The use of model systems to study biological functions of Nox/Duox enzymes

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Abstract

ROS (reactive oxygen species; including superoxide and H₂O₂) are conventionally thought of as being broadly reactive and cytotoxic. Phagocytes utilize an NADPH oxidase to generate large amounts of ROS, and exploit their toxic properties as a host-defence mechanism to kill invading microbes. However, the recent discovery of the Nox and Duox enzymes that are expressed in many non-phagocytic cells implies that the ‘deliberate’ generation of ROS has additional cellular roles, which are currently incompletely understood. Functions of ROS in mammals have been inferred primarily from cell-culture experiments, and include signalling for mitogenic growth, apoptosis and angiogenesis. Nox/Duox enzymes may also provide H₂O₂ as a substrate for peroxidase enzymes (or, in the case of Duox, for its own peroxidase domain), thereby supporting peroxidative reactions. A broad comparison of biological functions of ROS and Nox enzymes across species and kingdoms provides insights into possible functions in mammals. To further understand novel biological roles for Nox/Duox enzymes, we are manipulating the expression of Nox/Duox enzymes in model organisms including Caenorhabditis elegans, Drosophila melanogaster and mouse. This chapter focuses on new insights into the roles of Nox enzymes gained from these approaches.

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Introduction

ROS (reactive oxygen species; superoxide, H$_2$O$_2$, hydroxyl radical) are conventionally regarded as being cytotoxic and mutagenic. According to this view, the occurrence of ROS in biological systems is unintended and deleterious. A preponderance of ROS literature has focused on ‘accidental’ sources of ROS, the mechanisms by which cells eliminate ROS, and the damage that ROS cause to DNA, lipids and proteins. The mitochondrial electron transport chain is frequently regarded as the major source of such ROS, leading to the idea that ROS are largely an accidental byproduct of aerobic metabolism [1,2]. ROS also originates, via the effects of UV radiation, drugs and toxins, and have been implicated in their toxic and mutagenic effects [3].

While such unintended ROS production does occur, ROS are also generated in a regulated, enzymic manner, implying that ROS serve useful biological functions. The classical example of deliberate ROS production is the NADPH oxidase of professional phagocytes. This oxidase was originally identified as a haem pigment, cytochrome b$_{558}$, that was absent in neutrophils from individuals with an X-linked form of CGD (chronic granulomatous disease), a rare genetic disorder that can also be inherited as an autosomal recessive condition [4,5]. Phagocytes from CGD patients are defective in bacterial killing and in their ability to mount a respiratory burst, the rapid production of superoxide, H$_2$O$_2$, perchlorous acid and hydroxyl radical that accompanies phagocytosis. The gene for X-linked CGD was identified by positional cloning and subtractive hybridization using RNA from a CGD patient with an Xp21 deletion, and encodes gp91phox, a membrane glycoprotein that migrates as a broad band at around 91 kDa [6]. This subunit is the catalytic moiety of the oxidase, and contains binding sites for FAD, haem and NADPH. An additional membrane protein, p22phox, was subsequently identified and together with gp91phox forms cytochrome b$_{558}$, or more correctly flavocytochrome b$_{558}$ [7]. Additional regulatory proteins of the phagocyte oxidase were identified and their role in activation was determined in cell-free systems, based on their ability to support superoxide generation by neutrophil membrane preparations [8]. These components include p47phox, p67phox, p40phox and the small GTPase Rac (Rac2 and/or Rac1, depending on cell type and activation stimulus). Upon exposure of phagocytes to bacteria or immune mediators, these regulatory proteins activate NADPH oxidase activity catalysed by gp91phox by assembling with this enzymic moiety at the plasma membrane. Phosphorylation of p47phox, p67phox and possibly other components as well as GTP loading of Rac trigger the assembly process.

It has recently been appreciated that a variety of cell types in addition to phagocytes produce ROS, many in response to ligand stimulation. In many cases, ROS production is inhibited by DPI (diphenylene iodinium), an inhibitor of the phagocyte NADPH oxidase [9,10]. These observations led to the identification and cloning of several homologues of gp91phox, termed the Nox/Duox family of NADPH oxidases [11–13]. The dendrogram in Figure 1, constructed based on identity with the gp91phox flavoprotein domain, illustrates evolutionary relationships among Nox family members. Nox1 and Nox3 are closely
related to gp91phox (Nox2), whereas Nox4 seems to be closer to a primordial Nox. Nox5 is more distantly related, followed by Duox1 and Duox2.

The domain complexity of this family is consistent with the predicted evolutionary distance from a primordial Nox, and points to a minimum of two gene-fusion events occurring very early, prior to the split between the Plant and Animal Kingdoms. Nox5 contains an EF-hand calcium-binding domain in addition to a C-terminal gp91phox homology domain that confers activity in response to calcium [14]. The Duox enzymes build on the Nox5 structure by the addition of an N-terminal peroxidase domain and thus contain a catalytic moiety that can potentially use H2O2 generated by the oxidase domain as a substrate for peroxidative reactions. Hence, the name Duox refers to its dual oxidase nature. Nox enzymes are expressed in a variety of tissues, with Nox1 highest in colon, Nox4 highest in kidney, Nox5 highest in spleen and testis, and Duox1 and Duox2 highest in thyroid, lung and colon. Nox3 is expressed in several fetal tissues but is not found in adult tissues tested thus far [15].

The existence of NADPH oxidases in multiple non-phagocytic tissues implies that the function of the deliberate generation of ROS extends beyond bacterial killing by phagocytes. Several hypotheses exist concerning the function of Nox enzymes, including host defence, signal transduction, oxygen sensing and various metabolic roles. While the function of gp91phox and its associated regulatory proteins was revealed by forward genetics in which the inherited condition CGD revealed the molecular functions of these proteins, there are currently no known disease associations with the Nox family enzymes. Therefore, the identification of functions for Nox/Duox enzymes in

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**Figure 1** Phylogenetic relationships among Nox/Duox oxidases. This dendrogram demonstrates evolutionary relationships of Nox/Duox enzymes based on identity with the flavocytochrome domain of gp91phox.
mammals will rely heavily both on examples from simpler organisms and on reverse genetic approaches. The existence of Nox homologues in genetically amenable model systems will therefore be informative in identifying functions for these enzymes. In this chapter, we discuss findings from yeast, plants and *Caenorhabditis elegans*, as well as ongoing studies in *Drosophila melanogaster* and *Mus musculus* model systems.

**Yeast**

The amino acid sequence encoded by the *fre-1* gene is sufficiently distant from that of *gp91phox* that the relationship is far from obvious. However, the domain structure is similar to that of *gp91phox*, and includes an apparent binding site for pyridine nucleotide, for FAD and for conserved histidine residues that are properly spaced as haem-binding sites. The enzyme functions in pyridine nucleotide-dependent reduction of extracellular ferric iron to form ferrous iron, forming part of an iron-uptake system in yeast. Thus the electron-transport function is conserved in this enzyme, but the substrate is iron rather than molecular oxygen. It is not clear that the ferric reductase activity is a primordial function of this enzyme class, since this function does not appear to be conserved in other species.

**Plants**

Homologues of Nox enzymes have been discovered in plant species including *Arabidopsis thaliana* [16,17], tobacco [18], tomato [19], potato [20] and rice [16]. These homologues are referred to as *rbohs* (respiratory burst oxidase homologues) because of their identity with *gp91phox*. However, the presence of an N-terminal domain containing EF-hand motifs reflects closer similarity to Nox5. The potato and tobacco genomes encode two *rboh* isogenes, and *Arabidopsis* has 10 isogenes, suggesting multiple functions in plants.

The recognition of pathogens by plant cells initiates an oxidative burst that participates in multiple aspects of the plant host-defence response, including direct bactericidal activity, cell-wall glycoprotein cross-linking [21], induction of defence-related genes [22,23] and induction of an apoptosis-like programme known as the HR (hypersensitive response) [24,25]. Hypersensitive cell death contributes to pathogen restriction by preventing the spread of the lesion to surrounding healthy tissue.

The finding that DPI inhibits the oxidative burst of plants provided the first evidence that NADPH oxidases might be involved [26,27]. Subsequent studies in tobacco and *Arabidopsis* linked *rboh* genes to plant host-defence responses and have begun to define distinct roles for individual isogenes. In *Arabidopsis*, combined insertional mutagenesis of *AtrbohD* and *AtrbohF* (where *At* denotes *A. thaliana*) eliminates extracellular ROS production in response to avirulent *Pseudomonas syringae* and *Peronospora parasitica* [28]. Individual mutagenesis of *AtrbohD* had the greater effect on extracellular ROS production, whereas *AtrbohF* mutants were more defective in HR-induced cell death.
A similar dichotomy has been observed for two Nbrboh genes of tobacco (where Nbrboh indicates Nicotiana benthamiana) [18]. NbrbohA gene expression is induced by leaf infiltration, while the NbrbohB gene is induced by interferon 1 and salicylic acid. Virus-induced gene silencing of NbrbohA and NbrbohB in tobacco leaves demonstrated that they are both required for ROS production and resistance to Phytophthora infestans. The activation of mitogen-activated protein kinase pathways leads to HR-like cell death that is preceded by the generation of ROS. While a pathogen-induced HR required both NbrbohA and NbrbohB, mitogen-activated protein kinase-mediated HR-like cell death was only dependent on NbrbohB. Correspondingly, transcription of NbrbohB, but not NbrbohA, is up-regulated in response to mitogen-activated protein kinase activation. Thus Nox homologues have both overlapping and unique functions in plant host defence.

In addition to host defence, a Nox homologue in Arabidopsis functions in calcium homoeostasis and cell growth. In Arabidopsis, specialized epidermal cells in roots extend tip-growing root hairs into the soil following the cessation of epidermal cell elongation. The extension of these root hairs is accompanied by transport of calcium across the plasma membrane into the tip, where calcium remains elevated for the duration of root-hair growth. Arabidopsis rhd2 (root hair defective 2) mutants fail to elevate calcium and develop stunted root hairs [29]. Foreman and colleagues [30] used transposon tagging to identify a mutation in AtrbohC as the cause of the rhd2 phenotype and demonstrated that treatment with DPI results in the same phenotype as rhd2 mutants. AtrbohC mutants fail to accumulate ROS in root hairs and treatment of rhd2 roots with ROS partly restores calcium influx and growth. The authors conclude that ROS derived from AtrbohC activate calcium channels that support root-cell elongation. In summary, data from plants implicate Nox enzymes in innate immune responses, regulation of cell growth and signalling to ion channels.

C. elegans

A search of the C. elegans genomic database identified sequence identity between two regions of the genome and the Nox enzymes [31], and these are referred to as Ce-Duox1 and Ce-Duox2 since they are predicted to also encode a peroxidase domain. Analysis of the genome revealed that Ce-Duox1 and Ce-Duox2 are oriented in opposite directions near the end of chromosome I, separated by only 6 kb. The cDNAs for Ce-Duox1 and Ce-Duox2 share approx. 30% identity with human Duox1 and Duox2, and 94% identity with one another. However, Ce-Duox2 lacks a portion of the C-terminus corresponding to the pyridine nucleotide-binding site and is therefore not functional as an NADPH oxidase. Because of the location and orientation with respect to Ce-Duox1, as well as the high level of sequence identity, Ce-Duox2 appears to be the product of a recent gene duplication that was partially truncated. No homologue of the smaller Nox forms was identified suggesting that whatever function the small Nox enzymes may have performed in early evolution is either no longer needed or is carried out by other gene products in C. elegans.
RNAi (RNA interference) of Ce-Duox1 eliminated Ce-Duox1 expression and revealed several phenotypes related to cuticle development, and provided the key clue to the function of Ce-Duox1. Immunofluorescence showed Duox in hypodermal cells, the outermost layer of cells in *C. elegans* and the cell layer that synthesizes the cuticle. RNAi phenotypes included blistering, translucence, immobility and improper development leading to a ‘dumpy’ (short, fat) animal. These are similar to phenotypes seen in mutants of collagen biosynthesis that are associated with defects in cuticle development, and suggested that Ce-Duox1 is also involved in cuticle biogenesis or maintenance. *C. elegans* cuticle is composed of three layers, the cortical (outer), the median and the basal (inner) layers. The median layer is a fluid-filled space that connects the cortical and basal layers via a system of struts. Electron microscopy of Ce-Duox1 RNAi animals revealed that the blister phenotype was caused by a rupture of the struts, causing separation of the cortical layer from the lower layers of the cuticle (Figure 2). Remnants of the struts were observed attached to the cortical and basal layers, suggesting a general structural failure of the struts rather than a failure of strut attachment.

Identity between the peroxidase domain of Duox enzymes and several peroxidases, especially sea urchin ovoperoxidase, suggested that Ce-Duox1 might stabilize the cuticle by catalysing tyrosine cross-linking. Ovoperoxidase functions in the cortical reaction, preventing polyspermy by cross-linking tyrosine residues in the egg wall following fertilization [32]. The products of this cross-linking, dityrosine and trityrosine, were readily detectable in wild-type worms, but RNAi worms showed no detectable cross-links. These data suggested that Ce-Duox1 participates in stabilization of the cuticle by cross-linking tyrosine residues in cuticle proteins. This was verified by demonstrating that the recombinant peroxidase domains of Duox1 (from both *C. elegans* and human) catalysed **H₂O₂**-dependent formation of di- and trityrosine. Because Duox partially purified from human thyroid had been shown previously to catalyse NADPH-dependent **H₂O₂** formation [33], we have proposed a model in which Duox generates **H₂O₂** via its gp91phox homology domain, and the **H₂O₂** is then utilized by the peroxidase domain to carry out peroxidative cross-linking of tyrosine residues in extracellular matrix proteins. This results in stabilization of the cuticle structures, including the struts.

The function of Duox in *C. elegans* to modify the extracellular matrix suggests similar functions in higher organisms. Human Duox1 and Duox2 are expressed in thyroid gland, the site of generation of thyroid hormone (thyroxine). The latter is produced through the iodination and cross-linking (coupling) of tyrosine residues within the matrix protein thyroglobulin. A recent study of variants of inherited hypothyroidism showed a link between mis-expression of human Duox2 and the inability of patients to produce thyroxine, consistent with thyroglobulin being a direct or indirect substrate for human Duox [34]. It has been suggested that this role involves exclusively the generation of **H₂O₂** by Duox for use by thyroid peroxidase to catalyse both iodination and coupling reactions. However, this proposal fails to account for any function for the peroxidase domain of human Duox, which as discussed above can catalyse tyrosine cross-linking and perform other peroxidative reactions. Therefore, the
peroxidase domain of Duox might also participate directly in one or both of these reactions. What function might Duox serve in other tissues where it is expressed (e.g. lung, salivary duct, brain, colon and testis)? The expression in lung and salivary ducts has been suggested by Leto and colleagues [35a] to implicate Duox in generation of \( \text{H}_2\text{O}_2 \) related to innate immune function, but this proposal too fails to account for a function for the peroxidase domain. Its role in tyrosine cross-linking of cuticle might suggest a similar role in cross-

Figure 2 Electron micrograph of Ce-Duox RNAi animals. Cross-sectional view of a Ce-Duox RNAi animal demonstrating separation of the outer cortical layer from the inner basal layer. Arrows indicate remnants of broken struts that normally function to separate these layers. Magnification, ×10752.
linking collagen or elastin in mammals [35], since these are major extracellular matrix constituents. However, tyrosine cross-linking occurs in very low abundance in mammalian collagen and elastin [36], and is usually thought of as a by-product of inflammation catalysed by phagocyte myeloperoxidase. A possible exception is the rather extensive tyrosine cross-linking of brain proteins that is associated with neurodegenerative disorders, including Alzheimer’s [37,38] and Parkinson’s diseases [39–41]. Oxidative stress has been implicated in this process [42], but the sources of ROS are incompletely understood. The localization of Duox in brain raises the intriguing possibility that Duox might participate in neurodegenerative diseases. Thus the established function of Duox in C. elegans suggests functions in higher organisms related to modification of the extracellular matrix. However, the lack of adequate animal model systems makes these proposals speculative at present.

D. melanogaster

D. melanogaster provides a powerful genetic system that is highly amenable to reverse genetics approaches. A search of the D. melanogaster genome on FlyBase revealed the existence of a homologue of Nox5 (CG3896) and another of Duox (CG3131) that we have termed D-Nox and D-Duox, respectively [42a]. Like the plant Nox proteins, D-Nox is comprised of a C-terminal gp91phox-homology domain and an N-terminal calcium-binding domain. The presence of only two homologues makes Drosophila appealing for the use of reverse genetics approaches, because of the reduced possibility of compensation by isoforms with overlapping functions.

We have cloned the genes for D-Nox and D-Duox and their expression and functions are under investigation (W.A. Edens and D.R. Ritsick, unpublished work). Western-blot analysis shows that D-Duox is highly expressed in embryos and during later developmental stages, whereas D-Nox is absent in embryos but is expressed at all other stages. Differential expression profiles are corroborated by DNA microarray analysis of mRNA expression as determined by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/). D-Duox is found on the apical surface of the plasma membrane of epidermal cells of embryos, whereas both D-Nox and D-Duox are located at apical plasma membranes of epithelial structures of larvae.

We are using the reverse-genetic RNAi approach to define functions for D-Nox and D-Duox. The RNAi approach in Drosophila has recently been adapted for use in combination with the UAS (upstream activating sequence)/GAL4 binary system: a long inverted repeat separated by a spacer is constructed in a Drosophila transformation vector containing a promoter with multiple UAS enhancer elements. When crossed with GAL4 driver lines, flies that have stably integrated such a construct produce hairpin double-stranded RNA capable of sequence-specific RNAi. The GAL4 protein is expressed under the control of gene-specific or -inducible (i.e. heat-shock) promoters. Therefore, expression of the double-stranded RNA can be tightly temporally and spatially controlled. Since early severe phenotypes can be avoided, this system is ideally suited to study functions of early-acting essential genes at later
times in development. Using this strategy, we have created transgenic flies that express D-Nox- or D-Duox-specific inverted repeats driven by a ubiquitous daughterless-GAL4 driver.

Surprisingly, preliminary studies reveal developmental roles for D-Nox and D-Duox. D-Nox RNAi animals develop defects in mouthparts, while D-Duox RNAi animals develop wing abnormalities. The underlying mechanism by which D-Nox RNAi produces mouthpart abnormalities is under investigation, but is suggestive of a signalling role related to reactive oxygen. We are also evaluating the hypothesis that, as in *C. elegans*, D-Duox RNAi-generated wing abnormalities result from a failure of tyrosine cross-linking, which is expected to rigidify and stabilize the wing structure. In addition to analysis of these developmental roles, *Drosophila* provides a powerful system in which to investigate other proposed functions for Nox enzymes including host defence and signal transduction [43]. In this regard NO has both bactericidal and signalling functions in the *Drosophila* immune response [44], and its production is frequently accompanied by generation of superoxide. This suggests that Noxs may also be involved in these NO-related immune functions. By manipulating ROS catabolic enzymes, ROS have also been directly linked to aging in *Drosophila* [45–47]. It is therefore possible that D-Nox and/or D-Duox contribute to aging through induction of oxidative stress. *Drosophila* has also been used as a model system to study the effects of hypoxia/re-oxygenation and hyperoxia [48], conditions that cause cell injury via NADPH oxidase-dependent production of ROS [49]. Thus important *Drosophila* models already exist that can be readily applied to testing the hypotheses concerning Nox/Duox function.

**M. musculus**

A major function for gp91phox in innate immunity was revealed by a natural case of forward genetics, and mouse models of CGD have been useful in refining our knowledge of function in phagocytes and in defining new functions in other cell types [50,51]. The distinctive distribution of Nox/Duox enzymes in human tissues suggests that they have tissue-specific functions. While *C. elegans* and *Drosophila* represent superior systems in terms of ease of genetic manipulation, they cannot provide clues to the function of the short Nox enzymes (Nox1, Nox3 and Nox4), nor can they directly demonstrate a mammalian function for any of the Nox enzymes. Additionally, it is not always possible directly to correlate fly and human anatomical structures and physiology. Therefore, mouse models should prove particularly informative. We have begun to explore the effects of manipulating murine Nox1 expression in a tissue-specific manner. Using the fatty acid-binding protein promoter [52] to drive Cre recombinase expression, we have developed mice that overexpress Nox1 specifically in the colon. Using immunofluorescence, Nox1 was highly overexpressed in colon epithelium, where its expression correlated with a marked increase in reactive oxygen generation. We are using the same strategy to develop mice overexpressing Nox1 in vascular smooth muscle, and these animals are being evaluated in hypertension and atherosclerosis model systems.
Using this approach, Nox1 can be overexpressed in any tissue for which a tissue-specific promoter is known.

In addition to transgenic Nox1-overexpressing mice, we have generated conditional Nox1-knockout mice in which exon 13 (the last exon) is disrupted. This eliminates the NADPH oxidase function of Nox1 without interfering with other molecular properties such as protein–protein interactions or the proposed function of the membrane domain as an ion channel. While the utility of these model systems has yet to be fully realized, they will permit the testing of proposals regarding normal and pathological functions for Nox1 in cell growth, angiogenesis, immune function, cancer development, atherosclerosis and hypertension, functions that have been suggested to a large degree on the basis of studies in cell culture [12,53].

Conclusions

Despite their well-documented toxic properties, ROS are now becoming widely recognized as molecules that have useful biological roles in a variety of processes including host defence, signal transduction and various metabolic reactions. The recently described Nox/Duox family of NADPH oxidases provides likely molecular sources for the production of these ‘useful’ ROS. Reverse genetic approaches, increasingly important in the post-genomic era, will be essential for determining specific functions for this family of enzymes. The increasing utility of RNAi, along with advances in the development of conditional transgenics, will provide important tools for this purpose. Application of these approaches to Nox/Duox homologues in genetic model systems such as D. melanogaster and M. musculus will provide unique insights into functions for ROS in biological systems.

References


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