DUOX Defects and Their Roles in Congenital Hypothyroidism

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Abstract

Extracellular hydrogen peroxide is required for thyroperoxidase-mediated thyroid hormone synthesis in the follicular lumen of the thyroid gland. Among the NADPH oxidases, dual oxidases, DUOX1 and DUOX2, constitute a distinct subfamily initially identified as thyroid oxidases, based on their level of expression in the thyroid. Despite their high sequence similarity, the two isoforms present distinct regulations, tissue expression, and catalytic functions. Inactivating mutations in many of the genes involved in thyroid hormone synthesis cause thyroid dyshormonogenesis associated with iodide organification defect. This chapter provides an overview of the genetic alterations in *DUOX2* and its maturation factor, *DUOXA2*, causing inherited severe hypothyroidism that clearly demonstrate the physiological implication of this oxidase in thyroid hormonogenesis. Mutations in the *DUOX2* gene have been described in permanent but also in transient forms of congenital hypothyroidism. Moreover, accumulating evidence demonstrates that the high phenotypic variability associated with altered DUOX2 function is not directly related to the number of inactivated *DUOX2* alleles, suggesting the existence of other pathophysiological factors. The presence of two DUOX isoforms and their corresponding maturation factors in the same organ could certainly constitute an efficient redundant mechanism to maintain sufficient H_2O_2 supply for iodide organification. Many of the reported *DUOX2* missense variants have not been functionally characterized, their clinical impact in the observed phenotype remaining unresolved, especially in mild transient congenital hypothyroidism. DUOX2 function should be carefully evaluated using an in vitro assay wherein (1) DUOXA2 is co-expressed, (2) H_2O_2 production is activated, (3) and DUOX2 membrane expression is precisely analyzed.

Key words NADPH oxidase, Thyroid, Congenital hypothyroidism, DUOX, DUOXA, H_2O_2, Dual oxidase, DUOX maturation factor, Inherited disease

1 Introduction

In 1908, a huge oxidative burst was reported upon sea urchin egg fertilization [1]. Hydrogen peroxide (H_2O_2) produced during this process has been shown later to mediate the formation of covalent dityrosine bounds by the ovoperoxidase in the extracellular matrix protecting the egg from polyspermy [2]. The discovery in 1986 of the molecular nature of the phagocyte oxidase, NOX2/gp91[phox], responsible for the "respiratory burst" [3] revealed that molecular complexes present from fungi to mammals have been selected to

produce ROS dedicated to specialized cellular functions in a variety of subcellular compartments [4]. The existence of a thyroid H_2O_2-generating system was postulated in the early seventies to be necessary for thyroid hormone (TH) synthesis by the enzyme thyroperoxidase (TPO) [5]. Further biochemical studies demonstrated that H_2O_2 was produced in the thyroid follicular space by a membrane-bound NADPH-dependent flavoprotein using calcium ions to be fully activated [6, 7]. Thirty years later, the molecular nature of the entity responsible of the thyroid H_2O_2-generating system was discovered by two independent groups following different strategies. Starting from purified membranes from pig thyroid follicles, the group of C. Dupuy isolated the p138^{Tox} corresponding to the dual oxidase DUOX2 lacking the first 338 amino acids [8]. Meanwhile, based on functional similarities with NOX2, our group cloned two cDNAs coding for the complete sequences of DUOX1 and DUOX2 after the molecular screening of cDNA libraries generated from human thyrocytes in primary culture [9]. These new members of the NOX family present, respectively, 53% and 47% similarities with the NOX2 catalytic core, including the six transmembrane regions harboring the four histidines and the arginine involved in the heme binding and the COOH intracellular extremity containing the FAD- and NADPH-binding sites characteristic of the NADPH oxidase enzymes. The primary structure of the DUOX proteins (1551 and 1548 amino acids for DUOX1 and DUOX2) is extended at the NH_2-extremity of the conserved catalytic domain by an intracellular loop containing two EF-hand motifs and an additional transmembrane segment followed by an extracellular peroxidase homology domain (PHD) presenting 43% similarities with TPO. The final demonstration of DUOX NADPH oxidase activity has to wait six more additional years and the discovery of the DUOX maturation factors, DUOXA1 and DUOXA2, allowing the reconstitution of a DUOX-based functional H_2O_2-generating complex in heterologous cell systems [10]. Up to now, DUOX/DUOXA expression has been documented in a growing list of non-thyroid tissues, among which the salivary glands, the airways, and the intestinal tract, revealing additional cellular functions associated with DUOX-related H_2O_2 generation [11, 12]. In the present review, we will mainly focus on the role of hydrogen peroxide and DUOXs in thyroid function and their implication in inherited congenital hypothyroidism (CH).

2   The Thyroid Gland

2.1   The Follicular Structure

During evolution, the thyroid function emerges with the capacity to concentrate iodide and to synthesize iodoproteins. Multiple tissues of insects can accumulate radioiodide, but instead of TH synthesis, these iodo-compounds mostly reflect by-products

generated during the cuticle formation [13]. An important evolutionary event was the development of iodination units in the endostyle of protochordate species presenting a peroxidase activity [14]. Most vertebrates possess a thyroid able to generate iodothyronines. The thyroid architecture is characterized by follicular units dispersed along the ventral aorta (between the first gill arch and the bulbus arteriosus) like in the zebrafish [15] or encapsulated in a compact glandular structure often divided in two lobes like in mammals. These thyroid functional units are composed of a monolayer of cuboidal cells, the thyrocytes, surrounding a colloidal lumen full of thyroglobulin (TG), the backbone of THs. The polarized organization of the thyroid follicles is critical for iodide concentration and TH storage as iodinated TG. These ovoid 3D structures are embedded in a dense network of blood capillaries allowing intense metabolite exchanges with the thyrocytes: iodide uptake and TH secretion [16]. Thyroid hormones play major roles in the regulation of multiple biological processes including development, growth, and metabolism [17]. Their critical role in embryogenesis is conserved in all vertebrates, especially for neuronal and skeleton development.

Dietary iodine is reduced into iodide before its absorption in the small intestine. About 20% of the iodide perfusing the thyroid is removed at each passage by the basolateral sodium/iodide symporter protein (NIS) allowing iodide to be concentrated 20–50 times in the thyroid (Fig. 1) [18]. However, under sufficient iodine intake, 90% of ingested iodide is lost in urine excretion [19]. To maintain a normal thyroid function, the recommended daily intake of iodine is around 150 μg in human [20]. However, iodine deficiency still remains a worldwide health problem promoting the development of multinodular goiters and thyroid nodules and, in case of severe iodine deficiency, causing hypothyroidism with mental retardation and cretinism. The main sources of iodine are seafood products, iodized salts, and bakery products [21, 22]. After NIS-mediated active transport from the blood, iodide is passively transported across the apical membrane in the follicular lumen. The transmembrane protein anion exchanger pendrin that exchanges chloride for bicarbonate, iodide, or thiocyanate (SCN^-) has been suggested to be involved in this transport [23]. Pendrin protein is expressed not only in the thyroid but also in other tissues, including the kidney, the airways, the mammary gland, and the inner ear. Pendred syndrome, an autosomal recessive disorder, is mainly characterized by deafness, but some patients also suffer from hypothyroidism. However, no thyroid phenotype has been reported in the pendrin knockout mice [24]. Recently, the calcium-activated chloride channel, anoctamin-1, has been demonstrated to mediate iodide efflux across the apical membrane of thyrocytes [25].
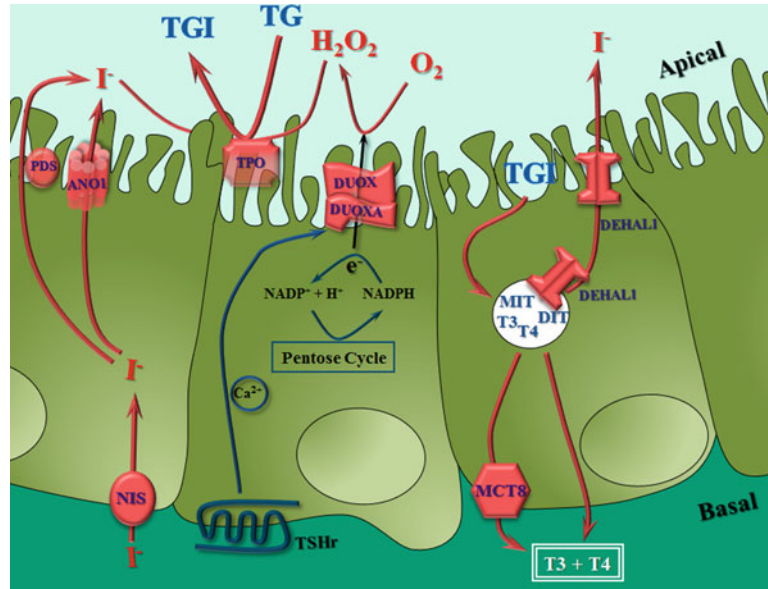
Fig. 1 Thyroid hormone synthesis. At the basal pole of thyrocytes, iodide uptake from the blood is mediated by the symporter NIS. Iodide is transported in the follicular lumen via the iodide channel anoctamin-1 (ANO1) and the anion exchanger pendrin (PDS). At the apex, thyroperoxidase (TPO) catalyzes iodide oxidation and coupling to tyrosine residues of thyroglobulin (TG) in the presence of H_2O_2 generated by DUOX/DUOXA complex. After endocytosis, thyroid hormones (T3 and T4) are released from iodinated TG (TGI) by proteolytic cleavage. Iodotyrosines (MITs and DITs) are deiodinated to recycle iodine by the iodotyrosine dehalogenase (DEHAL1). Thyroid hormones are secreted in the blood via dedicated transporters like the monocarboxylate transporter 8 (MCT8). The thyroid function is under the control of thyrotropin via its protein G-coupled receptor (TSHr)

2.2 Thyroid Hormone Biosynthesis

In the follicular lumen, iodide is oxidized and covalently linked to tyrosine residues of the macromolecule thyroglobulin [5]. This so-called organification process is catalyzed by the transmembrane thyroperoxidase at the apex of the thyrocytes in the presence of H_2O_2 generated by DUOX (Fig. 1). A close proximity between DUOX and TPO has been demonstrated at the plasma membrane of the follicular cells [26, 27]. This apical membrane complex named thyroxisome favors the hormonogenesis and limits H_2O_2 leakage [28]. Under sufficient iodine supply, the amount of H_2O_2 produced constitutes the limiting factor for TH synthesis [29]. The 933 residues of TPO present 44% sequence similarity with the myeloperoxidase and is composed of a short intracellular COOH-extremity, one transmembrane region, and a long catalytic ectodomain containing the heme moiety [30]. The final step of TH biosynthesis consists of the TPO-mediated coupling reactions. The assembly of two diiodotyrosines (DIT) forms the

3,5,3′,5′-tetraiodothyronine or thyroxin (T4), while the coupling of one monoiodotyrosine (MIT) with one DIT generates the 3,5,3′-triiodothyronine (T3), the active form of THs [31].

TG is the most abundant protein in the thyroid at a concentration of 200–300 mg/mL in the follicular lumen. Its main function is to provide the polypeptide backbone for TH synthesis, as well as TH storage and iodine depot when iodine availability is limited. The TG transcript encodes a protein of 2767 amino acids containing numerous proline and cysteine residues in constant position participating in the secondary structure of the protein via intramolecular disulfide bonds [32]. Mature TG is mainly found as homodimers with a molecular weight of 660 kDa, the carbohydrates comprising about 10% of its weight. Among the 132 tyrosyl residues of TG dimers, only 25–30 participate in the iodination reactions. These hormonogenic residues will be further used for the coupling reactions. One iodophenoxyl group from a MIT or a DIT residue called the "donor" is transferred onto a DIT residue called the "acceptor" [33]. Only 5–16 can be associated to generate 2–8 molecules of T4 and T3 [34]. A typical distribution for a TG containing 0.5% iodine is 5 residues of MIT, 5 of DIT, 2.5 of T4, and 0.7 of T3 [35].

Before being delivered into the bloodstream, THs must be released from TG after its internalization by endocytosis and fusion in lysosomal compartments [36]. Proteolytic cleavage is mediated by multiple glycohydrolases, phosphatases, sulfatases, and proteases including the cathepsins D, H, and L [37]. Nevertheless, about 70% of the TG iodine contents are in the forms of MITs and DITs which are deiodinated to recycle iodine in the intrathyroidal iodide pool. An iodotyrosine dehalogenase (DEHAL1) present at the apical plasma membrane and in endocytic vesicles of the thyrocytes has been characterized [38]. Patients carrying biallelic loss-of-function mutations in the corresponding gene present high levels of MIT/DIT in the urines and may develop a goiter under iodine deficiency [39]. For decades, the lipophilic nature of T3/T4 suggested that TH secretion was mediated mainly by simple diffusion across the basal plasma membrane. However, recent data clearly demonstrated that TH efflux from the thyroid in the blood and their uptake by the targeted cells are mediated by dedicated transporters like the monocarboxylate transporter 8 (MCT8) [40].

Thyroid physiology is mainly controlled by iodide availability and the plasma level of THs. A negative feedback loop controls TH synthesis and secretion via the thyrotropin (TSH) secreted by the pituitary. The TSH receptor (TSHr), a G-protein-coupled receptor, activates in humans two signaling pathways [41]: (1) The cAMP cascade stimulates TH secretion as well as the expression of genes involved in TH synthesis (*NIS, TPO, TG*); and (2) The Gq/phospholipase C cascade activates TH synthesis mainly via the activation of the thyroid H_2O_2-generating system. Under intense and

chronic thyroid stimulation by TSH, thyroid cell proliferation is increased leading to the classical goiter formation. Iodine excess can also rapidly induce an inhibition of thyroid function [42] called the Wolff-Chaikoff effect mediated by an iodo-compound, 2-iodo-hexadecanal, that is able to inhibit H_2O_2 generation, blocking iodide organification [43].

3   The Thyroid H_2O_2-Generating Complex: DUOX/DUOXA

3.1   The DUOX/DUOXA Gene Locus

DUOX and *DUOXA* genes are oriented head to head in an operon-like unit, each couple of genes being located in tandem on the long arm of chromosome 15 (Fig. 2A) and sharing the same bidirectional promoter [10, 44, 45]. *DUOX1* spans 36 kb and is composed of 35 exons, *DUOX2* spans 21.5 kb containing 34 exons, and both genes are composed of 33 coding exons [46]. Interestingly, the length and position of the exons coding for the functional domains (EF-hands, FAD- and NADPH-binding sites) are well conserved between the two genes as well as with *NOX2*, reflecting their common molecular evolution (Fig. 2B) [4]. The DUOXA2 open reading frame spans 6 exons encoding a 320 amino acid protein composed of five transmembrane segments, the first extracellular loop presenting N-glycosylation sites, and a C-terminal cytoplasmic region. Four alternative DUOXA1 splicing variants have been identified. DUOXA1α (343 residues) corresponds to the closest homolog of DUOXA2 and is the major
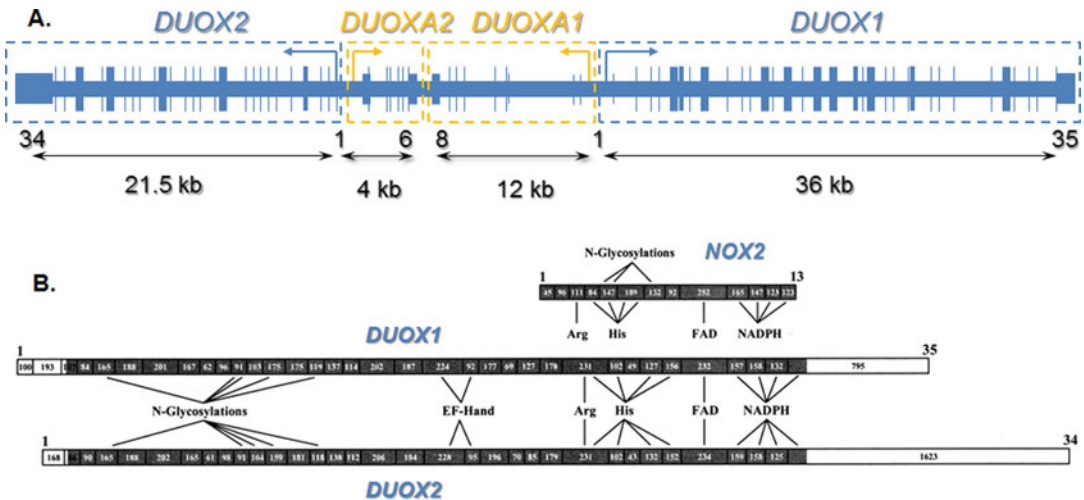
Fig. 2 A. Schematic structure of the genomic organization of the *DUOX/DUOXA* gene locus on chromosome 15q15.3 with the number of exons represented as vertical bars. B. Comparison of the transcripts encoding DUOX1, DUOX2, and NOX2. The length of each exon is mentioned and the corresponding coding region is represented in gray. Exons coding for N-glycosylation sites, EF-hand motifs, and heme-, FAD-, and NADPH-binding sites are indicated

variant in DUOX1/DUOXA1 expressing tissues [47, 48]. Due to their extra-thyroid expression, *DUOX/DUOXA* could not be defined as thyroid-specific genes. However, their expression appears only at the late stages of cell differentiation during thyroid embryogenesis in mice and fish, when the follicular structure is functionally specified making them important thyroid differentiation markers [15, 49]. In human thyroid, the *DUOX2* transcript is 2–5 times more expressed than *DUOX1* [46]. Transcriptional regulation by TSH seems to be species dependent. In dog, rat, and pig, *DUOX2* mRNA expression is positively controlled through the activation of the cAMP pathway [50–52], whereas in mouse and human, no significant modulation of *DUOX* transcription is observed [46, 49, 53].

3.2 Maturation of DUOX Proteins

The oxidases are fully functional when properly addressed at the apical membrane of the thyroid cell. When traveling to the apex, DUOX proteins undergo N-linked glycosylation in the Golgi apparatus to adopt the active 190 kDa form [50, 51]. Complete glycosyl-defective DUOX mutants generated by site-directed mutagenesis demonstrate impairment of cell surface expression and ROS production in reconstituted cellular system [54]. However, inhibition of the Golgi complex α-mannosidase II by swainsonine results in a fully active enzyme targeted at the membrane, demonstrating that the maturation of the N-glycan moieties in the Golgi is dispensable for the function of the oxidases [55]. In the absence of DUOX maturation factors, the oxidases are retained in the endoplasmic reticulum (ER) compartment where only low levels of superoxide ($O_2^{.-}$) are detected [56]. Their critical role for DUOX function has been clearly demonstrated in DUOXA1/DUOXA2 double knockout mice [57] showing a hypothyroid phenotype characterized by the impairment of T4 production in thyroid follicles caused by the absence of DUOX cell surface expression and loss of H_2O_2 generation (Fig. 3; personal communication and [58]). Initially, DUOXA proteins were characterized as ER-resident proteins allowing ER-to-Golgi transition of mature DUOX enzymes [10]. However, accumulating evidence suggests now that they most probably act as organizing elements required for surface expression but also regulation of DUOX activity [47, 48, 59]. Their role can be related to the p22[phox] function for the NOX enzymes.

3.3 Control of DUOX Catalytic Activity

As NOX5, DUOX isoenzymes are obligate Ca^{2+}-dependent NADPH oxidases via the two EF-hand Ca^{2+}-binding motifs [60]. Contrary to the other NOX proteins, Rac1 activation is not required for DUOX-mediated thyroid H_2O_2 generation [61]. The intrinsic activity of DUOX enzymes can be also modulated via direct serine/threonine phosphorylation. In DUOX/DUOXA reconstituted Cos-7 cells, protein kinase A-mediated
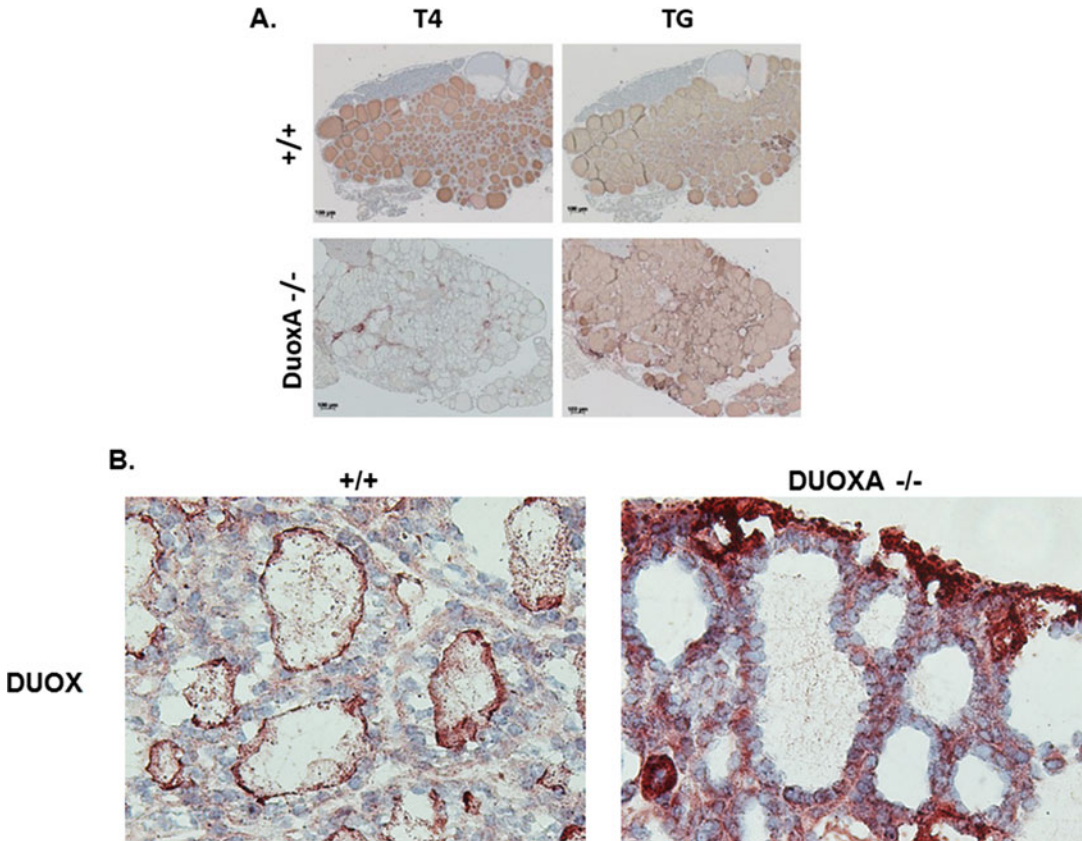
Fig. 3 A. T4 and TG immunostaining on serial thyroid sections (5 μm thick) from wild-type (+/+) and DUOXA-deficient (DUOXA−/−) mice. B. DUOX immunodetection in thyroid sections (5 μm thick) from wild-type and DUOXA −/− mice. DUOX immunostaining was localized at the apical membrane of wild-type mice and in the cytoplasm for DUOXA −/− mice

phosphorylation on serine 955 activates DUOX1, while DUOX2 is stimulated by nanomolar concentrations of phorbol 12-myristate 13-acetate (PMA), associated with protein kinase C-dependent phosphorylation [60]. Primary cultured human thyrocytes and bronchial epithelial cells show an increase in H_2O_2 generation after PMA treatment [60, 62, 63]. Finally, micromolar concentrations of iodide are also able to trigger H_2O_2 generation in human, pig, and dog thyroid slices [64], whereas higher concentrations inhibit H_2O_2 production via the Wolff-Chaikoff effect that represses iodide metabolism, preventing thyrotoxicosis [42].

Based on their sequence homology with NOX2 and the obligate one-electron transfer from the heme, the dual oxidases should primarily produce superoxide [65]. However, DUOX1 and DUOX2 co-expressed with their corresponding partner produce mainly hydrogen peroxide. Structure/function studies have demonstrated that the second intracellular loop and the COOH-terminal tail of DUOXA1 are required for H_2O_2 production by

DUOX1, while active DUOX2 depends on the integrity of the NH_2-terminal extremity of its maturation factor [66]. Moreover, exchanging the first N-linked glycosylated extracellular loop between DUOXA1 and DUOXA2 does not alter DUOX maturation, suggesting that this region could rather be involved in DUOX cell surface expression, a common feature shared by the two maturation factors. The existence of cysteine disulfide bridges for intermolecular protein-protein interactions between DUOX1 and DUOXA1 has been postulated [67]. In addition, recent studies by the group of C. Dupuy demonstrated the implication of two cysteine residues (Cys-124 and Cys-1162) in the formation of an intramolecular disulfide bound that stabilizes the conformation of DUOX2 supporting its interaction with DUOXA2 [68].

DUOX2, but not DUOX1, generates superoxide when co-expressed with the DUOXA1 maturation factor [47, 69]. Moreover, alterations of the NH_2-terminal end of DUOXA2 by deletion or exchange with the NH_2-extremity of DUOXA1 are sufficient to turn DUOX2 to a superoxide-generating enzyme [66]. Likewise, addition of a small unrelated sequence in front of this region in wild-type DUOXA2 converts DUOX2 to a dual-generating oxidase producing H_2O_2 and superoxide. A similar switch to $O_2^{\bullet-}$ production has been reported for NOX4 after the replacement of its signal peptide with the corresponding NOX1 sequence [70]. An elegant structure/function study has been conducted to delineate the domain in DUOX1 that constrains H_2O_2 production [54]. Using chimeric constructs between DUOX1 and DUOX2, Ueyama et al. identified the first extracellular loop of DUOX1 responsible for the reduction of $O_2^{\bullet-}$ leakage when transferred in DUOX2. However, the purified corresponding peptides did not show any superoxide dismutase activity. NOX4 also possesses a unique third extracellular loop involved in its hydrogen peroxide-generating capacity [71]. Mutational analysis identified an essential residue, His^{222}, involved in this process, suggesting that it could be an important proton donor to facilitate the formation of H_2O_2 [72]. Interestingly, Ueyama et al. identified in the first extracellular loop of DUOX1 two critical histidines, His^{1017} and His^{1072}, for the reduction of superoxide release.
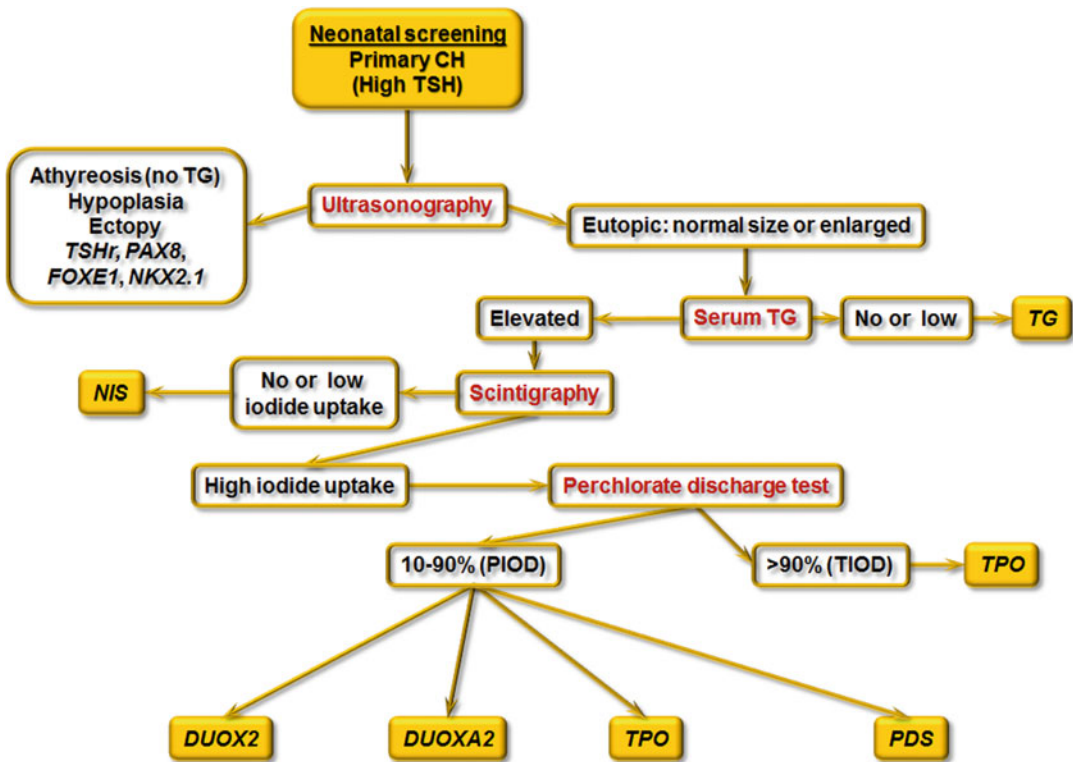
4 Congenital Hypothyroidism

4.1 Genetic Causes of Congenital Hypothyroidism

Congenital hypothyroidism, characterized by high TSH and low T4 serum levels, is one of the most frequent inherited endocrine disorders affecting one in 3000 newborns [73]. The most frequent cause of sporadic CH is iodine deficiency. During human embryogenesis, iodide trapping and TH synthesis begin only at 10–12 days of gestation [74]. Before, transplacental passages of maternal THs ensure the normal fetal development, especially the maturation of

the central nervous system. The implementation of neonatal screening for CH with blood spot assays in the early seventies allowed early treatment of newborn babies with thyroxin supplementation reducing the risk of mental retardation [75].

Thyroid dysgenesis (TD) is another major cause (85%) of CH resulting in thyroid ectopy, athyreosis, or hypoplasia. Loss-of-function mutations in the TSHr encoding gene have been shown to cause thyroid hypoplasia and TSH resistance in humans and in the hyt/hyt mouse model [76, 77]. Germline mutations in thyroid-related transcription factors have also been described in patients suffering from TD with athyreosis associated with cleft palate and spiky hair (*FOXE1*) [78] or thyroid gland hypoplasia sometimes mislocalized (*NKX2*.1 and *PAX8*) [79, 80]. However, the genetic causes of the majority of TD familial cases are still unknown. High prevalence of congenital heart diseases co-occurring with TD suggested that cardiovascular and thyroid developments could be linked [81]. Recent studies performed in the zebrafish have beautifully demonstrated the relationship between cardiovascular development and thyroid morphogenesis, the former probably being used as tissue guidance for correct thyroid migration [82, 83].

About 15% of CH cases are due to defects in thyroid hormone synthesis causing thyroid dyshormonogenesis (TDH), a group of disorders often inherited in an autosomal recessive manner [84]. Inactivating mutations in many of the genes involved in TH synthesis cause TDH associated with iodide organification defect (IOD). After thyroid trapping, free iodide is rapidly covalently bound to TG tyrosyl residues, remaining in the thyroid gland even after the blocking of its transport by the NIS-competitive inhibitor perchlorate. In the perchlorate discharge test, the amount of radioiodide in the neck is followed using a gamma camera after its uptake by the thyroid was blocked by perchlorate, 2 hours after radioisotope administration [85]. In healthy patients, less than 10% of the isotope initially present in the thyroid is washed out 1 hour after perchlorate injection [86]. A discharge value between 10 and 90% reflects a partial IOD. A summary of the etiologic classification of primary CH is presented in Fig. 4 [75, 85]. When ultrasonography revealed an eutopic thyroid gland, high TSH serum levels associated with low serum TG concentrations often reflect inactivating mutations in the *TG* gene. An absence or low iodide uptake detected by scintigraphy most probably suggests a NIS defect. Goiter is not always present in the affected patients, and the severity of hypothyroidism will be dependent on the dietary iodine intake [87]. The most prevalent cause of TDH is TPO deficiency usually associated with a total IOD (perchlorate discharge value >90%) [88]. Alterations of genes encoding TG, NIS, DUOX2, DUOXA2, or pendrin have been identified in patients with partial IOD [69, 86, 89–91].

Fig. 4 Etiologic classification of primary congenital hypothyroidism (adapted from [75, 85]). *PIOD* partial iodide organification defect, *TIOD* total iodide organification defect

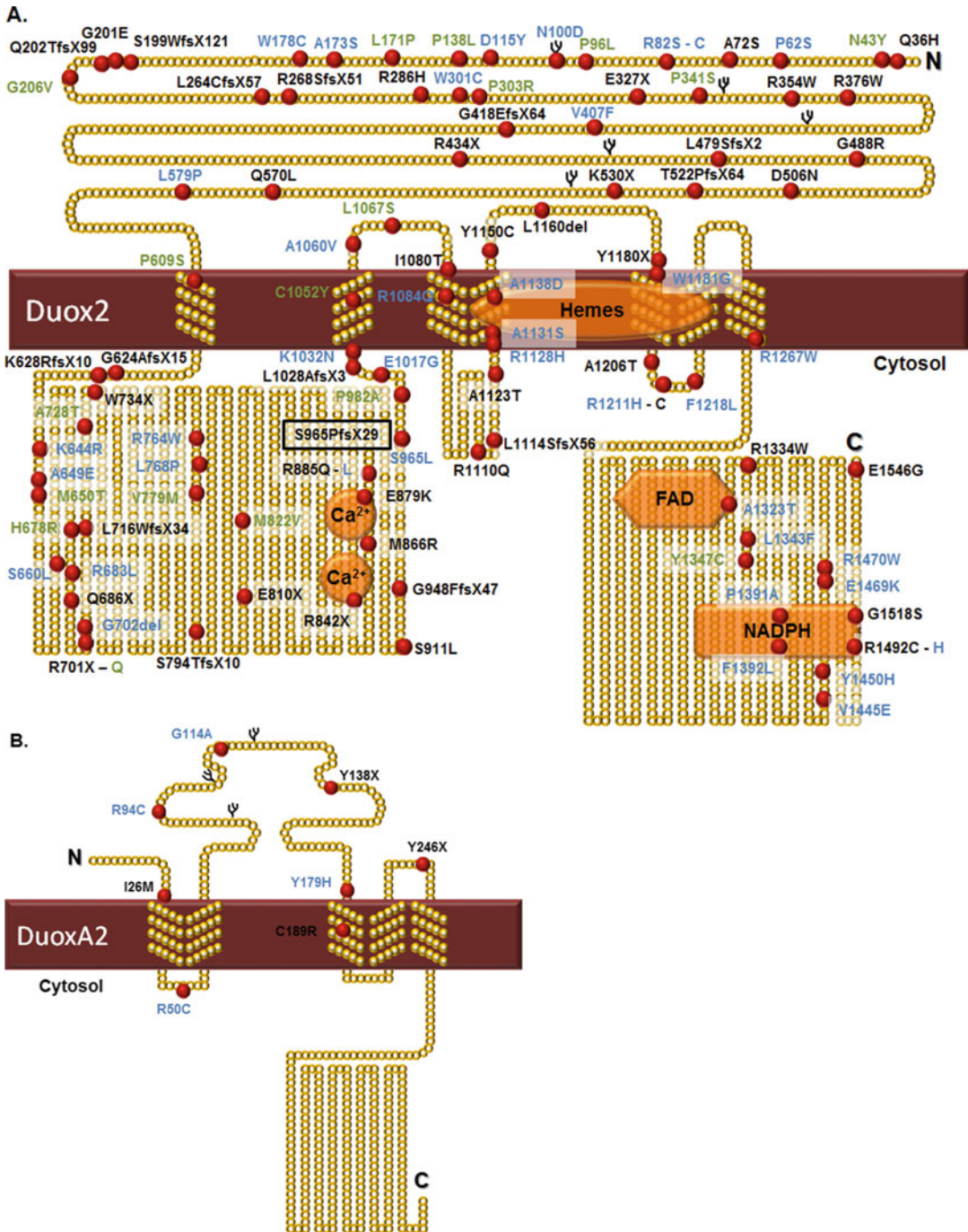**4.2   DUOX Defects in Congenital Hypothyroidism**

Biallelic inactivation of *TPO*, *NIS*, *TG*, or *pendrin* causes permanent CH. Transient CH is frequently associated with a temporally limited exposure to external factors during pregnancy such as a lack or an excess of iodide intake [92], transplacental antibodies [93], or antithyroid drug treatment [94]. In 2002, the characterization of the first inactivating *DUOX2* mutations causing IOD undoubtedly demonstrates the essential role played by DUOX2 in TH synthesis [86]. Furthermore, the authors established the first genetic cause for transient CH with mono-allelic inactivation of *DUOX2* and postulated that *DUOX2* biallelic mutations would be associated with a permanent form of CH. However, numerous subsequent studies provide further evidence that the permanent or transient nature of congenital hypothyroidism is not directly related to the number of inactivated *DUOX2* alleles, suggesting the existence of other pathophysiological factors [95–98].

Multiple interesting reviews about *DUOX2/DUOXA2* genetic disorders have been published in the last 10 years [85, 99, 100], including the recent publication by Muzza et al. [101] analyzing the numerous described DUOX2/DUOXA2 variants. We have completed their extensive analysis of the literature with

additional reported cases of novel *DUOX2/DUOXA2* deficient patients [102–111]. In summary and to the best of our knowledge, about 105 *DUOX2* variants including in-frame deletions, missense, nonsense, splice site, and frameshift mutations have been described in more than 200 unrelated CH patients. One third of the mutations are found in the ectodomain, one third in the first intracellular loop, and one third in the NOX catalytic domain (Fig. 5A). Interestingly, very few nonsense or frameshift mutations are present in the catalytic core of the protein. The p.S965PfsX29 variant, a frameshift mutation localized in the first intracellular loop of the protein, is the most prevalent *DUOX2* mutation. Multiple reported cases are not compatible with the initial hypothesis, showing transient CH with homozygous *DUOX2* inactivation and permanent CH with heterozygous mutations. Intrafamilial variabilities have also been reported in siblings presenting the same genetic defects. For example, four affected siblings of a family carrying the same compound heterozygous *DUOX2* mutations, p.L479SfsX2 and p.K628RfsX10, present permanent or transient CH [96].

The prevalence of *DUOX2* mutations among CH patients is quite variable but generally high with 29–83% in China [112–115], 43% in Japan [116], 30–45% in Italy [98, 117], 44% in Netherlands [86], and 35% in Korea [118]. In case of suspicion of an inherited congenital hypothyroidism, the best criteria for a *DUOX2* genetic screening are the presence of a goiter, a partial IOD, a low T4/TSH serum ratio, high serum TG levels, and a transient phenotype [119]. The prevalence of *DUOXA2* variants is much lower with less than 1%. Six missense, two nonsense, and two splice site mutations have been described (Fig. 5B) [69, 105, 107, 109, 120–125]. The first homozygous missense p.Y246X mutation was reported in 2008 in a Chinese patient suffering from a mild permanent CH [69]. This mutation has been reported in four additional cases but with various severities in the clinical outcome [107, 121, 122, 124]. Intrafamilial variabilities have also been reported for *DUOXA2*-affected patients. Among two dizygotic twins with a mono-allelic p.Y246X *DUOXA2* mutation and a heterozygous p.R885Q *DUOX2* mutation, the girl presented a more severe hypothyroid phenotype than the brother [124]. Another case of two siblings with biallelic *DUOXA2* p.Y138X mutation showed again that the girl suffered from a permanent CH while her brother was not clinically affected [105].

What would be the possible mechanisms to explain this high phenotypic variability? The presence of two DUOX isoforms and their corresponding maturation factors in the same organ could certainly constitute an efficient redundant mechanism to maintain sufficient H_2O_2 supply for iodide organification. Furthermore, the transient nature of CH could be related to the different requirement of THs with age, from the neonatal period (10–15 μg/kg/day) to adulthood (2 μg/kg/day) [126]. In the presence of

Fig. 5 Schematic structures of DUOX2 (A) and DUOXA2 (B) proteins with the genetic alterations (red dots) identified in congenital hypothyroid-affected patients. For the clarity of the figure, only the missense, nonsense, frameshift mutations and in-frame deletions have been localized. Black, damaging mutations (<60% residual activity); green, functional missense mutants (>60% residual activity); blue, not tested mutations

complete *DUOX2* deficiency, H_2O_2 supply by DUOX1 would be sufficient only after the infantile period when the need of TH decreases. Two independent studies performed in Italian and Japanese populations with CH-affected patients carrying *DUOX2* defects showed a majority of cases where thyroxin supplementation could be stopped after puberty [117, 127]. However, the reduction of T3/T4 production will be permanent in these affected children implying their continuous follow-up throughout their life, especially during pregnancy [99]. Recently, the first biallelic *DUOX1* splice site mutation c.1823-1G>C resulting in a truncated protein (p.V607DfsX43) has been reported in two siblings suffering from a particular severe form of permanent CH [108]. These children presented an additional homozygous p.R434X *DUOX2* mutation that has been originally associated with total IOD [86]. The severity of the phenotype could reflect the absence of compensatory mechanism played by DUOX1 in these affected patients. A mild congenital hypothyroid phenotype in a patient with only one *DUOX2* allele, two *DUOX1* alleles, and one remaining *DUOXA1* functional allele supports also the existence of a compensatory mechanism with DUOXA1 [120]. Another possible source of hormonogenic H_2O_2 could be NOX4. Its expression has been shown to be positively controlled by TSH, but its localization, mainly found in intracellular vesicles, is obviously incompatible with TH synthesis [128]. Finally, the dietary iodide intake was clearly demonstrated to be a disease modifier retarding the appearance of the hypothyroid phenotype [129, 130]. The percentage of households having access to iodide salts is higher in North America and Japan than in Europe where the hypothyroid phenotype seems to be more severe [131].

4.3 DUOX Functional Characterization in Heterologous Cell Systems

With the development of next-generation sequencing techniques, analyses of multiple genetic alterations associated with CH-affected patients have been largely facilitated. The coexistence of multiple genetic alterations in the *DUOX2* gene such as tri-allelic mutations has been associated with an increase in the severity of the disease [106, 112, 113]. In addition, increasing number of clinical case studies report *DUOX2* pathogenic variants concomitant with genetic alterations in other genes involved in TH synthesis including *TG* [125, 127], *TSHr* [102, 109, 118, 132], *TPO* [133], *Pendrin* [115], and *DUOXA2* [107, 123]. Additional studies would clarify their functional relevance in the evolution of the pathology. However, many of the reported *DUOX2* missense variants have not been functionally characterized raising the issue of their real functional impact in the observed phenotype, especially in mild transient CH.

To date, about 78 *DUOX2* missense mutations have been described. Only 41 have been functionally characterized in various heterologous systems, 23 of them showing a reduced catalytic activity by more than 60% (summarized in Table 1). However, an

Table 1
Summary of published functional DUOX2/DUOXA2 assays performed in heterologous cell systems to test the activity of *DUOX2/DUOXA2* missense genetic variants identified in congenital hypothyroid-affected patients

		Enzymatic activity				Surface expression			
Date	DUOX2/DUOXA2 constructs	Cells	Activators	Tests	DUOX2 mutants with <60% activity	Cells	FACS/IF	DUOX2 mutant classification	References
2007	HA-DUOX2 DUOXA2-Myc	Hela/Cos-7	Ionomycin	Amplex Red Homovanillic acid	p.Q36H (0%) p.R376W (0%) p.D506N (50%)	Hela/Cos-7	FACS/IF	p.Q36H (0) p.R376W (0) p.D506N (−)	[55]
2008	HA-DUOX2 Myc-DUOXA2	Hela	NM	Amplex Red Diogenes Reagent		Hela	IF		[69][a]
2009	HA-DUOX2 DUOXA2-Myc	Hela	NM	Amplex Red	p.S911 L (50%)	Hela	FACS	p.S911 L (−) **p.C1052Y (−)**	[138]
2010	HA-DUOX2 DUOXA2-Myc	CHO	Ionomycin PMA	Homovanillic acid Diogenes Reagent	p.G1518S (0%)	CHO	FACS	p.G1518S (+)	[135]
2011	HA-DUOX2 DUOXA2-Myc	Hek293	Ionomycin	Amplex Red	p.I1080T (50%) p.R1110Q (15%)	NT	NT		[116]
2011	HA-DUOX2 Myc-DUOXA2	Hela	NM	Amplex Red		NT	NT		[120][a]
2011	HA-DUOX2 DUOXA2-Myc	Hela	NM	Amplex Red	p.Y1150C (10%) **p.A728T (10%)**	Hela	FACS	p.Y1150C (+) **p.A728T (+)**	[98]

(continued)

Table 1
(continued)

		Enzymatic activity				Surface expression			
Date	DUOX2/DUOXA2 constructs	Cells	Activators	Tests	DUOX2 mutants with <60% activity	Cells	FACS/IF	DUOX2 mutant classification	References
2014	GFP-DUOX2 V5-DUOXA2	A549	NM	Amplex Red	p.A72S (25%) p.G488R (0%) p.E879K (5%) p.R885Q (5%) p.R1110Q (10%) p.A1123T (10%) p.R1334W (40%)	NT	NT		[118]
2014	HA-DUOX2 DUOXA2-Myc	Hela	NM	Amplex Red	p.Q570L (20%) p.M866R (0%) **p.C1052Y (20%)** p.E1546G (50%)	NT	NT		[119]
2015	HA-DUOX2 DUOXA2-Myc	Hek293	Ionomycin	Amplex Red		Hek293	IF	p.Y1347C (−)	[134]
2015	HA-DUOX2 DUOXA2	H661 clone DUOXA2	Thapsigargin	Homovanillic Ac	p.R1211C (55%) p.R1492C (15%)	H661 clone DUOXA2	FACS/IF	p.R1211C (+) p.R1492C (+)	[111]
2016	HA-DUOX2 DUOXA2-Myc	Hek293	Ionomycin	Amplex Red	p.R1110Q (5%) p.R1334W (25%)	NT	NT		[133]
2016	HA-DUOX2 Flag-DUOX2 DUOXA2-GFP	Hek293/ Hela	Ionomycin + PMA	Amplex Red Diogenes Reagent	**p.P303R (40%)**	Hek293	FACS	**p.P303R (−)**	[110]

2017	HA-DUOX2 DUOXA2	H661 clone DUOXA2	Thapsigargin	Homovanillic acid	p.R286H (0%)	H661 clone DUOXA2	FACS	p.R286H (0) **p.P609S (−)**	[104]
2017	HA-DUOX2 DUOXA2-Myc	Hek293	Ionomycin	Amplex Red	p.G201E (0%)	Hek293	FACS/IF	p.G201E (0)	[106]
2018	HA-DUOX2 DUOXA2-Myc	Hek293	Ionomycin	Amplex Red		NT	NT		[102]
2018	DUOX2 DUOXA2	Hela/ Hek293	NM	Amplex Red	p.R354W (0%) p.A1206T (0%)	NT	NT		[103]

Proposed *DUOX2* mutant classification: DUOX2^0: Absence of H_2O_2 generation associated with no DUOX2 cell surface expression; DUOX2$^-$: Low H_2O_2 generation associated with reduced DUOX2 cell surface expression; DUOX2$^+$: No or reduced H_2O_2 generation associated with DUOX2 cell surface expression. *FACS* flow cytometry, *IF* immunofluorescence, *NT* not tested, *NM* not mentioned. DUOX2 variants with conflicting reported data are presented in bold

[a]Only DUOXA2 missense variants tested

ideal test to carefully assess DUOX2 function must include specific quantification of H_2O_2 production but also evaluate the expression of the enzyme at the cell surface by flow cytometry using NH_2-terminally epitope-tagged proteins. From the 17 publications including DUOX2 functional assays, only 10 analyze the membrane expression of the protein. Reagents specific for H_2O_2 detection must be systematically used in the assays as the Amplex Red or the homovanillic acid. Moreover, as the basal activity of the oxidase is very low, agents increasing the intracellular Ca^{2+} concentration must be added in the assay. The calcium ionophore ionomycin [55, 106, 110, 116, 133–135] and the Ca^{2+}-ATPase inhibitor thapsigargin [104, 111] have been successfully used in different assays. The cell type could also modify the final outcome of the assay in terms of activity or maturation of the protein. The Hek293 and Hela cells are the two main cell lines used in the published assays.

A reduction of H_2O_2 generation for a particular DUOX2 variant has to be normalized to the level of protein expression but foremost compared to the cell surface expression of the oxidase. Based on this assay, we have characterized the functional consequences of the first DUOX2 variant in the NOX catalytic core [135]. The DUOX2 p.G1518S mutant was nonfunctional but correctly processed at the cell surface. A NOX2 p.C537R variant affecting a highly conserved cysteine localized in the fourth NADPH-binding site, next to the DUOX2 mutated glycine, presented similar functional consequences [136]. We already proposed to apply a comparable classification used for NOX2 mutants depending on the behavior of the protein in heterologous system (DUOX2 mutant classification mentioned in Table 1): $DUOX2^0$ variants showing a loss of H_2O_2 generation associated with no surface expression, $DUOX2^-$ characterized by a low oxidase activity and a reduced membrane expression, and the $DUOX2^+$ mutants which are expressed at the cell surface but less or not active [135, 137]. Additional mutations affecting functional domains of DUOX2 have been described: in the second EF-hand (p.E879K [96, 112, 114, 118, 127]) and in the FAD- (p.A1323T [113, 132]) and NADPH-binding sites (p.P1391A, p.F1392L, p.R1492C, p. R1492H [109, 111, 113, 114, 127]). Interestingly, the three mutants in DUOX2 functional domains that have been fully characterized (activity and membrane expression) to date (p.G1518S, p.R1492C, and p.E879Q generated by site-directed mutagenesis [60]) all belong to the $DUOX2^+$ class.

About 18 tested missense DUOX2 variants show residual activity with more than 60% of the wild-type protein (displayed in green in Fig. 5A), raising the question of their clinical relevance in the pathology of the affected CH patient. The following mutants could be considered as functional single-nucleotide polymorphism (SNP) with limited damaging impact: p.P138L, p.P341S [119], p.H678R

[116, 118, 119, 127], p.R701Q [119], p.P982A [98, 119], p. L1067S [119], p.A728T [98, 106], p.M650T, p.M822V, p. P609S [104, 106], p.C1052Y [138], p.Y1347C [83], p.V779M [102], p.L171P, p.P303R [110, 119], p.G206V, p.N43Y, and p. P96L [118]. The p.H678R mutant has been functionally characterized as a non-deleterious variant, even if the mutation was combined with two other DUOX2 variants, p.R701Q and p.P982A, in the same expressed protein [98].

Conflicting data about the functional analysis of some DUOX2 variants have also been reported. The p.A728T mutant has been shown to be a $DUOX2^+$ nonfunctional variant when expressed in Hela cells [98]. But the same mutant showed unaffected H_2O_2 production and cell surface expression in Hek293 cells [106]. The possible absence of stimulating agents (not mentioned) in the first study could explain this discrepancy as well as the different cell types used. The p.C1052Y DUOX2 variant expressed in Hela cells presented a residual activity of more than 60% in one study [138] but only 20% in another [119]. The p.P303R mutant presented about 75% enzymatic activity of the wild type in one study [119] but less than 50% with reduced cell surface expression in another study ($DUOX2^-$) [110]. Additional conflicting data concern a DUOX2 variant reported in a boy suffering from very early-onset inflammatory bowel disease (IBD) with compound heterozygous mutations [104]. After expression in the large cell lung carcinoma H661 cell line and stimulation with thapsigargin, the p.R286H mutant was undetectable at the cell surface ($DUOX2^0$), while p.P609S expressing cells showed a limited reduction of H_2O_2 generation with lower protein surface expression ($DUOX2^-$). When expressed in Hek293 cells, the p.P609S DUOX2 mutant presented unaffected H_2O_2 generation as well as normal cell surface expression compared to the wild-type protein [106].

5   Conclusions

The implication of DUOX-mediated H_2O_2 in thyroid function is undoubtedly established. The DUOX2/DUOXA2 system represents the major H_2O_2 provider for TPO in thyroid hormonogenesis, but it could be supplemented and compensated by DUOX1/DUOXA1 under pathological circumstances. Recently, additional DUOX-related diseases have been reported in familial cases of IBD with mono- or biallelic *DUOX2* mutations. Even if no strong thyroid phenotype was identified in these patients, a recent meta-analysis-based study suggested that patients with transient CH with partial defects in DUOX2 are at higher risk of developing IBD [139]. The phenotypic characterization of new tissue-targeted DUOX- and DUOXA-deficient animal models will be very useful to study the DUOX function in extra-thyroid tissues. Better

functional characterization of the novel *DUOX2* missense mutations using standardized DUOX functional assays combining quantifications of extracellular H_2O_2 generation and DUOX cell surface expression will be required to better evaluate their functional consequences in the clinical outcome of the affected patients. However, the different cell types used in the assays are not of thyroid origin and would therefore not reflect the physiological behavior of the protein in a real thyrocyte.

Acknowledgments

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