains that are critical for haem binding and peroxidase activity, and their function most likely relates to their critical role in DUOX maturation, as these domains contain several *N*-glycosylation sites (Morand *et al.*, 2004; De Deken *et al.*, 2014). Alternatively, the PHD domains may also be crucial for appropriate interactions of DUOX with their partnering haem peroxidases, such as with TPO within the thyroid (Fortunato *et al.*, 2010). The functional importance of the DUOX2 PHD is illustrated by the fact that several mutations within this domain are associated with hypothyroidism (Grasberger, 2010). Another critical area where DUOX PHD domains differ from mammalian peroxidases concerns their cysteine residues. Whereas mammalian haem peroxidases contain a conserved set of structurally important disulfide bonds (six in MPO and seven in LPO), human DUOX enzymes only contain six cysteine residues within their PHD, and sequence alignment indicates that none of them correspond to conserved cysteines within mammalian peroxidases that are important for structural stability (Meitzler *et al.*, 2013). Five of these cysteines (Cys^{118}, Cys^{345}, Cys^{364}, Cys^{564} and Cys^{579} in DUOX1; Cys^{124}, Cys^{351}, Cys^{370}, Cys^{568} and Cys^{582} in DUOX2) are conserved between both isoforms and also across organisms (Meitzler *et al.*, 2013), and mutation studies indicated that four of these (Cys^{351}, Cys^{370}, Cys^{568} and Cys^{582} within DUOX2) are important for DUOX maturation and emergence from the ER (Morand *et al.*, 2004). Homology modelling studies with bovine LPO suggested that these four cysteines are solvent exposed, and subsequent mutation studies suggested their involvement in intermolecular disulfide bonding with other DUOX monomers or with their DUOXAs (Meitzler *et al.*, 2013). More recent studies indicated that Cys^{568} and Cys^{582} within the DUOX2 PHD domain engage in intermolecular disulfide linking with DUOXA2 and that another conserved PHD cysteine (Cys^{124}) likely engages in intramolecular cross-linking with Cys^{1162} located in one of the extracellular loops in the transmembrane region of DUOX2 (Carre *et al.*, 2015), which might also apply to homologous cysteines in DUOX1 (Figure 4). A potential function for the other two PHD cysteines (Cys^{351} and Cys^{370}) was suggested by studies demonstrating that overexpression of TPO enhanced DUOX2-mediated H_2O_2 production in HEK293 cells, which was not observed with DUOX2 mutants lacking Cys^{351} or Cys^{370} (Fortunato *et al.*, 2010). These studies imply that DUOX2-generated H_2O_2 inhibits DUOX2 activity in a negative feedback fashion, which was prevented by enhancing H_2O_2

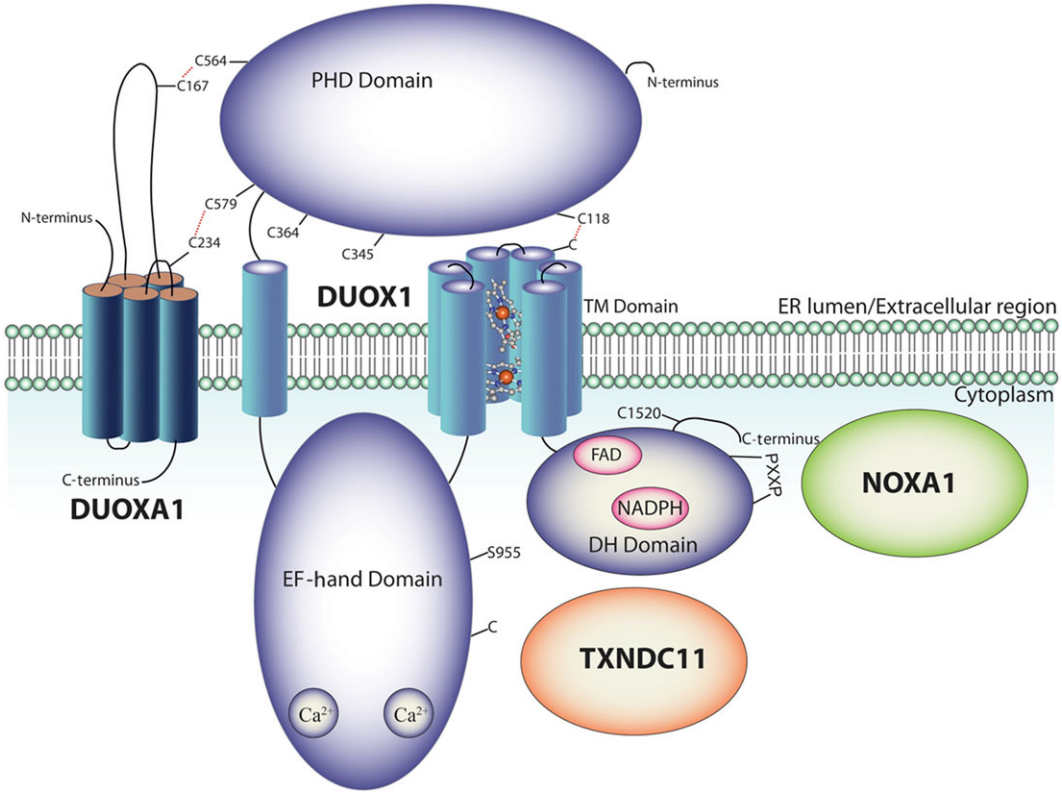

Figure 4

Schematic illustration of DUOX1 protein and potentially unique features for potential drug targeting. Topographical model of the DUOX1 protein highlighting specific amino acid residues that are involved in regulating DUOX1 activity or interactions with other regulatory proteins, and may represent druggable targets. Details are clarified in the text. Note that many of these amino acids are also conserved in DUOX2.

metabolism by TPO. The importance of the corresponding cysteines in DUOX1 (Cys^{345} and Cys^{364}) is not presently known, but based on their solvent exposure and homology modelling studies (Figure 5A), they likely have a similar function.

EF-hand domains

DUOX proteins do not require additional co-factors, and their activation relies primarily on their Ca^{2+}-binding EF-hand domains. A yeast two-hybrid screen of the EF-hand fragment of DUOX1 has identified the thioredoxin (TRX)-related protein EF-hand binding protein 1 (EFP1) as a potential partner for DUOX1 (Wang *et al.*, 2005). EFP1, also known as TXNDC11, has shared reductase activities with other TRX-related proteins and has recently been implicated in ER-localized degradation of glycoproteins (Timms *et al.*, 2016) and may thus be involved in the folding process of DUOX1. The cytoplasmic region (containing the EF-hand binding regions) of DUOX1 (aa618–aa1044) possesses a number of cysteine residues, some of which are also present in DUOX2 but others being isoform specific. While nothing is known with respect to the functional importance of these cysteines, their thiol–disulfide status may conceivably be regulated by TXDNC11 (which may apply particularly to the region between aa951 and aa988, which contains four cysteines in relative close proximity), to alter local protein structure and/or binding of regulatory factors such as protein kinases (see next paragraph). Intriguingly, a genome-wide screen in horses has identified associations of a single nucleotide polymorphism in the TXNDC11 gene with heaves, an equine recurrent airway obstruction in horses with asthma-like features (Schnider *et al.*, 2017), which might point to a role for TXNDC11 in regulating DUOX1 maturation or activation.

Other unique features of DUOX

Although very little is known regarding other post-translational modifications in DUOX regulation, observations of divergent activation of DUOX1 and DUOX2 by different signalling pathways has spurred analysis of phosphorylation sites in DUOX1 and DUOX2, which identified Ser^{955}, Thr^{1007} and Ser^{1217} within DUOX1 as direct phosphorylation targets, of which Ser^{955} (located adjacent to the EF-hand binding regions) was found to be critical for DUOX1 activation by PKA (Rigutto *et al.*, 2009). Studies by Knaus and coworkers indicated that DUOX activation by Ca^{2+}-activating triggers was associated with dissociation of NOX activator 1 (NOXA1, a homologue of p67phox or NCF2) from the DUOX protein (Pacquelet *et al.*, 2008). Mutation and deletion studies indicated that NOXA1 interacts with the C-terminal region in DUOX1 to suppress basal oxidase activity, and this in part involves interactions of a PXXP motif (residues 1497–1500 in

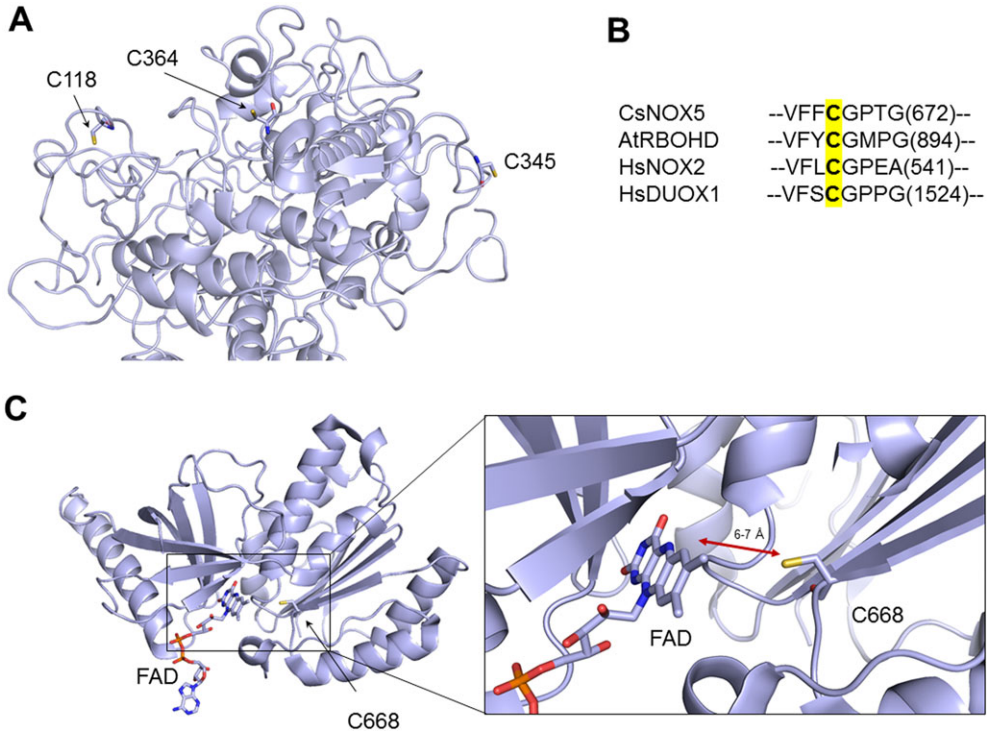

Figure 5

Location of functionally relevant cysteines within NOX/DUOX. (A) SWISS model of DUOX1 PHD based on homology mapping with the crystal structure of LPO (PDB ID 3BXI), revealing the solvent exposure of C345 and C364, which are present in their reduced state in the mature enzyme. (B) Sequence alignment of amino acids adjacent to a conserved cysteine within the C-terminal region of csNOX5(C668), AtRBOHD(C890), HsNOX2(C537) and HsDUOX1(1520). (C) Visualization of C668 in proximity to FAD with the dehydrogenase domain of csNOX5 (PDB ID: 5O0X). The close proximity suggests that covalent cysteine modifications likely disrupt FAD binding or its electron transfer properties.

DUOX1) with the Src homology 3 domain within NOXA1 (Pacquelet *et al.*, 2008). The C-terminal region of DUOX1 also contains various cysteines that may be involved in regulating activity, and recent studies in *Arabidopsis* demonstrated the ability of NO to suppress activation of its NOX homologue AtRBOHD *via S*-nitrosylation at a single cysteine residue, Cys^{890}, within the C-terminal region (Yun *et al.*, 2011). Sequence alignment indicates that this cysteine is conserved in mammalian NOX2 and also in DUOX proteins (Cys^{1520} in DUOX1) (Figure 5B), suggesting the potential general importance of such reversible cysteine modifications for mammalian NOXs. Mapping of this cysteine within the crystal structure of csNOX5 (PDB ID: 5O0X) indicates its close proximity to FAD (Figure 5C), which would suggest a potential general function of this Cys in NOX activity, perhaps by participating in electron transfer between NADPH and FAD. Intriguingly, a recent study implicates a homologous cysteine within NOX4, as well as an adjacent cysteine, in regulating electron transfer (Nisimoto *et al.*, 2018).

Thiol-reactive compounds inhibit DUOX1

The previous sections highlight the presence of several cysteines within DUOX and other NOX enzymes that appear to be non-essential for catalytic activity but may be involved in regulating NOX/DUOX location or activity. Hence, pharmacological approaches that selectively target these cysteines may enable the development of useful and potentially isoform-specific inhibitors. Targeting of non-catalytic cysteines by covalently bonded or irreversible inhibitors is increasingly recognized as an attractive strategy in drug design, since covalent inhibitors may have increased biochemical efficiency compared with non-covalent inhibitors, due to non-equilibrium binding, reduced sensitivity to pharmacokinetic parameters (e.g. clearance) and increased duration of action dependent on the biological lifespan of the targeted protein (Singh *et al.*, 2011). Recent studies by our group indicated that several thiol-reactive electrophiles can potently inhibit innate allergen-induced responses in airway epithelial cells *in vitro* as well as *in vivo*, and these inhibitory effects were mediated (in part) by covalent modification of DUOX1 and inhibition of DUOX1 activity (Danyal *et al.*, 2016). Soft electrophiles have attracted much recent interest because of their presence in certain health-promoting food groups (e.g. curcumin and sulforaphane) and their well-documented anti-inflammatory properties, which are typically attributed to their ability to target important protein cysteine residues in, for example, NF-κB or Keap1/nuclear factor (erythroid-derived 2)-like 2 (Nrf2), might also involve direct targeting of alternative proteins such as DUOX1 (Danyal *et al.*, 2016). The identity of the DUOX1 cysteines targeted by these electrophiles is yet to be established, but these studies offer the exciting prospect that selective targeting of specific functionally important cysteines within DUOX1 may lead to inhibition of DUOX1, and could be exploited for the development of DUOX-selective inhibitors to treat allergic disorders such as asthma, allergic rhinitis, atopic dermatitis and conjunctivitis.

Concluding remarks and future perspectives

In this review, we summarized the current knowledge with respect to the importance of DUOX enzymes in innate host defence mechanisms and their potential contribution to disease pathology that is associated with dysregulated immune pathways. In contrast to ongoing efforts to develop inhibitors targeting other NOX isoforms, the importance of DUOX as a therapeutic target has so far not been considered. However, recent evidence highlights the importance of DUOX1 in the context of allergic disease, the incidence of which is rapidly increasing in Westernized societies due to the relative elimination of helminth infections, alterations in microbiome diversity and increased success in treating chronic infectious diseases, and provides a strong rationale for efforts to develop DUOX1-selective inhibitors. Since the DUOX enzymes are quite distinct from other NOX isoforms, it should be possible to design selective inhibitors based on known unique functional or structural features of DUOX, even in the absence of crystal structure data to aid in rational drug design. It will be more challenging to develop inhibitors that distinguish between DUOX1 and DUOX2, because of their high homology, and the best opportunity for developing such specific inhibitors will likely be based on targeting unique and variant regulatory features within their intracellular EF-hand-containing domains. Alternatively, unwanted inhibition of DUOX2 and its negative consequences for thyroid function could be minimized by developing local administration strategies, for example, in the form of inhalers or ointments. High-throughput drug screening efforts may be useful but typically rely on ectopic expression systems and generally do not monitor critical functional outcomes associated with NOX/DUOX activation. Continued efforts to obtain structural data of DUOX enzymes and the development of improved genetically engineered models to evaluate specific aspects of DUOX biology will facilitate new rationalized strategies for DUOX-specific targeting approaches and testing in relevant preclinical models.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY (Alexander *et al.*, 2017a,b,c,d).

Acknowledgements

A.V.D.V. gratefully acknowledges research support by grants from NHLBI (R01 HL085646 and HL138708) and NIA (R21 AG055325). D.E.H. acknowledges previous support from an NRSA Fellowship from NIH (F32 HL129706). A.V.D.V. would also like to thank David Seward for help with generating Figure 1 and Nathalie Grandvaux, Miklos Geiszt, Xavier de Deken and Ulla Knaus for frequent informal discussions with respect to the biology of DUOX enzymes.

Conflict of interest

The authors are co-inventors on a US patent application no. 15/299848, entitled Covalent Inhibitors of Dual Oxidase 1.

References

Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA *et al*. (2017a). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. Br J Pharmacol 174 (Suppl 1): S17–S129.

Alexander SPH, Fabbro D, Kelly E, Marrion NV, Peters JA, Faccenda E *et al*. (2017b). The Concise Guide to PHARMACOLOGY 2017/18: Catalytic receptors. Br J Pharmacol 174: S225–S271.

Alexander SPH, Fabbro D, Kelly E, Marrion NV, Peters JA, Faccenda E *et al*. (2017c). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. Br J Pharmacol 174 (Suppl 1): S272–S359.

Alexander SPH, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD *et al*. (2017d). The Concise Guide to PHARMACOLOGY 2017/18: Other ion channels. Br J Pharmacol 174: S195–S207.

Altenhofer S, Radermacher KA, Kleikers PW, Wingler K, Schmidt HH (2015). Evolution of NADPH oxidase inhibitors: selectivity and mechanisms for target engagement. Antioxid Redox Signal 23: 406–427.

Ameziane-El-Hassani R, Morand S, Boucher JL, Frapart YM, Apostolou D, Agnandji D *et al*. (2005). Dual oxidase-2 has an intrinsic Ca^{2+}-dependent H_2O_2-generating activity. J Biol Chem 280: 30046–30054.

Ameziane-El-Hassani R, Talbot M, de Souza Dos Santos MC, Al Ghuzlan A, Hartl D, Bidart JM *et al*. (2015). NADPH oxidase DUOX1 promotes long-term persistence of oxidative stress after an exposure to irradiation. Proc Natl Acad Sci U S A 112: 5051–5056.

Anh NT, Nishitani M, Harada S, Yamaguchi M, Kamei K (2011). Essential role of Duox in stabilization of *Drosophila* wing. J Biol Chem 286: 33244–33251.

Aviello G, Knaus UG (2017). ROS in gastrointestinal inflammation: rescue or sabotage? Br J Pharmacol 174: 1704–1718.

Baum GL, Schotz SA, Gumpel RC, Osgood C (1957). The role of chlorpromazine in the treatment of bronchial asthma and chronic pulmonary emphysema. Dis Chest 32: 574–579.

Bedard K, Krause KH (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87: 245–313.

Bernard K, Hecker L, Luckhardt TR, Cheng G, Thannickal VJ (2014). NADPH oxidases in lung health and disease. Antioxid Redox Signal 20: 2838–2853.

Bertolotti M, Farinelli G, Galli M, Aiuti A, Sitia R (2016). AQP8 transports NOX2-generated H_2O_2 across the plasma membrane to promote signaling in B cells. J Leukoc Biol 100: 1071–1079.

Boots AW, Hristova M, Kasahara DI, Haenen GR, Bast A, van der Vliet A (2009). ATP-mediated activation of the NADPH oxidase DUOX1 mediates airway epithelial responses to bacterial stimuli. J Biol Chem 284: 17858–17867.

Bouchery T, Kyle R, Camberis M, Shepherd A, Filbey K, Smith A *et al*. (2015). ILC2s and T cells cooperate to ensure maintenance of M2 macrophages for lung immunity against hookworms. Nat Commun 6: 6970.

Candel S, de Oliveira S, Lopez-Munoz A, Garcia-Moreno D, Espin-Palazon R, Tyrkalska SD *et al*. (2014). Tnfa signaling through Tnfr2 protects skin against oxidative stress-induced inflammation. PLoS Biol 12: e1001855.

Carre A, Louzada RA, Fortunato RS, Ameziane-El-Hassani R, Morand S, Ogryzko V *et al*. (2015). When an intramolecular disulfide bridge governs the interaction of DUOX2 with its partner DUOXA2. Antioxid Redox Signal 23: 724–733.

Cayrol C, Girard JP (2014). IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr Opin Immunol 31: 31–37.

Chang S, Linderholm A, Franzi L, Kenyon N, Grasberger H, Harper R (2013). Dual oxidase regulates neutrophil recruitment in allergic airways. Free Radic Biol Med 65C: 38–46.

Chang S, Linderholm A, Harper R (2015). DUOX-mediated signaling is not required for LPS-induced neutrophilic response in the airways. PLoS One 10: e0131810.

Cho DY, Nayak JV, Bravo DT, Le W, Nguyen A, Edward JA *et al*. (2013). Expression of dual oxidases and secreted cytokines in chronic rhinosinusitis. Int Forum Allergy Rhinol 3: 376–383.

Choi H, Park JY, Kim HJ, Noh M, Ueyama T, Bae Y *et al*. (2014). Hydrogen peroxide generated by DUOX1 regulates the expression levels of specific differentiation markers in normal human keratinocytes. J Dermatol Sci 74: 56–63.

Christophe-Hobertus C, Christophe D (2010). Delimitation and functional characterization of the bidirectional THOX–DUOXA promoter regions in thyrocytes. Mol Cell Endocrinol 317: 161–167.

Cifuentes-Pagano ME, Meijles DN, Pagano PJ (2015). Nox inhibitors & therapies: rational design of peptidic and small molecule inhibitors. Curr Pharm Des 21: 6023–6035.

Conner GE, Wijkstrom-Frei C, Randell SH, Fernandez VE, Salathe M (2007). The lactoperoxidase system links anion transport to host defense in cystic fibrosis. FEBS Lett 581: 271–278.

Daniel JA, Chau N, Abdel-Hamid MK, Hu L, von Kleist L, Whiting A *et al*. (2015). Phenothiazine-derived antipsychotic drugs inhibit dynamin and clathrin-mediated endocytosis. Traffic 16: 635–654.

Danyal K, de Jong W, O'Brien E, Bauer RA, Heppner DE, Little AC *et al*. (2016). Acrolein and thiol-reactive electrophiles suppress allergen-induced innate airway epithelial responses by inhibition of DUOX1 and EGFR. Am J Physiol Lung Cell Mol Physiol 311: L913–L923.

De Deken X, Corvilain B, Dumont JE, Miot F (2014). Roles of DUOX-mediated hydrogen peroxide in metabolism, host defense, and signaling. Antioxid Redox Signal 20: 2776–2793.

De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G *et al*. (2000). Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. J Biol Chem 275: 23227–23233.

de Kouchkovsky DA, Ghosh S, Rothlin CV (2017). Negative regulation of type 2 immunity. Trends Immunol 38: 154–167.

Donko A, Ruisanchez E, Orient A, Enyedi B, Kapui R, Peterfi Z *et al*. (2010). Urothelial cells produce hydrogen peroxide through the activation of Duox1. Free Radic Biol Med 49: 2040–2048.

Drummond GR, Selemidis S, Griendling KK, Sobey CG (2011). Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. Nat Rev Drug Discov 10: 453–471.

Dupuy C, Ohayon R, Valent A, Noel-Hudson MS, Deme D, Virion A (1999). Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cdnas. J Biol Chem 274: 37265–37269.

Edens WA, Sharling L, Cheng G, Shapira R, Kinkade JM, Lee T *et al*. (2001). Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. J Cell Biol 154: 879–891.

El Hassani RA, Benfares N, Caillou B, Talbot M, Sabourin JC, Belotte V *et al*. (2005). Dual oxidase2 is expressed all along the digestive tract. Am J Physiol Gastrointest Liver Physiol 288: G933–G942.

Feng Z, Jia X, Adams MD, Ghosh SK, Bonomo RA, Weinberg A (2014). Epithelial innate immune response to *Acinetobacter baumannii* challenge. Infect Immun 82: 4458–4465.

Fink K, Martin L, Mukawera E, Chartier S, De Deken X, Brochiero E *et al*. (2013). IFNβ/TNFα synergism induces a non-canonical STAT2/IRF9-dependent pathway triggering a novel DUOX2 NADPH oxidase-mediated airway antiviral response. Cell Res 23: 673–690.

Fischer H (2009). Mechanisms and function of DUOX in epithelia of the lung. Antioxid Redox Signal 11: 2453–2465.

Fischer H, Gonzales LK, Kolla V, Schwarzer C, Miot F, Illek B *et al*. (2007). Developmental regulation of DUOX1 expression and function in human fetal lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 292: L1506–L1514.

Flores MV, Crawford KC, Pullin LM, Hall CJ, Crosier KE, Crosier PS (2010). Dual oxidase in the intestinal epithelium of zebrafish larvae has anti-bacterial properties. Biochem Biophys Res Commun 400: 164–168.

Forteza R, Salathe M, Miot F, Conner GE (2005). Regulated hydrogen peroxide production by Duox in human airway epithelial cells. Am J Respir Cell Mol Biol 32: 462–469.

Fortunato RS, Lima de Souza EC, Ameziane-el Hassani R, Boufraqech M, Weyemi U, Talbot M *et al*. (2010). Functional consequences of dual oxidase–thyroperoxidase interaction at the plasma membrane. J Clin Endocrinol Metab 95: 5403–5411.

Gallo RL, Hooper LV (2012). Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 12: 503–516.

Gattas MV, Forteza R, Fragoso MA, Fregien N, Salas P, Salathe M *et al*. (2009). Oxidative epithelial host defense is regulated by infectious and inflammatory stimuli. Free Radic Biol Med 47: 1450–1458.

Gatto GJ Jr, Ao Z, Kearse MG, Zhou M, Morales CR, Daniels E *et al*. (2013). NADPH oxidase-dependent and -independent mechanisms of reported inhibitors of reactive oxygen generation. J Enzyme Inhib Med Chem 28: 95–104.

Gause WC, Wynn TA, Allen JE (2013). Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths. Nat Rev Immunol 13: 607–614.

Geiszt M, Witta J, Baffi J, Lekstrom K, Leto TL (2003). Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. FASEB J 17: 1502–1504.

Ghezzi P, Jaquet V, Marcucci F, Schmidt H (2017). The oxidative stress theory of disease: levels of evidence and epistemological aspects. Br J Pharmacol 174: 1784–1796.

Gianni D, Taulet N, Zhang H, DerMardirossian C, Kister J, Martinez L *et al*. (2010). A novel and specific NADPH oxidase-1 (Nox1) small-molecule inhibitor blocks the formation of functional invadopodia in human colon cancer cells. ACS Chem Biol 5: 981–993.

Gorissen SH, Hristova M, Habibovic A, Sipsey LM, Spiess PC, Janssen-Heininger YM *et al*. (2013). Dual oxidase-1 is required for airway epithelial cell migration and bronchiolar reepithelialization after injury. Am J Respir Cell Mol Biol 48: 337–345.

Grandvaux N, Mariani M, Fink K (2015). Lung epithelial NOX/DUOX and respiratory virus infections. Clin Sci (Lond) 128: 337–347.

Grasberger H (2010). Defects of thyroidal hydrogen peroxide generation in congenital hypothyroidism. Mol Cell Endocrinol 322: 99–106.

Grasberger H, Gao J, Nagao-Kitamoto H, Kitamoto S, Zhang M, Kamada N *et al*. (2015). Increased expression of DUOX2 is an epithelial response to mucosal dysbiosis required for immune homeostasis in mouse intestine. Gastroenterology 149: 1849–1859.

Grasberger H, Refetoff S (2006). Identification of the maturation factor for dual oxidase. Evolution of an eukaryotic operon equivalent. J Biol Chem 281: 18269–18272.

Ha EM, Lee KA, Seo YY, Kim SH, Lim JH, Oh BH *et al*. (2009). Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in *Drosophila* gut. Nat Immunol 10: 949–957.

Ha EM, Oh CT, Bae YS, Lee WJ (2005). A direct role for dual oxidase in *Drosophila* gut immunity. Science 310: 847–850.

Habibovic A, Hristova M, Heppner DE, Danyal K, Ather JL, Janssen-Heininger YMW *et al*. (2016). DUOX1 mediates persistent epithelial EGFR activation, mucous cell metaplasia, and airway remodeling during allergic asthma. JCI Insight 1: e88811.

Han H, Roan F, Ziegler SF (2017). The atopic march: current insights into skin barrier dysfunction and epithelial cell-derived cytokines. Immunol Rev 278: 116–130.

Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S *et al*. (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res 46: D1091–D1106.

Harper RW, Xu C, Eiserich JP, Chen Y, Kao CY, Thai P *et al*. (2005). Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. FEBS Lett 579: 4911–4917.

Hayes P, Dhillon S, O'Neill K, Thoeni C, Hui KY, Elkadri A *et al*. (2015). Defects in NADPH oxidase genes NOX1 and DUOX2 in very early onset inflammatory bowel disease. Cell Mol Gastroenterol Hepatol 1: 489–502.

Heppner DE, Hristova M, Dustin CM, Danyal K, Habibovic A, van der Vliet A (2016). The NADPH oxidases DUOX1 and NOX2 play distinct roles in redox regulation of epidermal growth factor receptor signaling. J Biol Chem 291: 23282–23293.

Hirakawa S, Saito R, Ohara H, Okuyama R, Aiba S (2011). Dual oxidase 1 induced by Th2 cytokines promotes STAT6 phosphorylation via oxidative inactivation of protein tyrosine phosphatase 1B in human epidermal keratinocytes. J Immunol 186: 4762–4770.

Hirano K, Chen WS, Chueng AL, Dunne AA, Seredenina T, Filippova A *et al*. (2015). Discovery of GSK2795039, a novel small molecule NADPH oxidase 2 inhibitor. Antioxid Redox Signal 23: 358–374.

Hoeven R, McCallum KC, Cruz MR, Garsin DA (2011). Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in *C. elegans*. PLoS Pathog 7: e1002453.

Hoffmann JA (2003). The immune response of *Drosophila*. Nature 426: 33–38.

Holmstrom KM, Finkel T (2014). Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 15: 411–421.

Holtzman MJ, Byers DE, Alexander-Brett J, Wang X (2014). The role of airway epithelial cells and innate immune cells in chronic respiratory disease. Nat Rev Immunol 14: 686–698.

Hong SN, Kim JY, Kim H, Kim DY, Won TB, Han DH *et al*. (2016). Duox2 is required for the transcription of pattern recognition receptors in acute viral lung infection: an interferon-independent regulatory mechanism. Antiviral Res 134: 1–5.

Hristova M, Habibovic A, Veith C, Janssen-Heininger YM, Dixon AE, Geiszt M *et al*. (2016). Airway epithelial dual oxidase 1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses. J Allergy Clin Immunol 137: 1545, e1511–1556.

Hristova M, Veith C, Habibovic A, Lam YW, Deng B, Geiszt M *et al*. (2014). Identification of DUOX1-dependent redox signaling through protein *S*-glutathionylation in airway epithelial cells. Redox Biol 2: 436–446.

Hurd TR, Liang FX, Lehmann R (2015). Curly encodes dual oxidase, which acts with heme peroxidase curly Su to shape the adult *Drosophila* wing. PLoS Genet 11: e1005625.

Janeway CA Jr, Medzhitov R (2002). Innate immune recognition. Annu Rev Immunol 20: 197–216.

Janssen-Heininger YM, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, Finkel T *et al*. (2008). Redox-based regulation of signal transduction: principles, pitfalls, and promises. Free Radic Biol Med 45: 1–17.

Joo JH, Huh JE, Lee JH, Park DR, Lee Y, Lee SG *et al*. (2016). A novel pyrazole derivative protects from ovariectomy-induced osteoporosis through the inhibition of NADPH oxidase. Sci Rep 6: 22389.

Joo JH, Ryu JH, Kim CH, Kim HJ, Suh MS, Kim JO *et al*. (2012). Dual oxidase 2 is essential for the toll-like receptor 5-mediated inflammatory response in airway mucosa. Antioxid Redox Signal 16: 57–70.

Juarez MT, Patterson RA, Sandoval-Guillen E, McGinnis W (2011). Duox, flotillin-2, and Src42A are required to activate or delimit the spread of the transcriptional response to epidermal wounds in *Drosophila*. PLoS Genet 7: e1002424.

Kawahara T, Quinn MT, Lambeth JD (2007). Molecular evolution of the reactive oxygen-generating NADPH oxidase (Nox/Duox) family of enzymes. BMC Evol Biol 7: 109.

Kim BJ, Cho SW, Jeon YJ, An S, Jo A, Lim JH *et al*. (2017). Intranasal delivery of Duox2 DNA using cationic polymer can prevent acute influenza A viral infection *in vivo* lung. Appl Microbiol Biotechnol 102: 105–115.

Kim HJ, Kim CH, Kim MJ, Ryu JH, Seong SY, Kim S *et al*. (2015). The induction of pattern-recognition receptor expression against influenza A virus through Duox2-derived reactive oxygen species in nasal mucosa. Am J Respir Cell Mol Biol 53: 525–535.

Kim MJ, Ryu JC, Kwon Y, Lee S, Bae YS, Yoon JH *et al*. (2014). Dual oxidase 2 in lung epithelia is essential for hyperoxia-induced acute lung injury in mice. Antioxid Redox Signal 21: 1803–1818.

Kim SH, Lee WJ (2014). Role of DUOX in gut inflammation: lessons from *Drosophila* model of gut–microbiota interactions. Front Cell Infect Microbiol 3: 116.

Ko E, Choi H, Park KN, Park JY, Lee TR, Shin DW *et al*. (2015). Dual oxidase 2 is essential for house dust mite-induced pro-inflammatory cytokine production in human keratinocytes. Exp Dermatol 24: 936–941.

Koff JL, Shao MX, Ueki IF, Nadel JA (2008). Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. Am J Physiol Lung Cell Mol Physiol 294: L1068–L1075.

Krick S, Wang J, St-Pierre M, Gonzalez C, Dahl G, Salathe M (2016). Dual oxidase 2 (Duox2) regulates pannexin 1-mediated ATP release in primary human airway epithelial cells via changes in intracellular pH and not H_2O_2 production. J Biol Chem 291: 6423–6432.

Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C (2010). A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. Science 327: 1644–1648.

Kwon J, Shatynski KE, Chen H, Morand S, de Deken X, Miot F *et al*. (2010). The nonphagocytic NADPH oxidase Duox1 mediates a positive feedback loop during T cell receptor signaling. Sci Signal 3: ra59.

Lambeth JD (2004). NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4: 181–189.

Lambrecht BN, Hammad H (2017). The immunology of the allergy epidemic and the hygiene hypothesis. Nat Immunol 18: 1076–1083.

Lassegue B, San Martin A, Griendling KK (2012). Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. Circ Res 110: 1364–1390.

Levine AP, Pontikos N, Schiff ER, Jostins L, Speed D, Consortium NIBDG *et al*. (2016). Genetic complexity of Crohn's disease in two large Ashkenazi Jewish families. Gastroenterology 151: 698–709.

Li W, Yan F, Zhou H, Lin X, Wu Y, Chen C *et al*. (2013). *P. aeruginosa* lipopolysaccharide-induced MUC5AC and CLCA3 expression is partly through Duox1 *in vitro* and *in vivo*. PLoS One 8: e63945.

Linderholm AL, Onitsuka J, Xu C, Chiu M, Lee WM, Harper RW (2010). All-*trans* retinoic acid mediates DUOX2 expression and function in respiratory tract epithelium. Am J Physiol Lung Cell Mol Physiol 299: L215–L221.

Lipinski S, Till A, Sina C, Arlt A, Grasberger H, Schreiber S *et al*. (2009). DUOX2-derived reactive oxygen species are effectors of NOD2-mediated antibacterial responses. J Cell Sci 122: 3522–3530.

Little AC, Sham D, Hristova M, Danyal K, Heppner DE, Bauer RA *et al*. (2016). DUOX1 silencing in lung cancer promotes EMT, cancer stem cell characteristics and invasive properties. Oncogene 5: e261.

Little AC, Sulovari A, Danyal K, Heppner DE, Seward DJ, van der Vliet A (2017). Paradoxical roles of dual oxidases in cancer biology. Free Radic Biol Med 110: 117–132.

Lu J, Risbood P, Kane CT, Jr., Hossain MT, Anderson L, Hill K *et al*. (2017). Characterization of potent and selective iodonium-class inhibitors of NADPH oxidases. Biochem Pharmacol 143: 25–38.

MacFie TS, Poulsom R, Parker A, Warnes G, Boitsova T, Nijhuis A *et al*. (2014). DUOX2 and DUOXA2 form the predominant enzyme system capable of producing the reactive oxygen species H_2O_2 in active ulcerative colitis and are modulated by 5-aminosalicylic acid. Inflamm Bowel Dis 20: 514–524.

Maghzal GJ, Krause KH, Stocker R, Jaquet V (2012). Detection of reactive oxygen species derived from the family of NOX NADPH oxidases. Free Radic Biol Med 53: 1903–1918.

Magnani F, Nenci S, Millana Fananas E, Ceccon M, Romero E, Fraaije MW *et al*. (2017). Crystal structures and atomic model of NADPH oxidase. Proc Natl Acad Sci U S A 114: 6764–6769.

Meitzler JL, Hinde S, Banfi B, Nauseef WM, Ortiz de Montellano PR (2013). Conserved cysteine residues provide a protein–protein interaction surface in dual oxidase (DUOX) proteins. J Biol Chem 288: 7147–7157.

Meitzler JL, Ortiz de Montellano PR (2009). *Caenorhabditis elegans* and human dual oxidase 1 (DUOX1) "peroxidase" domains: insights into heme binding and catalytic activity. J Biol Chem 284: 18634–18643.

Molofsky AB, Savage AK, Locksley RM (2015). Interleukin-33 in tissue homeostasis, injury, and inflammation. Immunity 42: 1005–1019.

Monticelli LA, Osborne LC, Noti M, Tran SV, Zaiss DM, Artis D (2015). IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin–EGFR interactions. Proc Natl Acad Sci U S A 112: 10762–10767.

Morand S, Agnandji D, Noel-Hudson MS, Nicolas V, Buisson S, Macon-Lemaitre L *et al*. (2004). Targeting of the dual oxidase 2 N-terminal region to the plasma membrane. J Biol Chem 279: 30244–30251.

Moskwa P, Lorentzen D, Excoffon KJ, Zabner J, McCray PB Jr, Nauseef WM *et al*. (2007). A novel host defense system of airways is defective in cystic fibrosis. Am J Respir Crit Care Med 175: 174–183.

Nadeem A, Alharbi NO, Vliagoftis H, Tyagi M, Ahmad SF, Sayed-Ahmed MM (2015). Proteinase activated receptor-2-mediated dual oxidase-2 up-regulation is involved in enhanced airway reactivity and inflammation in a mouse model of allergic asthma. Immunology 145: 391–403.

Niethammer P, Grabher C, Look AT, Mitchison TJ (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature 459: 996–999.

Nisimoto Y, Ogawa H, Kadokawa Y, Qiao S (2018). NADPH oxidase 4 function as a hydrogen peroxide sensor. J Biochem. https://doi.org/10.1093/jb/mvy014 [Epub ahead of priint].

O'Donnell BV, Tew DG, Jones OT, England PJ (1993). Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem J 290 (Pt 1): 41–49.

Ohlow MJ, Moosmann B (2011). Phenothiazine: the seven lives of pharmacology's first lead structure. Drug Discov Today 16: 119–131.

Pacquelet S, Lehmann M, Luxen S, Regazzoni K, Frausto M, Noack D *et al*. (2008). Inhibitory action of NoxA1 on dual oxidase activity in airway cells. J Biol Chem 283: 24649–24658.

Parlato M, Charbit-Henrion F, Hayes P, Tiberti A, Aloi M, Cucchiara S *et al*. (2017). First identification of biallelic inherited DUOX2 inactivating mutations as a cause of very early onset inflammatory bowel disease. Gastroenterology 153 (609–611): e603.

Pongnimitprasert N, Hurtado M, Lamari F, El Benna J, Dupuy C, Fay M *et al*. (2012). Implication of NADPH oxidases in the early inflammation process generated by cystic fibrosis cells. ISRN Inflamm 2012 481432, 1,11.

Qi R, Zhou Y, Li X, Guo H, Gao L, Wu L *et al*. (2016). DUOX2 expression is increased in Barrett esophagus and cancerous tissues of stomach and colon. Gastroenterol Res Pract 2016 1835684.

Raad H, Eskalli Z, Corvilain B, Miot F, De Deken X (2013). Thyroid hydrogen peroxide production is enhanced by the Th2 cytokines, IL-4 and IL-13, through increased expression of the dual oxidase 2 and its maturation factor DUOXA2. Free Radic Biol Med 56: 216–225.

Rada B, Boudreau HE, Park JJ, Leto TL (2014a). Histamine stimulates hydrogen peroxide production by bronchial epithelial cells via histamine H1 receptor and dual oxidase. Am J Respir Cell Mol Biol 50: 125–134.

Rada B, Leto TL (2010). Characterization of hydrogen peroxide production by Duox in bronchial epithelial cells exposed to *Pseudomonas aeruginosa*. FEBS Lett 584: 917–922.

Rada B, Park JJ, Sil P, Geiszt M, Leto TL (2014b). NLRP3 inflammasome activation and interleukin-1β release in macrophages require calcium but are independent of calcium-activated NADPH oxidases. Inflamm Res 63: 821–830.

Razzell W, Evans IR, Martin P, Wood W (2013). Calcium flashes orchestrate the wound inflammatory response through DUOX activation and hydrogen peroxide release. Curr Biol 23: 424–429.

Rigutto S, Hoste C, Grasberger H, Milenkovic M, Communi D, Dumont JE *et al*. (2009). Activation of dual oxidases Duox1 and Duox2: differential regulation mediated by camp-dependent protein kinase and protein kinase C-dependent phosphorylation. J Biol Chem 284: 6725–6734.

Rivera A, Siracusa MC, Yap GS, Gause WC (2016). Innate cell communication kick-starts pathogen-specific immunity. Nat Immunol 17: 356–363.

Ryu JH, Yoo JY, Kim MJ, Hwang SG, Ahn KC, Ryu JC *et al*. (2013). Distinct TLR-mediated pathways regulate house dust mite-induced allergic disease in the upper and lower airways. J Allergy Clin Immunol 131: 549–561.

Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X *et al*. (2013). A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. J Exp Med 210: 2939–2950.

Savinko T, Matikainen S, Saarialho-Kere U, Lehto M, Wang G, Lehtimaki S *et al*. (2012). IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. J Invest Dermatol 132: 1392–1400.

Schnider D, Rieder S, Leeb T, Gerber V, Neuditschko M (2017). A genome-wide association study for equine recurrent airway obstruction in European warmblood horses reveals a suggestive new quantitative trait locus on chromosome 13. Anim Genet 48: 691–693.

Segal AW (2016). NADPH oxidases as electrochemical generators to produce ion fluxes and turgor in fungi, plants and humans. Open Biol 6: 160028.

Seredenina T, Chiriano G, Filippova A, Nayernia Z, Mahiout Z, Fioraso-Cartier L *et al*. (2015). A subset of N-substituted phenothiazines inhibits NADPH oxidases. Free Radic Biol Med 86: 239–249.

Sham D, Wesley UV, Hristova M, van der Vliet A (2013). ATP-mediated transactivation of the epidermal growth factor receptor in airway epithelial cells involves DUOX1-dependent oxidation of Src and ADAM17. PLoS One 8: e54391.

Shao MX, Nadel JA (2005). Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. Proc Natl Acad Sci U S A 102: 767–772.

Singh DK, Kumar D, Siddiqui Z, Basu SK, Kumar V, Rao KV (2005). The strength of receptor signaling is centrally controlled through a cooperative loop between Ca^{2+} and an oxidant signal. Cell 121: 281–293.

Singh J, Petter RC, Baillie TA, Whitty A (2011). The resurgence of covalent drugs. Nat Rev Drug Discov 10: 307–317.

Sommer F, Backhed F (2015). The gut microbiota engages different signaling pathways to induce Duox2 expression in the ileum and colon epithelium. Mucosal Immunol 8: 372–379.

Strengert M, Jennings R, Davanture S, Hayes P, Gabriel G, Knaus UG (2014). Mucosal reactive oxygen species are required for antiviral response: role of Duox in influenza A virus infection. Antioxid Redox Signal 20: 2695–2709.

Svenningsen S, Nair P (2017). Asthma endotypes and an overview of targeted therapy for asthma. Front Med (Lausanne) 4: 158.

Teixeira G, Szyndralewiez C, Molango S, Carnesecchi S, Heitz F, Wiesel P *et al*. (2017). Therapeutic potential of NADPH oxidase 1/4 inhibitors. Br J Pharmacol 174: 1647–1669.

Thiagarajah JR, Chang J, Goettel JA, Verkman AS, Lencer WI (2017). Aquaporin-3 mediates hydrogen peroxide-dependent responses to environmental stress in colonic epithelia. Proc Natl Acad Sci U S A 114: 568–573.

Timms RT, Menzies SA, Tchasovnikarova IA, Christensen LC, Williamson JC, Antrobus R *et al*. (2016). Genetic dissection of mammalian ERAD through comparative haploid and CRISPR forward genetic screens. Nat Commun 7: 11786.

Vallath S, Hynds RE, Succony L, Janes SM, Giangreco A (2014). Targeting EGFR signalling in chronic lung disease: therapeutic challenges and opportunities. Eur Respir J 44: 513–522.

van der Hoeven R, Cruz MR, Chavez V, Garsin DA (2015). Localization of the dual oxidase BLI-3 and characterization of its NADPH oxidase domain during infection of *Caenorhabditis elegans*. PLoS One 10: e0124091.

van der Vliet A (2011). Nox enzymes in allergic airway inflammation. Biochim Biophys Acta 1810: 1035–1044.

van der Vliet A, Janssen-Heininger YM (2014). Hydrogen peroxide as a damage signal in tissue injury and inflammation: murderer, mediator, or messenger? J Cell Biochem 115: 427–435.

Voraphani N, Gladwin MT, Contreras AU, Kaminski N, Tedrow JR, Milosevic J *et al*. (2014). An airway epithelial iNOS–DUOX2–thyroid peroxidase metabolome drives Th1/Th2 nitrative stress in human severe asthma. Mucosal Immunol 7: 1175–1185.

Wan WY, Hollins F, Haste L, Woodman L, Hirst RA, Bolton S *et al*. (2016). NADPH oxidase-4 overexpression is associated with epithelial ciliary dysfunction in neutrophilic asthma. Chest 149: 1445–1459.

Wang D, De Deken X, Milenkovic M, Song Y, Pirson I, Dumont JE *et al*. (2005). Identification of a novel partner of duox: EFP1, a thioredoxin-related protein. J Biol Chem 280: 3096–3103.

Wesley UV, Bove PF, Hristova M, McCarthy S, van der Vliet A (2007). Airway epithelial cell migration and wound repair by ATP-mediated activation of dual oxidase 1. J Biol Chem 282: 3213–3220.

Winterbourn CC, Kettle AJ, Hampton MB (2016). Reactive oxygen species and neutrophil function. Annu Rev Biochem 85: 765–792.

Wu Y, Antony S, Hewitt SM, Jiang G, Yang SX, Meitzler JL *et al*. (2013). Functional activity and tumor-specific expression of dual oxidase 2 in pancreatic cancer cells and human malignancies characterized with a novel monoclonal antibody. Int J Oncol 42: 1229–1238.

Xiao X, Yang L, Pang X, Zhang R, Zhu Y, Wang P *et al*. (2017). A Mesh–Duox pathway regulates homeostasis in the insect gut. Nat Microbiol 2: 17020.

Xu C, Linderholm A, Grasberger H, Harper RW (2012). Dual oxidase 2 bidirectional promoter polymorphisms confer differential immune responses in airway epithelia. Am J Respir Cell Mol Biol 47: 484–490.

Yang X, Smith AA, Williams MS, Pal U (2014). A dityrosine network mediated by dual oxidase and peroxidase influences the persistence of Lyme disease pathogens within the vector. J Biol Chem 289: 12813–12822.

Yu M, Lam J, Rada B, Leto TL, Levine SJ (2011). Double-stranded RNA induces shedding of the 34-kDa soluble TNFR1 from human airway epithelial cells via TLR3-TRIF-RIP1-dependent signaling: roles for dual oxidase 2- and caspase-dependent pathways. J Immunol 186: 1180–1188.

Yun BW, Feechan A, Yin M, Saidi NB, Le Bihan T, Yu M *et al*. (2011). *S*-nitrosylation of NADPH oxidase regulates cell death in plant immunity. Nature 478: 264–268.