Structures of mouse DUOX1–DUOXA1 provide mechanistic insights into enzyme activation and regulation

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DUOX1, an NADPH oxidase family member, catalyzes the production of hydrogen peroxide. DUOX1 is expressed in various tissues, including the thyroid and respiratory tract, and plays a crucial role in processes such as thyroid hormone biosynthesis and innate host defense. DUOX1 co-assembles with its maturation factor DUOXA1 to form an active enzyme complex. However, the molecular mechanisms for activation and regulation of DUOX1 remain mostly unclear. Here, I present cryo-EM structures of the mammalian DUOX1–DUOXA1 complex, in the absence and presence of substrate NADPH, as well as DUOX1–DUOXA1 in an unexpected dimer-of-dimers configuration. These structures reveal atomic details of the DUOX1–DUOXA1 interaction, a lipid-mediated NADPH-binding pocket and the electron transfer path. Furthermore, biochemical and structural analyses indicate that the dimer-of-dimers configuration represents an inactive state of DUOX1–DUOXA1, suggesting an oligomerization-dependent regulatory mechanism. Together, my work provides structural bases for DUOX1–DUOXA1 activation and regulation.

Maintenance of reactive oxygen species (ROS) homeostasis is essential to preserve cell integrity and is thus vital for the survival and growth of almost all life. ROS generation by NADPH oxidases (NOXs) is an essential aspect of ROS regulation. Enhanced NOX activity could cause oxidative stress and thus result in cell or tissue damage and, in some cases, even organ failure in mammals. For example, during viral infection, it is proposed that long-time viral stimulation is prone to eliciting intensive immunological reactions, which includes increased production of ROS through NOXs in macrophages and neutrophils. The excessive ROS will not only clear viruses but also damage healthy cells in the lung and even the heart, leading to organ failure.

As a member of the NADPH oxidase family, DUOX1 catalyzes the production of hydrogen peroxide (H₂O₂) and plays a crucial role in innate host defense and thyroid hormone biosynthesis. Mammals encode seven NADPH oxidases: NOX1–5 and DUOX1–2. All NOX members share conserved structural features, including an NADPH-binding domain (NBD), a FAD-binding domain (FBD) and a 6-TM ferric oxidoreductase domain (FRD) and catalyze cross-membrane electron transfer from NADPH to oxygen. Among the seven NOX proteins, DUOX1–2 and NOX4 generate H₂O₂, while the rest produce superoxide. DUOX1 and DUOX2 were the first identified thyroid NADPH oxidases that are important for thyroid hormone production. DUOX1 and DUOX2 share ~85% sequence homology and both contain additional N-terminal peroxidase homology domain (PHD) and cytosolic EF-hands. In addition to thyroid glands, DUOX1 is also found in airway epithelial cells and contributes to the innate host defense.

Maturation and activation of DUOX1 require DUOX activator 1 (DUOXA1). DUOXA1 is a transmembrane glycoprotein, expression of which rescues the catalytic function of DUOX1 in reconstituted systems. DUOXA1 regulates DUOX1 by promoting its endoplasmic reticulum (ER)-to-Golgi transition, maturation, translocation to the plasma membrane and protein stability. However, how DUOX1 associates with DUOX1 and regulates its function remains largely unclear.

The catalytic activity of DUOX1–DUOXA1 complexes is tightly regulated. DUOX1–DUOXA1-mediated H₂O₂ production is Ca²⁺-dependent. DUOX1 contains cytoplasmic EF-hand motifs, and mutations of calcium-binding residues abolish the enzymatic activities of DUOX1–DUOXA1 complexes. The function of DUOX1–DUOXA1 complexes is also modulated through post-translational modifications. For example, N-glycosylation of DUOX1 and DUOXA1 is essential for protein maturation, and phosphorylation of DUOX1 could boost H₂O₂ production. Furthermore, the dimerization of DUOX1 PHD domains has been reported, yet it is not clear whether full-length DUOX1–DUOXA1 complexes could also adopt a dimer-of-dimers configuration and, if so, what the physiological importance of such an arrangement is.

Here, I report cryo-EM structures of mouse DUOX1–DUOXA1 complexes in NADPH-free, NADPH-bound and dimer-of-dimers states, providing structural mechanisms underlying DUOX1–DUOXA1 activation and regulation and molecular insights into ROS production.

**Results**

**Structure of the mouse DUOX1–DUOXA1 complex.** Structural studies of the mouse DUOX1–DUOXA1 complex were first carried out in the absence of NADPH using single-particle cryo-EM analysis. The catalytic activity of DUOX1–DUOXA1 complexes is tightly regulated. DUOX1–DUOXA1-mediated H₂O₂ production is Ca²⁺-dependent. DUOX1 contains cytoplasmic EF-hand motifs, and mutations of calcium-binding residues abolish the enzymatic activities of DUOX1–DUOXA1 complexes. The function of DUOX1–DUOXA1 complexes is also modulated through post-translational modifications. For example, N-glycosylation of DUOX1 and DUOXA1 is essential for protein maturation, and phosphorylation of DUOX1 could boost H₂O₂ production. Furthermore, the dimerization of DUOX1 PHD domains has been reported, yet it is not clear whether full-length DUOX1–DUOXA1 complexes could also adopt a dimer-of-dimers configuration and, if so, what the physiological importance of such an arrangement is.

Here, I report cryo-EM structures of mouse DUOX1–DUOXA1 complexes in NADPH-free, NADPH-bound and dimer-of-dimers states, providing structural mechanisms underlying DUOX1–DUOXA1 activation and regulation and molecular insights into ROS production.
Here, I will first discuss the structure of the DUOX1–DUOXA1 heterodimer, which was resolved at an overall resolution of 3.2 Å. The final map allowed de novo model building of both DUOX1 and DUOXA1 (Fig. 1b). There are also sugar densities for several glycosylation sites in DUOXA1 (Asn84 and Asn109) and DUOX1 (N94, N324 and N534) (Extended Data Fig. 1d). The resulting complex structure has dimensions of ~145 Å × 80 Å × 50 Å (Fig. 1a and Extended Data Fig. 1b).

The structure of DUOX1 encompasses an extracellular PHD, a transmembrane domain (TMD) and the cytosolic part containing a Ca²⁺-binding domain (CaBD), an FBD and an NBD (Fig. 1b). The

### Table 1 | Cryo-EM data collection, refinement and validation statistics

|                | DUOX1–DUOXA1 (apo) (EMDB-21962, PDB 6WXR) | DUOX1–DUOXA1 (dimer of dimers) (EMDB-21963, PDB 6WXR) | DUOX1–DUOXA1 (with NADPH) (EMDB-21964, PDB 6WXR) |
|----------------|--------------------------------------------|--------------------------------------------------------|--------------------------------------------------|
| Data collection and processing | Magnification 81,000 81,000 | Voltage (kV) 300 300 | Electron exposure (e⁻/Å²) 65.4 80.5 |
|                     | Defocus range (μm) 11.2.0 11.2.0 | Pixel size (Å) 1.08 1.08 | Symmetry imposed C1 C2 C1 |
| Initial particle images (no.) | -2,200,000 1,486,046 | Final particle images (no.) 534,337 302,097 667,038 | FSC threshold 0.143 0.143 0.143 |
| Map resolution (Å) | 3.2 2.7 3.3 | Map resolution range (Å) 3.2–200 2.7–200 3.3–200 | Map sharpening B factor (Å²) −135 −105 −125 |
| Refinement | Initial model used (PDB code) De novo De novo De novo | Model resolution (Å) 3.2 2.7 3.3 | Model composition Nonhydrogen atoms 10,410 18,419 10,610 |
|                     | FSC threshold 0.143 0.143 0.143 | Model resolution range (Å) 3.2–200 2.7–200 3.3–200 | Protein residues 1,303 2,212 1,319 |
|                     | Map sharpening B factor (Å²) −135 −105 −125 | Map sharpening B factor (Å²) 36.06 67.61 41.73 | Ligands 4 8 5 |
|                     | Model composition | R.m.s. deviations Bond lengths (Å) 0.003 0.006 0.004 | B factors (Å²) Protein 36.06 67.61 41.73 |
|                     | | Bond angles (°) 0.716 0.694 0.702 | Ligand - - 30.07 |
|                     | | Validation | R.m.s. deviations Bond lengths (Å) 0.003 0.006 0.004 |
|                     | | MolProbity score 1.90 1.71 1.82 | Bond angles (°) 0.716 0.694 0.702 |
|                     | | Clashscore 8.68 3.62 8.23 | Poor rotamers (%) 0.19 1.95 0 |
|                     | | Poor rotamers (%) 0.19 1.95 0 | Ramachandran plot Favored (%) 93.31 95.18 94.46 |
|                     | | Ramachandran plot Favored (%) 93.31 95.18 94.46 | Allowed (%) 6.69 4.82 5.54 |
|                     | | Ramachandran plot Favored (%) 93.31 95.18 94.46 | Disallowed (%) 0 0 0 |

**Fig. 1 | Structure of DUOX1–DUOXA1 in the absence of NADPH.**

a, Cryo-EM map and structural model of the DUOX1–DUOXA1 complex. DUOX1 and DUOXA1 are colored in blue and orange, respectively (the same color code will be used unless otherwise noted). The membrane boundary is indicated using gray lines. b, Top: structure and cartoon model of DUOX1. The PHD, TMD, FBD and NBD of DUOX1 are colored in light blue, blue, pink and gray, respectively. The CaBD is indicated by a dashed circle. FAD and hemes are shown as sticks and colored in magenta and green, respectively. Bottom: scheme of DUOX1 with domain boundaries shown. FRD, S1–S6 of DUOX1. c, Structure of DUOX1. The secondary structures between TM2 and TM3 are labeled with β1–4 and H1–2.
The cryo-EM density was too weak for model building. Instead, Cys579 but not Cys364 has a marked functional consequence. It remains puzzling why mutation of Cys579 could disrupt the interaction between DUOX2 and DUOXA2. Interface 2 mainly involves the S5-S6 linker of DUOX1 and TM3 of DUOXA1, with contribution from four other residues: Gly1169 and Val1190 of DUOX1 and Leu134 and Asn171 of DUOXA1.

The structure of the DUOX1–DUOXA1 complex revealed all the vital small molecules (two heme groups, one FAD) involved in electron transfer except NADPH. The binding pocket strongly suggests that the PHD domain of DUOX1 lacks heme-dependent peroxidase activity. This is consistent with previous biochemical analyses (Extended Data Fig. 1d). The PHD of DUOX1 contains seven transmembrane segments (S0–S6): S0 has a kink in the middle, and S1–S6 form a ferric oxidoreductase domain (Fig. 1b). The other cation binding site is located in the same position where the calcium-binding site of DUOXA1 is surrounded by the carboxyl group of Asp397, the hydroxyl group of Thr332, and main-chain carbonyl groups of Thr332, Arg395 and Val399 (Extended Data Fig. 2d). The TMD of DUOX1 contains seven transmembrane segments (S0–S6): S0 has a kink in the middle, and S1–S6 form a ferric oxidoreductase domain (Fig. 1b). The other cation binding site is located in the same position where the calcium-binding site of DUOXA1 is surrounded by the carboxyl group of Asp397, the hydroxyl group of Thr332, and main-chain carbonyl groups of Thr332, Arg395 and Val399 (Extended Data Fig. 2d).

The TM3 of DUOX1 interacts with TM3 and TM4 of DUOXA1, mainly through hydrophobic interactions (Fig. 2). Four intramolecular disulfide bonds are observed: Cys118-Cys1165, Cys345-Cys565 and Cys364-Cys579 of DUOX1 and Cys167-Cys234 of DUOXA1 (Extended Data Fig. 1d). The Cys118-Cys165 pair has been reported, but previously proposed intramolecular disulfide bridges, including the one involving Cys579 of DUOX1 that would substantially affect the trafficking and H2O2-generating activity of DUOX1–DUOXA1 complexes, were not observed in the current structure. Instead, Cys579 is spatially distant from the DUOXA1 subunit and forms an intramolecular disulfide bond with Cys364. It remains puzzling why mutation of Cys579 but not Cys364 has a marked functional consequence.

Heme- and FAD-binding sites. The structure of the DUOX1–DUOXA1 complex revealed all the vital small molecules (two hemes and one FAD) involved in electron transfer except NADPH. Two heme groups were observed in the TMD of DUOX1.

Fig. 2 Interaction between DUOX1 and DUOXA1. Three major interfaces between DUOX1 and DUOXA1 are magnified and shown in the dashed boxes. Left: interface 1, between extracellular domains with two 170° rotational views. Q30 is shown as a red sphere. Top right: interface 2 at the extracellular side of the cell membrane. Bottom right: interface 3 at the cytosolic side of the cell membrane.
They are positioned almost orthogonally to the lipid bilayer and sandwiched within four continuous transmembrane helices S2–S5. A similar binding mode was also seen in different ferric oxidoreductases (Extended Data Fig. 3b)\textsuperscript{23,27,28}. The heme group on the extracellular side (HEME #1) is coordinated between His\textsubscript{1144} of S3 and His\textsubscript{1238} of S5, whereas the other one on the cytoplasmic side (HEME #2) is sandwiched between His\textsubscript{1130} of S3 and His\textsubscript{1225} of S5 (Fig. 3b). His\textsubscript{1225} is part of a 310 helix within S5, which is probably a conserved feature and has also been observed in the \textit{csNOX5} structure\textsuperscript{23}. The metal-to-metal distance of the two hemes is \( \approx 20.6 \text{Å} \) and the shortest interatomic distance is \( \approx 8.9 \text{Å} \) (Fig. 3b).

Cryo-EM density consistent with a FAD molecule was identified in a positively charged pocket at the interface between the transmembrane and cytoplasmic domains (Fig. 3c,d and Extended Data Fig. 3c). On the cytoplasmic side, the FAD-binding site is mainly formed by residues from the FBD (Fig. 3d). On the TMD side, the FAD resides in a pocket composed of the S2-S3 linker, the S4-S5 linker and HEME #2 (Fig. 3d).

**NADPH-binding site and the electron transfer pathway.** To map the complete electron transfer pathway, the cryo-EM structure of the mouse DUOX1–DUOXA1 complex in the presence of NADPH was determined at an overall resolution of 3.3 Å (Table 1). The map shows a potential density corresponding to an NADPH molecule in the NBD (Fig. 4a and Extended Data Fig. 4a). This extra density permits modeling of the ‘phosphor-ADP-ribose’ part of NADPH, while there is little density for the nicotinamide group (Fig. 4a and Extended Data Fig. 4b,c).

Binding of NADPH involves both the TMD and cytoplasmic NBD of DUOX1 (Fig. 4a,b). The NBD adopts a ‘Rossmann fold’ characterized by an alternating motif of \( \beta\alpha\beta \) secondary structures (Extended Data Fig. 4d)\textsuperscript{29}. A conserved ‘GXGXG’ motif is located in the tight loop connecting the first \( \beta \)-strand and \( \alpha \)-helix, lying under the diphosphate group of the NADPH (Extended Data Fig. 4d). On the membrane side, a lipid molecule with well-resolved density mediates the interaction between the TMD and the phospho-ADP-ribose part of NADPH (Fig. 4a,b and Extended Data Fig. 4c). Without knowing the lipid identity, a phosphatidylcholine (PC) molecule, the most abundant lipid in cell membranes, was modeled into the cryo-EM density (Fig. 4a,b). One alkyl chain of the lipid runs along with the S1 of DUOX1 and the other inserts into the gap between S1 and S3, lying on the top of the membrane.
S2-S3 linker (Extended Data Fig. 4e). Intriguingly, two alkyl chains have also been observed in the csNOX5 structure in similar locations, suggesting an evolutionarily conserved lipid-binding pocket (Extended Data Fig. 4e)\(^2\). The choline group of the lipid molecule points towards NADPH and directly interacts with its ribose group (Fig. 4b and Extended Data Fig. 4c).

The electron transfer path from the cytoplasmic NADPH to HEME #1 was next explored using the Pathways plugin in VMD\(^3\). One possible electron transfer pathway was identified as follows: NADPH $\rightarrow$ FAD $\rightarrow$ HEME #2 $\rightarrow$ Phe1097 $\rightarrow$ HEME #1. Accordingly, mutations of Phe1097 reduced H$_2$O$_2$ production (Extended Data Fig. 4f). A similar path was proposed in the csNOX5 study\(^2\) based on a structural model that was generated using two separately solved crystal structures of transmembrane (PDB 5O0T) and cytoplasmic (PDB 5O0X) domains of csNOX5. Therefore, a quantitative analysis of the complete electron transfer path was not possible.

Here, by presenting the full-length structure of DUOX1, the position of structural elements, as well as the distances between them in the electron transfer path, are mapped in atomic detail (Fig. 4c). As such, a complete picture depicting cross-membrane electron transfer from cytosolic NADPH to a specific oxygen-binding cavity is presented at the molecular level (Fig. 4c). Of note, the distance between the NADPH and FAD (~7.8 Å) was measured in the absence of the nicotinamide group of NADPH. I believe this distance would be much shorter and in the range of electron transfer if a complete NADPH were present.

Oxygen-reacting center and potential O$_2$ entering/H$_2$O$_2$ exiting paths were then analyzed. Similar to csNOX5\(^2\), I was able to map a potential oxygen-binding pocket lined by His1144, His1148, Arg1087 and the propionate group of HEME #1 (Extended Data Fig. 3d). In DUOX1, the presence of the long S3-S4 linker largely restricted O$_2$ entry or H$_2$O$_2$ exit. Using the oxygen-binding cavity as a starting point, I explored the possible O$_2$ entering/H$_2$O$_2$ exiting paths using a probe radius of 1 Å and revealed two possible routes\(^3\) (Fig. 4d and Extended Data Fig. 3e). One path (P1) is surrounded by the S1-S2 linker, S2, the S3-S4 linker and the S5-S6 linker, while the other path (P2) goes through the tunnel shaped by the S3-S4 linker, S4 and the S5-S6 linker. Closer examination of both paths suggests that P1 is more likely to be physiologically relevant for the following reasons: first, the exit of P1, not P2, passes through the S1-S2 linker—a structural motif important for substrate release—mutations in which have been shown to affect the release of ROS by altering product specificity\(^3\) (Fig. 4d); second, the exit of P2 is partially blocked by the membrane bilayer (Extended Data Fig. 3e); finally,

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**Fig. 4 | The NADPH-binding site and electron transfer path.**
- **a**, The density of NADPH. Densities of NADPH and a nearby lipid molecule are shown. The FAD, NADPH and lipid molecules are shown as sticks and balls and colored in magenta, cyan and yellow, respectively.
- **b**, A lipid-mediated NADPH-binding site. Left: the NADPH-binding site. Right: the lipid-binding site. The NADPH and lipid are colored in cyan and yellow, respectively. The side chains of residues surrounding the NADPH and lipid molecules within 4 Å and the last residue of DUOX1 (F1551) are shown as sticks and balls.
- **c**, The electron transfer path. Left: calculated distances between players in the electron transfer path. Right: a model of electron transfer and the H$_2$O$_2$-production reaction.
- **d**, The potential O$_2$ entering and H$_2$O$_2$ exiting pathway (P1). Paths were calculated using the Caver 3.0.3 plugin in PyMOL.
the tunnel lengths from the proposed oxygen-binding site to extracellular solvent are calculated to be 13.4 Å and 22.0 Å for P1 and P2, respectively, suggesting that P1 is a shorter path for substrate entry and product release with lower energy cost32.

Structure of DUOX1–DUOXA1 in a dimer-of-dimers configuration. A structure of DUOX1–DUOXA1 in a dimer-of-dimers configuration was determined at an overall resolution of 2.7 Å (Extended Data Fig. 1b and Table 1). The cryo-EM map permits modeling of extracellular domains and TMDs, while cytoplasmic domains were too flexible to be resolved (Fig. 5a and Supplementary Video 1). Structurally, DUOX1 in the dimer-of-dimers state shows two major differences: first, the cytoplasmic domain is flexible; second, the TMD displaces by about 6° (Extended Data Fig. 5a), a movement that could potentially disrupt the interface between TM1–TM2 and TM3 of DUOX1 and TM1 of DUOXA1, all of which are structural motifs that contribute to the formation of the DUOX1–DUOXA1 dimer of dimers (Extended Data Fig. 5b).

The dimer-of-dimers state adopts an X–A–X′–A′ arrangement with a two-fold symmetry axis running perpendicular to the cell membrane (Fig. 5a). Here, X–A or X′–A′ stands for one DUOX1–DUOXA1 heterodimer. A new interface between DUOX1 and DUOXA1 subunits is formed by multiple structural motifs involving protein, lipid and glycans. First, the N-terminal extracellular loop of DUOX1 and DUOXA1, which was disordered in the DUOX1–DUOXA1 heterodimer, wraps around the ‘neck’ of DUOX1 (the region between the PHD and TMD; Fig. 5b,c and Extended Data Fig. 5c). Second, there are two interfaces in the transmembrane region: one between the TM1–TM2 linker of DUOX1 and the amphipathic helix priming S1 of DUOX1 and the other between TM1 of DUOXA1 and S1 of DUOX1 mediated by a lipid molecule (Extended Data Fig. 5d). Again, a PC molecule was modeled here in the cryo-EM map without knowing the lipid identity. Third, linkers connecting β1–β2, β3–β4 and H1–H2 of DUOX1 are touching the PHD of DUOX1 (Fig. 5c). The N-acetylgalactosamine modification on Asn121 of the β3–β4 linker is also located on the interface (Fig. 5c). Finally, the glycans chain linked to Asn109 of DUOXA1, which displays a high-mannose linkage with 11 sugar moieties, inserts into the neck region of DUOX1 (Fig. 5c and Extended Data Fig. 5c). Furthermore, this long glycans chain is localized in a position where it bridges together the N-terminal loop, the lipid molecule and the β3–β4 linker of DUOX1, all of which are structural motifs that contribute to the DUOX1–DUOXA1 (X–A′ or X′–A) interaction (Fig. 5c).

In addition to the interface between DUOX1 and DUOXA1, there are direct interactions between the PHDs of DUOX1 (Extended Data Fig. 5e). The PHDs of DUOX1 form a homodimer resembling the dimeric myeloperoxidase (MPO, PDB 1MYP) with an inter-subunit rotation of ~40° (Extended Data Fig. 5e)14.

A closer examination of the dimer-of-dimers configuration of the DUOX1–DUOXA1 complex strongly indicates that this conformation represents an inactive state of the enzyme complex. This hypothesis is supported by the following observations. First, cytoplasmic domains are flexible and not primed for electron transfer (Fig. 5a and Supplementary Video 1). Second, the docking of two DUOX1–DUOXA1 heterodimers into the dimer-of-dimers structure will cause steric clashes between NBDs and between the NBD and FBD (Extended Data Fig. 6a). Third, formation of the dimer-of-dimers complex creates an almost completely sealed solvent cavity, which encapsulates the potential O3 entry/H2O2 out.
An activity assay was also carried out to examine the function of purified DUOX1–DUOXA1 complexes in different states. The assay monitors the fluorescence reduction coupled to NADPH oxidation and H₂O₂ production. As the two states could not be cleanly separated, DUOX1–DUOXA1 complexes from three different fractions of the size exclusion chromatography were used in the assay (Fig. 6a). Each fraction is a mixture of DUOX1–DUOXA1 in both heterodimeric and dimer-of-dimers states—the earlier the elution fraction, the higher the population of the dimer-of-dimers complexes. The resulting data showed that protein sample from earlier fractions was less active than later fractions when the same amount of DUOX1–DUOXA1 protein was used (Fig. 6a), supporting the hypothesis that the dimer-of-dimers configuration represents an inactive state of the enzyme complex.

Discussion
In this study, I have determined cryo-EM structures of the DUOX1–DUOXA1 complex in both heterodimeric and dimer-of-dimers states. Structural and biochemical analyses led to the hypothesis posing an additional regulatory mechanism on the DUOX1–DUOXA1 complex through protein oligomerization: activation of DUOX1–DUOXA1 undergoes conformational changes from an inactive dimer-of-dimers state to a heterodimer active state (Fig. 6b). In the inactive dimer-of-dimers state, the cytosolic domain of DUOX1 is flexible and not primed for electron transfer. In addition, the potential O₂ entering/H₂O₂ exiting path (P1 in Fig. 4d) is embedded in a large enclosed cavity. In the active heterodimeric state, the FBD and NBD of DUOX1 dock onto the TMD, which rotates ~6° away from the dimer-of-dimers interface, and the potential O₂ entering/H₂O₂ exiting path (P1) is fully exposed to the extracellular solvent. In addition, the N-terminal loop and TM1 of DUOXA1, which play a key role in dimer-of-dimers formation, become structurally less constrained. As DUOX1–DUOXA1 and DUOX2–DUOXA2 share high sequence and structural similarities, it is likely that such a regulation will also be relevant for the DUOX2–DUOXA2 complex. Indeed, an equilibrium between the DUOX2–DUOXA2 heterodimer and the dimer-of-dimers has also been observed using fluorescence detection size exclusion chromatography (FSEC) (Extended Data Fig. 6c).

Although the question regarding what controls the transition between the DUOX1–DUOXA1 heterodimer and the dimer-of-dimers remains to be answered, the observation that FAD and NADPH are both located at the interface between the TMD and the cytosolic domain (flexible in the dimer-of-dimers state) suggests that one or both of the two small molecules may function as ‘molecular glue’ (Fig. 3d and Fig. 4a,c). Binding of FAD, NADPH or both could contribute to the docking of cytosolic domains onto the TMDs of DUOX1 and facilitate the transition from dimer-of-dimers to heterodimeric state. Interestingly, the presence of excess NADPH largely increases the heterodimer to dimer-of-dimers ratio on FSEC (Extended Data Fig. 6d), indicating that NADPH could potentially play a role in regulating equilibrium between different oligomerization states of DUOX1–DUOXA1.
DUOX1–DUOXA1 and DUOX2–DUOXA2 differ from the other NOXs (except NOX4), because DUOX–DUOXA complexes generate H$_2$O$_2$ instead of superoxide (O$_2^-$) at the apical surface of epithelial cells. Compared with sNOX5, DUOX1–DUOXA1 has a similar electron transfer path, suggesting there are no notable differences during superoxide production. Therefore, it is likely that a dismutation reaction occurs in DUOX–DUOXA complexes to generate H$_2$O$_2$. My structural comparison has revealed that HEME #1 and the oxygen-reaction center of DUOX1–DUOXA1 are enclosed by the S1-S2, S3-S4 and S5-S6 linkers and thus have limited access to the extracellular space (Fig. 4d and Extended Data Fig. 3a,e). In contrast, the extracellular heme and oxygen-reaction center of sNOX5 are exposed (Extended Data Fig. 6f). Furthermore, the internal cavity formed by extracellular loops is highly positively charged (Extended Data Fig. 6g), creating an environment that could hinder the release of O$_2^-$ and facilitate dismutation reactions. The PHD could potentially contribute to H$_2$O$_2$ production by stabilizing the S3-S4 linker through a disulfide bond and by maintaining the structural integrity of the internal cavity needed for the dismutation reaction. This is consistent with the previous observation that the proper modification and folding of the PHD of DUOXS are important for specific production of H$_2$O$_2$ (refs. 8, 9). The S1-S2 linker, a key structural determinant in the conversion of superoxide to H$_2$O$_2$, is located at the exit of the H$_2$O$_2$-escaping path (P1). It is plausible that the histidine-containing S1-S2 linker functions as a ‘gatekeeper’ to tightly control H$_2$O$_2$ release, and perturbation of the S1-S2 linker leads to O$_2^-$ leakage

In summary, I have determined the cryo-EM structures of the DUOX1–DUOXA1 complex in three different states: an NADPH-free state, an NADPH-bound state and a dimer-of-dimers state. These structures uncovered the electron transfer path within an NADPH oxidase and revealed the structural mechanism underlying DUOX1–DUOXA1 regulation through protein oligomerization. Therefore, this work allows the mechanistic interpretation of mutations in DUOX–DUOXA associated with diseases (Extended Data Fig. 6e) and provides a structural foundation for novel drug development strategies by manipulating the oligomerization state of the enzyme complex.

Online content
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Methods

Cloning, expression and purification of DUOX1–DUOX1A. Complementary DNAs (cDNAs) encoding mouse DUOX1 (Mus musculus: NP_001092767.1) and DUOX1A (Mus musculus: NP_001292191.1) were purchased from Invitrogen. The N-terminal signal peptide region (1–19) was truncated and replaced by an interleukin-2 signal peptide linked GFP tag, and a preScission protease cleavage site was also engineered between the GFP tag and DUOX1A. DUOX1A was cloned into a BacMam expression vector without any tags (Extended Data Fig. 1a).

Recombinant baculoviruses of DUOX1 and DUOX1A were generated separately using the Bac-to-Bac system according to the manufacturer’s instructions (Invitrogen). A P3 virus mixture of DUOX1 and DUOXA1 (1:1) was used. Recombinant baculoviruses were amplified separately using the Bac-to-Bac system according to the manufacturer’s instructions (Invitrogen) and used for transfection of HEK293S GNTI cells (Invitrogen). A P3 virus mixture of DUOX1 and DUOXA1 (1:1) was used.

Fourier cropping, and corrected for beam-induced motion using MotionCor2. Images of all datasets were recorded with a SerialEM system in super-resolution mode with a super-resolution pixel size of 0.54 Å and a defocus range of −1.1 to −2.0 μm. Data without NADPH collection were acquired at a dose rate of 1.15 e− per Å−2 per frame, and images were recorded during a 3-s exposure with 50-ms subframes (60 total frames). The dataset with NADPH collection was acquired at a dose rate of 1.15 e− per Å−2 per frame, and images were recorded during a 4.9 s exposure with 70-ms subframes (70 total frames).

Super-resolution image stacks were gain-normalized, binned by 2 with Fourier cropping, and corrected for beam-induced motion using MotionCor2. Contrast transfer function parameters were estimated from motion-corrected summed images, without dose weighting, using GCTFF. All subsequent processing was performed on motion-corrected summed images with dose weighting. About 5,000 particles were manually picked and processed with reference-free two-dimensional (2D) classification in RELION to generate a template for auto-picking. Auto-picked particles were extracted in RELION and were further cleaned up by multiple rounds of 2D classification in cryoSPARC.

For 3D reconstruction, an initial reconstruction was carried out in cryoSPARC asking for three classes, which resulted in one DUOX1–DUOX1A heterodimer class, one DUOX1–DUOX1A dimer-of-dimers class and one ‘trash’ class. Multiple rounds of heterogeneous refinement were then carried out against these three classes to remove bad particles followed by CTF refinement and non-uniform refinement in cryoSPARC.

Model building and refinement. Models were built in Coot. First, homology models of the PDB and TMD of DUOX1 were generated using the I-TASSER server and docked into the cryo-EM map. From this starting point, manual building was carried out. The structural model was iteratively refined using phenix.real_space_refine with secondary structure restraints and Coot. Protein structure quality was monitored using the MolProbity server. The possible O−, entering and H2O, exiting paths were calculated using the Caver plugin in PyMOL, and the electron transfer path was predicted using the Pathways plugin in VMD. Figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger) and UCSF Chimera.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this Article.

Data availability

Cryo-EM maps and atomic models for mouse DUOX1–DUOX1A complexes have been deposited in the EMDB and wwPDB with the following accession numbers: EMDB-21962 and PDB 6WXR (apo state); EMDB-21963 and PDB 6WXU (dimer of dimers); EMDB-21964 and PDB 6WXV (with NADPH).

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Author contributions
J.S. designed and performed all the experiments, analyzed the results and prepared the manuscript.

Competing interests
The author declares no competing interests.

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Supplementary information is available for this paper at https://doi.org/10.1038/s41594-020-0501-x.
Correspondence and requests for materials should be addressed to J.S.
Peer review information Inês Chen was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
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Extended Data Fig. 1 | Structure determination of the DUOX1–DUOXA1 complex. a, Construct design of DUOX1 and DUOXA1 used for structural studies and size exclusion chromatography profile of the DUOX1–DUOXA1 complex. Fractions of second peak (PK2) in the red box are concentrated and used for single-particle analysis. b, Flowchart of DUOX1–DUOXA1 structure determination. The steps in blue are carried out in cryoSPARC, ones in green in RELION. In the 2D classification, dimer-of-dimer classes are indicated by red dashed cycles. c, Interaction between the S3-S4 linker and PHD of DUOX1. The outer leaflet of the membrane bilayer is indicated by a gray line. d, Disulfide bonds and glycosylation sites of the DUOX1–DUOXA1 complex. Sugars and their linked Asn side chains are shown as sticks and balls. Disulfide bonds are colored in green.
Extended Data Fig. 2 | Structure of the PHD of DUOX1. **a**, Superimposition between LPO and PHD of DUOX1. LPO and DUOX1 are colored in gray and blue, respectively. **b**, Putative heme-binding site. The possible heme-binding pocket indicated by a red oval. Positions of Ser326 and Ser108 (colored in brown) are where the heme-coordinating histidines are located. **c**, The putative calcium-binding site and surrounding residues. Calcium is indicated by a green sphere. **d**, A potential ion binding site and surrounding residues. Cryo-EM density is contoured by gray meshes.
Extended Data Fig. 3 | Heme- and FAD-binding sites. a, Structural comparison of heme-binding sites between DUOX1 (blue) and csNOX5 (gray). The transmembrane helices are labeled with S1–S6. b, Structural conservation of heme coordination in ferric oxidoreductases. DUOX1, csNOX5, Cyto b561 and Dcytb are colored in blue, gray, light blue and cyan, respectively. c, FAD-binding site in 2D representation. Hydrogen bonds, hydrophobic interactions and cation-π interactions are indicated by dashes, spokes and vertical dashes, respectively. Residues from FBD, NBD, TMD are colored in light pink, gray and blue, respectively. d, The putative oxygen-binding site. Oxygen is represented by a dashed red oval. e, The possible oxygen entering and hydrogen peroxide exiting paths.
Extended Data Fig. 4 | NADPH-binding site. a, Cryo-EM density of DUOX1–DUOXA1 with and without NADPH. The potential NADPH-binding site is indicated by red dashes. b, Structure of an NADPH molecule. c, The NADPH-binding site. Residues from TMD and NBD are colored in blue and gray, respectively. The “invisible” nicotinamide group is cycled in a cyan dashed oval. d, Cartoon and structure of the NADPH-binding site. The conserved glycines on the “GXGXG” motif are shown as magenta balls. e, The lipid-binding pocket of csNOX5 and DUOX1. Lipid or alkyl chains are colored in red. f, Functional analyses of F1097 mutations. F1097 is mutated to Tyr, Ala, Ile and Val, and the activity of the mutations are normalized to the wild-type protein (Data shown are mean and s.d. of n = 4 independent experiments).
Extended Data Fig. 5  |  Formation of the dimer-of-dimer interface.  a, b, Structural comparison of DUOX1 and DUOXA1 in heterodimeric and dimer-of-dimer states.  c, Interaction between DUOX1 and DUOXA1. Left: interactions between DUOX1 and the N-terminal loop and glycan chain linked to N109 of DUOXA1. Right: cryo-EM density and cartoon of the glycan chain on N109.  d, Interactions between transmembrane domains of DUOX1 and DUOXA1 mediated by a lipid molecule. Left: interaction details. Right: density of the lipid molecule.  e, PHD-PHD interactions in the dimer-of-dimer configuration. Left: interactions between PHDs of DUOX1. Right: comparison between MPO dimers and PHD dimers of DUOX1.
Extended Data Fig. 6 | The interface between DUOX1–DUOX1 heterodimers.  
a, Modeling of two DUOX1–DUOX1 dimers into the dimer-of-dimer state. Structural crashes are indicated by red arrows.  
b, The potential oxygen entering/hydrogen peroxide exiting paths in the DUOX1–DUOX1 dimer of dimers.  
c, FSEC curves of mouse DUOX1–DUOX1 (blue), human DUOX1–DUOX1 (gray) and human DUOX2–DUOX2 (orange).  
d, FSEC curves of mouse DUOX1–DUOX1 (gray), mouse DUOX1–DUOX1 with NADPH (green) and mouse DUOX1–DUOX1 with FAD (orange).  
e, Modeling of DUOX2–DUOX2 complex and mapping of the hypothyroidism disease mutations.  
f, Accessibility of the outer heme of csNOX5 to extracellular space. The heme molecule is colored in green, indicated by a red arrow. The csNOX5 is shown as gray surface.  
g, The positively charged environment surrounding the heme molecule (heme #1).
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about: availability of computer code

Data collection
Cryo-EM data collection was carried out using SerialEM

Data analysis
Cryo-EM data analysis was done in cryoSPARC (version 2.14) and Relion (version 3.1). Model was built in Coot (version 0.8.9.2) and refined using Phenix (1.18.1)

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CryoEM structures and atomic models are deposited in PDB and EMDB as follows:
DUOX1-DUOX1A1 in the absence of NADPH: EMD-21962, PDB ID 6WXR
DUOX1-DUOX1A1 in the dimer-of-dimer state: EMD-21963, PDB ID 6WXU
DUOX1-DUOX1A1 in the presence of NADPH: EMD-21964, PDB ID 6WXV
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to predetermine sample sizes. |
|-------------|------------------------------------------------------------------|
| Data exclusions | No exclusion criteria were pre-established. No data was excluded from the activity assays |
| Replication | All attempts to replicate the experiments were successful |
| Randomization | This is not relevant for our study because we were not comparing two sets of individuals who had been selected as control or sample group |
| Blinding | As we were not comparing animal or human populations with individuals assigned to groups, it was not relevant to do double blind tests |

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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| Eukaryotic cell lines | |
| Palaeontology and archaeology | |
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| Human research participants | |
| Clinical data | |
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| Methods | Involved in the study |
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Cell line source(s)

- HEK293S GnTI- (ATCC® CRL-3022) HEK cells
- Spodoptera frugiperda (SF9) cells (Thermo-Fisher Scientific, Cat. No. 11496015)

Authentication

The cell lines used are commercially available and monitored by regular visual inspection

Mycoplasma contamination

The HEK and SF9 cells were tested for mycoplasma contamination by the manufacturer.

Commonly misidentified lines

No commonly misidentified cell lines were used.