ORIGINAL RESEARCH

Defects in Nicotinamide-adenine Dinucleotide Phosphate Oxidase Genes NOX1 and DUOX2 in Very Early Onset Inflammatory Bowel Disease

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SUMMARY

NOX1 and DUOX2 are the predominant source of intestinal epithelial ROS. Here we identify novel NOX1 and DUOX2 variants associated with VEOIBD that result in reduced ROS production, Paneth cell metaplasia and defective host resistance to C. jejuni.

BACKGROUND & AIMS: Defects in intestinal innate defense systems predispose patients to inflammatory bowel disease (IBD). Reactive oxygen species (ROS) generated by nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases in the mucosal barrier maintain gut homeostasis and defend against pathogenic attack. We hypothesized that molecular genetic defects in intestinal NADPH oxidases might be present in children with IBD.

METHODS: After targeted exome sequencing of epithelial NADPH oxidases NOX1 and DUOX2 on 59 children with very early onset inflammatory bowel disease (VEOIBD), the identified mutations were validated using Sanger Sequencing. A structural analysis of NOX1 and DUOX2 variants was performed by homology in silico modeling. The functional characterization included ROS generation in model cell lines and in vivo transduced murine crypts, protein expression, intracellular localization, and cell-based infection studies with the enteric pathogens Campylobacter jejuni and enteropathogenic Escherichia coli.

RESULTS: We identified missense mutations in NOX1 (c.986G>A, p.Pro330Ser; c.967G>A, p.Asp360Asn) and DUOX2 (c.4474G>A, p.Arg1211Cys; c.3631C>T, p.Arg1492Cys) in 5 of 209 VEOIBD patients. The NOX1 p.Asp360Asn variant was replicated in a male Ashkenazi Jewish ulcerative colitis cohort. Patients with both NOX1 and DUOX2 variants showed abnormal Paneth cell metaplasia. All NOX1 and DUOX2 variants showed reduced ROS production compared with wild-type enzymes. Despite appropriate cellular localization and comparable pathogen-stimulated translocation of altered oxidases, cells harboring NOX1 or DUOX2 variants had defective host resistance to infection with C. jejuni.

CONCLUSIONS: This study identifies the first inactivating missense variants in NOX1 and DUOX2 associated with VEOIBD. Defective ROS production from intestinal epithelial cells constitutes a risk factor for developing VEOIBD. (Cell Mol Gastroenterol Hepatol 2015;4:252–263)
Inflammatory bowel disease (IBD), a complex disease associated with genetic predisposition and environmental factors, is characterized by recurrent intestinal inflammation and microbial dysbiosis. Genomewide association studies link adult IBD to alterations in genes involved in host-microbe interactions. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-generated reactive oxygen species (ROS) are intrinsic to the antimicrobial host defense system of professional phagocytes. Defective ROS production in patients with chronic granulomatous disease (CGD), a rare genetic disorder caused by inactivating variants in all components of the NOX2 phagocyte oxidase (NADPH oxidase 2, NOX2) function, confers susceptibility to life-threatening bacterial and fungal infections. Up to 40% of CGD patients develop inflammatory colitis that mimics Crohn’s disease. Genetic variants in NCF4 and NCF2 that lead to partial attenuation in phagocyte oxidase (NADPH oxidase 2, NOX2) function without causing CGD have been associated with adult and very early onset IBD (VEOIBD). We have recently shown that single-nucleotide polymorphisms (SNPs) and rare hypomorphic variants in all components of the NOX2 NADPH oxidase complex are associated with VEOIBD.

A role for ROS production by intestinal epithelial cells in mucosal barrier function and intestinal homeostasis is just emerging. The predominant sources of ROS in the lining of the gastrointestinal tract are the NADPH oxidases NOX1 (NADPH oxidase 1) and DUOX2 (dual oxidase 2), with NOX1 expression restricted mainly to colon, caecum, and ileum, whereas DUOX2 can be found in all segments of the gut. NOX1 and DUOX2 are the catalytic subunits of multimeric, membrane-bound enzymes that generate upon stimulation superoxide and hydrogen peroxide by transfer of electrons from NADPH to molecular oxygen. We and others have reported NOX1/DUOX2-mediated ROS production in the intestine and its effect on bacterial pathogenicity and barrier integrity. Here, we describe the identification and characterization of missense mutations in NOX1 (NM_007052.4, location Xq22) and in DUOX2 (NG_016992, location 15q15.3) in patients diagnosed with VEOIBD.

### Materials and Methods

#### Study Design

All results are presented according to the STrengthening the REporting of Genetic Association Studies (STREGA) guidelines. Fifty-nine IBD patients diagnosed under the age of 6 years were sequenced for NOX1 and DUOX2 by targeted exome sequencing using Agilent SureSelect target enrichment and sequencing (Agilent Technologies, Santa Clara, CA) on the Illumina HiSeq 2000/2500 (Illumina, San Diego, CA) with exon primer and sequencing protocols designed by the Beckman Coulter Genomics (beckmangenomics.com; Beckman Coulter, Brea, CA) as described previously elsewhere. Sanger sequencing was used to verify all genetic defects identified using targeted sequencing of the NOX1 and DUOX2 genes at the Centre for Applied Genomics (TCAG; http://www.tcag.ca; Hospital for Sick Children, Toronto, ON, Canada).

Single-nucleotide and insertion/deletion (indel) variants identified by targeted exome sequencing and validated by Sanger sequencing were automatically scanned and manually verified. Furthermore, all variants were also validated using Taqman performed by the Centre for Applied Genomics, Hospital for Sick Children. Function and minor allele frequency (MAF) were searched for using the National Heart, Lung, and Blood Institute Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/), the National Center for Biotechnology Information dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), the National Institute of Environmental Health Sciences FuncPred (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), FastSNP (http://fastsnp.ibms.sinica.edu.tw/), Human Splicing Finder (http://www.umd.be/HSF/), and pfsNP (http://pfs.nus.edu.sg/).

### Setting

Patients included in the study were recruited from the Inflammatory Bowel Disease Clinic at the Hospital from Sick Children, University of Toronto. They were diagnosed with VEOIBD between the years 1994 and 2012 and had a confirmed diagnosis of IBD before the age of 6 years. Although there is no consensus on the definition of VEOIBD, we have used the stricter definition based on our recent modification (diagnosis < 6 years of age) of the Paris classification. Our definition, which is more stringent and includes more severe cases that are more likely to cause monogenic forms of the disease, has been used to identify risk variants in this age group. There were no exclusion criteria for patients diagnosed with VEOIBD; however, patients with a known immunodeficiency or a clinical diagnosis of CGD were excluded because these patients were not defined as VEOIBD. The five identified patients were screened and were found negative for pathogenic mutations in IL10RA, IL10RB, IL10, XIAP, TTC7A, as well as genes involved in CGD (RAC1/2, NCF1/2/4, CYBB, CYBA) and NOD2 and ATG16L1 variants associated with IBD.

#### Participants

This was a cohort study that examined the genetics of VEOIBD patients. Fifty-five VEOIBD patients were recruited from the Hospital for Sick Children, Toronto, Canada. A second cohort of VEOIBD patients was recruited through NEOPICS (www.NEOPICS.org). The replication cohort comprised 1477 Crohn’s disease cases, 559 ulcerative colitis cases, and 2614 healthy controls, all with genetically verified Ashkenazi Jewish ancestry by principal components analysis.
Standard quality control procedures were applied, and we performed association testing using Fisher’s exact method, stratified by gender in 297 male ulcerative colitis (UC) cases, 262 female UC cases, 1708 male controls, and 906 female controls. Phenotypic information and DNA samples were obtained from the study participants with approval of the institutional review ethics board for IBD genetic studies at the Hospital for Sick Children and Mount Sinai Hospital Toronto.

Later onset UC cases were recruited through the National Institute of Diabetes and Digestive and Kidney Diseases Inflammatory Bowel Disease Genetics Consortium, the Cedars-Sinai Medical Center IBD Center in California and Mount Sinai Hospital in New York. Replication cohorts had ethics board approval for genetic and phenotypic studies at the individual institutions. Written informed consent was obtained from all participants/parents.

H&E and Periodic Acid–Schiff Staining in Patient Biopsy Samples

Colonic biopsy samples were fixed in 10% formaldehyde without methanol and afterward embedded in paraffin. For H&E staining, embedded paraffin tissues on slides were deparaffinized with xylene and afterward rehydrated with different percentages of ethanol. The slides were stained for 5 minutes with Meyer’s hematoxylin (Fisher Scientific, Fair Lawn, NJ) for nuclei and counterstained with eosin-Y (Fisher Scientific) for cytoplasm. Slides were mounted in Entellan (EMD Millipore, Billerica, MA). Photographs were taken using an epifluorescence light microscope (Leica Microsystems, Buffalo Grove, IL) and adjusted for brightness, contrast, and pixel size in Adobe Photoshop CS5 version 12.0 (Adobe System, San Jose, CA).

Modeling and Docking Procedure

Three-dimensional (3D) models of C-terminal domains of NOX1 and DUOX2 were generated using the homology modeling program Modeller 9v11 (http://www.salilab.org/modeller/). Blast of PDB was performed with the NOX1 FAD-binding domain, and a combination of several homologous structures served together with the 3D X-ray structure the NOX2 NADPH binding domain (PDB ID: 3A1F) as initial template. The modeling was performed with default parameters using the “allHmodel” protocol to include hydrogen atoms and the “HETATM” protocol to include FAD and NADPH. To compare the FAD and NADPH binding interaction between wild-type (WT) and sequence altered oxidases, the docking runs were performed with HADDOCK. Docking was performed with most of the parameters set to default using the Web server version of HADDOCK with a Guru interface. To gain the Van der Waals, electrostatic, and desolvation energy for each enzyme - FAD or -NADPH model, HADDOCK automatically performed the molecular dynamics before and after each docking trial by including water into the calculation (detailed modeling procedure, publication in preparation).

Cell Culture and Transfection

Model cell lines were employed as intestinal epithelial cell lines, and primary colon cells express endogenous NOX1 and DUOX2. Cos7 cells are a suitable model system for NOX1-based oxidase reconstitution as they lack any functional NADPH oxidases, and NCI-H661 cells serve as a physiologically relevant model for DUOX oxidases. Cos7 cells stably expressing p22phox were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum; for NCI-H661 cells stably expressing DUOXA2, RPMI 1640 medium with 10% fetal bovine serum was used. NOX1 was cloned into pcDNA3.1 with and without the N-terminal Myc epitope tag including a linker sequence. Influenza hemagglutinin (HA)-tagged DUOX2 in pcDNA3.1 was prepared by cloning the HA tag between amino acids D27 and A28. Mutations were introduced using site-directed mutagenesis and were verified by sequencing. NOX1 WT and missense variants were transiently transfected with NOXA1 and Myc-NOXO1 into Cos-p22phox cells (24 hours). HA-tagged DUOX2 WT and missense variants were transiently transfected into H661-DUOXA2 cells or together with DUOXA2 into Cos7 cells using X-tremeGENE (Roche Applied Science, Indianapolis, IN) (48 hours). For analysis of DUOX2 localization upon bacterial challenge, HT29 colon epithelial cells expressing endogenous NOX1 and NOD2 were stably transduced with lentivirus encoding HA-tagged DUOX2 WT, DUOX2 R1211C, and DUOX2 R1492C in combination with WT DUOXA2.

Protein Isolation and Western Blotting

Cells were lysed in radioimmunoprecipitation assay buffer and after gel electrophoresis and blotting, membranes were probed with anti-HA (Covance Laboratories, Princeton, NJ), anti-DUOX2, anti-Myc (9E10), anti-NOX1, anti-p22phox FL-195 (Santa Cruz Biotechnology, Dallas, TX), anti-calnexin (BD Biosciences, San Jose, CA), and horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (SouthernBiotech, Birmingham, AL). Proteins were visualized using enhanced chemiluminescence reagent (Pierce Biotech, Rockford, IL). Immunoblotting of p22phox or calnexin served as control.

ROS Assays

Superoxide production (NOX1) was measured using luminol enhanced chemiluminescence and stimulation with 1 mg/mL phorbol 12-myristate 13-acetate (PMA) for 30 minutes. Luminescence was measured on a Berthold Centro 960 LB in white 96-well plates. The chemiluminescence (relative light units, ΔRLU) readings were standardized against cellular protein (BCA assay).

H2O2 production (DUOX2) was measured using the homovanillic acid assay and addition of 1 μM thapsigargin. H2O2 production was standardized to H2O2 standard curves and cell lysate protein concentration. Empty vector transfection served as the control. For crypt ROS assays, Nox1−/− mice (Jackson Laboratory, Bar Harbor, ME) were transduced with lentivirus encoding empty vector, NOX1, NOX1 D330N, and NOX1 P360S. Briefly, the lentiviral titer was determined.
1.5% paraformaldehyde and analyzed on an Accuri C6 cytometer (BD Biosciences). With anti-mouse Alexa Fluor 647, the cells were fixed in 0.5% Triton X-100, and permeabilized in 3% paraformaldehyde, permeabilized in 0.5% Triton X-100, and stained with anti-DUOX2 or anti-Myc antibody, followed by goat anti-rabbit or anti-mouse Alexa Fluor 488 (Invitrogen/Life Technologies, Carlsbad, CA). HT29 cells expressing DUOX2 WT or missense variants were seed on glass coverslips and treated with 300 µL of a clinical isolate of enteropathogenic Escherichia coli (EPEC) at optical density OD600 = 1 for 5 hours. Slides were washed, fixed, and permeabilized with 0.1% Triton X-100 and probed with antibodies against HA tag (Covance) and NOD2 (sc-30199, kind gift by P. Moynagh, National University of Ireland Maynooth), and 4′,6-diamidino-2-phenylindole (DAPI, blue). Images were acquired using a Zeiss LSM 700 microscope (Carl Zeiss, Thornwood, NY) and magnification 63× (oil) objective.

Colonies biopsies from control, disease control, and patients were fixed in 10% formaldehyde without methanol, embedded in paraffin, and processed for staining. Antigen retrieval was performed using high pressure-cooking with 1 mM EDTA at a pH 9.0 containing 0.05% Tween 20. Afterward, slides were blocked for 1 hour at room temperature with 5% bovine serum albumin in 1x phosphate-buffered saline (PBS) without calcium and magnesium containing 15% goat serum. Primary antibody incubation was performed overnight at 4°C. On the following day, the stained slides were washed three times for 10 minutes with 1x PBS without calcium and magnesium.

Secondary antibody incubation was performed at room temperature and in darkness for 1 hour. Slides were washed afterward three times for 10 minutes in darkness. Next, nuclear counterstaining with Hoechst 33342 Fluorescence Stain (Thermo Fisher Scientific, Waltham, MA) was performed at a dilution of 1:15,000. Finally, sections were mounted overnight with Vectorshield fluorescence mounting medium (Vector Laboratories, Burlingame, CA). Antibodies anti-beta catenin (BD Transduction Laboratories, BD Biosciences), anti-lysozyme (Abcam, Cambridge, MA), anti-CD24 (Abcam), and anti-EpCAM (Sigma-Aldrich, St. Louis, MO) were used at 1:100 dilution. Secondary antibodies were Alexa fluoro 568 goat anti-rabbit and Alexa fluoro 488 goat-anti mouse (both Invitrogen/Life Technologies). Images were acquired with an Olympus IX81 inverted fluorescence microscope (Olympus America, Center Valley, PA) equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera (Hamamatsu Photonics KK, Hamamatsu City, Japan) and Yokogawa CSU X1 spinning disc confocal scan head (Yokogawa Electric Corporation, Tokyo, Japan). Images were adjusted for contrast and brightness using the

### Table 1. List of Variants Found in NOX1 and DUOX2 in Very Early Onset Inflammatory Bowel Disease Patients

| Gene   | Variant             | rs #            | MAF/Minor Allele Count* | CADD Rank Score | Age at Diagnosis (y) | Gender | Diagnosis     | Patient Summary                      |
|--------|---------------------|-----------------|-------------------------|-----------------|----------------------|--------|--------------|--------------------------------------|
| NOX1   | c.988G>C, p.F330S   | Novel           | Novel—no data available | 0.40694         | 1.8                  | Male   | IBD-U        | Severe pancolitis Granuloma          |
| NOX1   | c.967G>A, p.D360N   | rs34688635      | T = 0.010/16            | 0.5415          | 5.3                  | Female | UC           | Pancolitis                           |
| DUOX2  | c.4474G>A, p.R1211C | Novel           | Novel—no data available | 0.90955         | 4.7                  | Male   | IBD-U        | Severe pancolitis Colectomy, perforation Recurrence of disease |
| DUOX2  | c.3631C>T, p.R1492C | rs374410986, Novel | Novel—no data available | 0.9002          | 4.3                  | Male   | UC           | Pancolitis                           |

Note: CADD, Combined Annotation Dependent Depletion; DUOX2, dual oxidase 2; IBD-U, inflammatory bowel disease unclassified; MAF, minor allele frequency; NOX1, NADPH oxidase 1; UC, ulcerative colitis.

*The minor allele frequencies are taken from 1000 Genomes of dbSNP.
Table 2: Computational Analysis of NOX1 and DUOX2 Variants

| Gene | Variant | Chromosome | Position       | rs#    | Chromosomal position | Minor Allele Frequency (dbSNP) | Minor Allele Frequency (1000G) | Minor Allele Frequency (NHLBI exome variant frequencies) | ExAC v0.3 MAF | SIFT | SIFT Prediction |
|------|---------|------------|----------------|-------|----------------------|-------------------------------|-------------------------------|--------------------------------------------------------|---------------|------|----------------|
| NOX1 | P330S   | X          | 100105285      | Novel | c.988G>A             | No MAF data                   | No MAF data                   | No MAF data                                             | 0.051         | Tolerated |
| NOX1 | D360N   | X          | 100105195      | rs34688635 | c.967G>A             | T=0.010/16 (1%)               | 0.019881                       | 0.018                                                  | 0.042         | Tolerated |
| DUOX2 | R1211C  | 15         | 45389874       | rs374410986 | c.3631C>T           | No MAF data                   | No MAF data                   | A=7.7e-05                                              | 0             | Damaging |
| DUOX2 | R1492C  | 15         | 45386811       | Novel  | c.4474G>A           | No MAF data                   | No MAF data                   | A=0.00004118                                           | 0             | Damaging |

Table 2. Continued

| Gene | PolyPhen2 Prediction | Mutation Taster Prediction | Mutation Taster | Mutation Assessor Prediction | Mutation Assessor | FATHMM Prediction | LRT Score | LRT Prediction | GERP ++ | PhyloP | CADD Rank Score |
|------|---------------------|---------------------------|----------------|-----------------------------|-----------------|-------------------|-----------|----------------|---------|--------|----------------|
| NOX1 | 1                   | Disease Causing           | 3.185          | Predicted Functional (Medium) | 2.44            | Tolerated         | 0         | Deleterious    | 3.87    | 1.767  | 0.40694       |
| NOX1 | 0.085               | Possibly Damaging         | 2.225          | Predicted Functional (Medium) | -3.09           | Damaging          | 0.000445  | Deleterious    | 3.87    | 1.767  | 0.54147       |
| DUOX2 | 1                   | Disease Causing           | 3.37           | Predicted Functional (Medium) | 0               | Deleterious       | 5.69      | 2.679         | 0.90955 |
| DUOX2 | 1                   | Disease Causing           | 3.97           | Predicted Functional (High)  | 0               | Deleterious       | 5.68      | 2.838         | 0.9002  |

ExAC, Exome Aggregation Consortium; CADD, Combined Annotation Dependent Depletion; FATHMM, Functional Analysis through Hidden Markov Models; GERP, Genomic Evolutionary Rate Profiling; LRT Score, likelihood ratio test; MAF: Minor allele frequency; PolyPhen2, Polymorphism Phenotyping v2; SIFT, Scale-invariant feature transform.
Virulence Assay
Adherence and invasion of *C. jejuni* 81-176 were assessed in NOX1 complex or DUOX2-DUOX2 expressing Cos7 cells using the gentamicin protection assay.25 Plate grown *C. jejuni* 81-176 was washed and resuspended in tissue culture medium at OD_{600}= 0.4 and added at multiplicity of infection 1000 to cells, followed by centrifugation at 250g for 5 minutes. After incubation for 3 hours at 37°C, the nonadherent and cell-associated bacteria were collected. For invasion, the infected and washed monolayers were incubated further with and without gentamicin (400 μg/mL) and incubated for an additional 2 hours at 37°C. The cells were lysed by the addition of 0.1% Triton X-100 in PBS for 10 minutes at 37°C. Bacterial counts for each assay were enumerated by serial dilution plating. All parameters were calculated as the average of the total number of colony-forming units/total initial inoculum.

Statistical Analysis
All functional experiments were conducted in triplicate with three repeats (n = 3), followed by an unpaired Student's *t* test.

Results
Identification of NOX1 and DUOX2 Variants in VEOIBD
NOX1 and DUOX2 missense mutations were identified in five of 59 VEOIBD patients (age ≤ 6 years). All five patients presented with pancolitis without small bowel or perianal disease at diagnosis. None of the patients had systemic disease including thyroid disease or chronic infections, suggesting that defects were confined to the intestinal epithelium. SNPs and insertion/deletion variants were confirmed using Sanger sequencing and analyzed for potential function. Exon sequencing (Table 1–2) identified a novel NOX1 variant (c.988G>A; p.P330S) in one male patient. Another rare variant (c.967G>A; rs34688635; p.D360N) was found in one male and one female patient. The missense variant NOX1 p.P330S is potentially damaging (PolyPhen2 score: 0.995) and unique according to the PhastCons program using 46 mammalian species. The missense variant NOX1 p.P330S is potentially damaging by PolyPhen2 and was given a maximum evolutionary conservation score of 1 by the PhastCons program using 46 mammalian species.

Variants in DUOX2 were also identified in VEOIBD patients (Table 1–2). One of the patients was heterozygous for DUOX2 p.R1211C (c.4474G>A) and developed severe disease that necessitated colonic resection. The disease subsequently recurred at the resection site, a finding consistent with Crohn's disease. The second variant was detected in a very early onset UC patient heterozygous for DUOX2 p.R1492C (c.3631C>T; rs374410986), who presented with pancolitis.

In an independent replication cohort of 150 VEOIBD patients, none of the NOX1 and DUOX2 missense variants were identified. Similarly, in the publicly available International IBD Genetics Consortium (http://www.ibdgenetics.org) database none of the NOX1 and DUOX2 missense variants were identified as this data set does not examine rare variants, only common polymorphisms, and the p.Asp360Asn variant is not analyzed by the immunochip.

Therefore, we took an alternate approach employing an array-based genotyping using the Illumina HumanExome v1.0 platform of 1477 Crohn's disease (CD) cases, 559 UC cases, and 2614 healthy controls, all with genetically verified Ashkenazi Jewish (AJ) ancestry by principal components analysis. Using this approach we detected an association between D360N NOX1 and male AJ UC (MAF_{case} 3.37%, MAF_{control} 0.82%; odds ratio 4.22; *P* = 1.25 × 10^{-3}). The association was not detected in either of the female AJ UC cases (MAF_{case} 1.53%, MAF_{control} 0.99%; odds ratio 1.55; *P* = .343), although the trend was in the same direction as observed in the AJ males cases. However, this trend was not observed in Crohn's disease cases (MAF_{CD} 0.97%). The finding in an adult UC cohort suggests that pathways/processes involved in VEOIBD will have implications for adult IBD patients.

Histologic Analysis of NOX1/DUOX2 Variants
Histopathology analysis using HE and PAS staining (Figure 1A) was performed in biopsies from patients with the identified DUOX2 p.R1211C variant as well as a patient with the NOX1 p.D360N variant and compared with the healthy control and an IBD control biopsy. The disease control showed features of chronic and regenerative IBD, demonstrated by metaplastic Paneth cells within colonic crypts. The patient with the NOX1 p.D360N variant showed focal inflammation, increased cellularity of inflammatory cells adjacent to normal areas of unaffected colonic mucosa. The patient with the DUOX2 p.R1211C variant demonstrated more severe morphologic changes, with severe inflammation and crypt damage in the colonic mucosa when compared with the NOX1 variant.
Immunofluorescence staining was performed on colonic biopsy samples to determine whether Paneth cell metaplasia, a feature of chronic and regenerative change as a consequence of continuous inflammation within the colon, has occurred. Both markers, lysozyme and CD24, were highly positive in metaplastic Paneth cells of colonic crypt enterocytes in the disease control (see Figure 1B). Altered NOX1 appears not to progress cells into full metaplasia as seen by the absence of CD24 within crypt cells of the patient harboring NOX1 p.D360N. In colonic crypts of the patient with the DUOX2 p.R1211C variant, both lysozyme and CD24 were expressed, albeit not as prominent as observed within metaplastic Paneth cells in the IBD control.

**Topologic Models of NOX1/DUOX2 Variants**

The NOX1 NADPH oxidase is formed by heterodimerization of NOX1 with p22phox, followed by assembly with the regulatory proteins NOXO1, NOXA1, and Rac1-GTP. The cytosolic carboxyl terminus of NADPH oxidases harbors NADPH and FAD-binding regions, which are required for electron transport across the membrane via hemes where molecular oxygen is reduced to form superoxide. The identified NOX1 variants are located either just in front of FAD1 (p.P330S) or inside FAD2 (p.D360N) (Figure 2A). Pro330 and Asp360 are conserved in NOX1–4 proteins identified in vertebrates and lower organisms. CYBB missense variants (X-CGD) leading to loss or diminished ROS generation in neutrophils are located in close vicinity to the identified NOX1 variants (http://bioinf.uta.fi/CYBBbase). Modeling of NOX1 WT, NOX1 (p.P330S), or NOX1 (p.D360N) dehydrogenase domains was performed by combining the crystal structures of FAD-binding domains homologous to the NOX FAD with the partial structure of the NOX1 enzyme.Debeurme et al reported disrupted FAD binding and diminished catalytic activity of NOX2 in selected CYBB variants.

**Functional Characterization of NOX1 Variants**

As structural analysis predicts that the catalytic activity of NOX1 variants will be compromised, we reconstituted WT and altered NOX1 complexes in an epithelial model cell system (Cos7) deficient in all NOX/DUOX isoforms. Both NOX1 p.P330S and NOX1 p.D360N variants displayed diminished catalytic activity (see Figure 2C). Basal and phorbol ester-stimulated ROS generation was significantly reduced for NOX1 missense variants (50%–60%), while the overall protein expression was comparable to WT NOX1 (see Figure 2D).

As patients could not be recalled for colon tissue evaluation, catalytic activity of NOX1 variants was also measured in a murine in vivo expression setting. Nox1 knockout mice were transduced with lentivirus encoding NOX1 WT and variants intrarectally, and ROS generation of isolated crypts was recorded 24 hours later. Similar to the results obtained in cell lines, ROS production in the crypts was reduced in the NOX1 variants when compared with NOX1 WT (see Figure 2E).

A reduction in epithelial ROS production will attenuate host protection from intestinal pathogens. Defective processing of responses to mucosal bacteria is recognized to play a central role in the development and perpetuation of intestinal inflammation in IBD. C. jejuni in particular has been associated with the initiation of IBD. C. jejuni uptake was used to visualize infection-associated translocation of NOX1 to membrane ruffles and to assess the antibacterial response. Stimulated membrane localization of NOX1 WT and NOX1 variants (NOX1 p.D360N shown) were comparable (see Figure 2F), but reduced ROS generation caused a 10-fold increase in bacterial invasion when cells harbored the NOX1 p.P330S or NOX1 p.D360N variants with reduced catalytic activity (see Figure 2G).

**Functional Characterization of DUOX2 Variants**

Inactivating mutations in DUOX2 or DUOX2 have been linked to inherited permanent or transient congenital inflammatory bowel disease (VEOIBD) variants. NOX1 and DUOX2 variants in VEOIBD

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**Figure 2.** (See previous page). Modeling and functional characterization of NOX1 variants. (A) Topologic model depicting NOX1 very early onset inflammatory bowel disease (VEOIBD) variants (red), selected X-CGD CYBB (NOX2) variants (green), conserved residues boxed (blue). (B) Three-dimensional model of NOX1 wild-type (WT) (grey), NOX1 P330S (green), or NOX1 D360N (pink) dehydrogenase domains. NADPH, FAD, residue H339, and variant positions are marked. (C) ROS production by NOX1 WT and variants. (D) Protein expression of NOX1 and variants, Myc-NOX1, NOX1A1, and p22phox as loading control. (E) ROS production in murine NOX1 WT or DUOX2 protein in C. jejuni (red) infected Cos-p22 cells. Scale bar: 10 μm; arrow indicates membrane localization. (F) Adhesion and invasion of Campylobacter jejuni in cells expressing NOX1 WT, P330S, or D360N. Error bars ± standard deviation n = 3; *P < .05; **P < .01; ***P < .001; comparing NOX1 WT to variants.
hypothyroidism, and to date over 23 DUOX2 mutations have been described in this context (HGMD, www.hgmd.cf.ac.uk/ac/gene) (Figure 3A). The two VEOIBD-associated DUOX2 variants are novel; in contrast to most of the reported DUOX2 variants, they are not located in the peroxidase homology domain or the EF hand regions. DUOX2 p.R1211C is placed in a polybasic region within an intracellular loop, and Arg1492 in DUOX2 is an integral part of the highly conserved GRP sequence in the NADPH3 domain (see Figure 3A).

As described for NOX1, the dehydrogenase domains of DUOX2 WT and DUOX2 p.R1492C were modeled onto the extended NOX2 structure; by use of HADDOCK, NADPH and FAD were docked to the structure (see Figure 3B). Structural analysis revealed that Arg1492 is part of the NADPH-binding pocket. NADPH binds to DUOX2 WT with strong electrostatic interactions to the residues Arg1421 and Arg1492 with a sum of −181.7 ± 76.4 kcal/mol and with weak Van der Waals interactions to Gly1385, Thr1463, Pro1520, Gly1521, and Met1520 with a sum of −30.9 ± 7.8 kcal/mol. Replacing Arg1492 with cysteine as in the DUOX2 p.R1492C variant does not change the DUOX2 structure or the position of other NADPH-interacting residues. However, the change is predicted to weaken the interaction between NADPH and DUOX2 by a factor of 2. How replacement of Arg1221 with cysteine will directly affect DUOX2 catalytic activity cannot be predicted because suitable structures for modeling do not exist, but in both NOX2 and NOX4 the analogous D loop participates in ROS production.

Functional analysis of DUOX2 variants was performed in the H661 cellular model system that represents a physiologic context for DUOX-DUOXA expression and is devoid of NOX1-5 activity. Both DUOX2 variants, when coexpressed with their dimerization partner DUOXA2, produced significantly less H2O2 than WT DUOX2 (see Figure 3C), although protein expression and cellular localization were not altered (see Figure 3D and E). DUOX2 has been functionally associated with NOD2 in transient overexpression conditions. HT29 colonic cells express endogenously a functional NOX1 complex and NOD2, and thus provide an appropriate context for analysis of putative DUOX2-NOD2 interactions.

DUOX2 or DUOX2 variants together with DUOXA2 were stably incorporated into HT29 cells, followed by exposure to entero-pathogenic E. coli. DUOX2 WT or variants, localized on internal membrane structures before the challenge, translocated to the plasma membrane and cell-cell junctions. NOD2, on the other hand, remained in the intracellular compartment, albeit NOD2 protein expression was up-regulated (Figure 4). Thus, DUOX2 and NOD2 were not recruited simultaneously upon E. coli challenge.

Stimulated H2O2 release in DUOX2 WT or variant-expressing HT29 cells mirrored the results obtained with H661 cells (data not shown). DUOX2-mediated H2O2 release at apical membranes has been linked to antimicrobial host defense and decreased C. jejuni virulence. Comparison of C. jejuni invasion in DUOX2 WT or DUOX2 variant-expressing (DUOX2 p.R1211C, DUOX2 p.R1492C) epithelial cells showed increased invasion when ROS generation was diminished (see Figure 3F).

Discussion

We have identified inactivating missense variants in each of the epithelial NADPH oxidases NOX1 (p.P330S, p.D360N) and DUOX2 (p.R1211C, p.R1492C) in five VEOIBD patients. Variants in X-linked NOX1 were found in two male VEOIBD patients, and NOX1 p.D360N was associated with male UC in an AJ ancestry case-control cohort, likely leading to increased or sustained disease severity.

The identification of rare functional variants contributing to the pathogenesis of VEOIBD has been observed with other genes, including the NOX2 NADPH oxidase complex, NOS2, IL10R, and XIAP. The variants we identified in both NOX1 and DUOX2 are rare and not found in a replication VEOIBD cohort or data sets of common variants. However, all variants showed both pathologic and functional defects, indicating that these variants may contribute to disease susceptibility or pathogenesis. Further large-scale sequencing of pediatric- and adult-onset IBD may indicate a broader role of both NOX1 and DUOX2 in IBD pathogenesis, as observed in our AJ population.

Recently, altered DUOX2 expression was identified in ileum biopsies from pediatric Crohn’s disease patients. Further, ROS derived from NADPH oxidases is critical to control mucin granule accumulation in colonic goblet cells, and NOX1 has been shown to control the balance between goblet and absorptive cell types in murine colon. Interestingly, colonic biopsies from patients carrying either NOX1 p.D360N or DUOX2 p.R1211C variants showed abnormal CD24 and lysozyme expression (see Figure 1B), suggesting a role for these proteins in Paneth cell metaplasia.

The thyroid function of the two male VEOIBD patients harboring DUOX2 mutations was normal, although

Figure 3. Modeling and functional characterization of DUOX2 variants. (A) Topologic model depicting DUOX2 very early onset inflammatory bowel disease (VEOIBD) variants (red), selected DUOX2 hypothyroidism variants (black), and conserved residues boxed (blue). (B) Three-dimensional model of DUOX2 wild-type (WT) (grey) and DUOX2 R1492C (pink) dehydrogenase domain. NADPH, FAD, and variant position are marked. (C) H2O2 release by DUOX2 WT and variants. (D) Protein expression of HA-DUOX2 WT and variants; calnexin served as loading control. (E) HA-DUOX2 WT and variant surface expression by flow cytometry (left) and localization by immunofluorescence (right) (green, arrow for membrane). Scale bar, 10 μm. (F) Adhesion and invasion of C. jejuni in cells expressing HA-DUOX2 WT, R1211C, or R1492C. Error bars ± standard deviation, n = 3; *P ≤ .05; **P ≤ .01; ***P ≤ .001; ****P ≤ .0001; comparing DUOX2 WT with variants.
inactivating monoallelic and biallelic \( \text{DUOX2} \) and \( \text{DUOXA2} \) variants have been linked to hypothyroidism.\(^{49}\) In contrast to adult onset IBD, VEOIBD frequently encompasses a unique clinical presentation, with severe disease limited to the colon and with poor response to standard therapies.\(^{24}\) VEOIBD variants (\( \text{NCF2} \),\(^{50}\) \( \text{NOS2} \),\(^{44}\) \( \text{IL10RA/B} \),\(^{15}\) \( \text{TTC7A} \)) have usually been rare, suggesting that these patients may have a unique genetic susceptibility. Furthermore, we have recently shown that SNPs and rare variants in all components of the NOX2 NADPH oxidase complex are associated with VEOIBD.\(^{7}\) Similar to our recent observations with NOX2 NADPH oxidase complex variants leading to decreased ROS production in neutrophils,\(^{7}\) reduced mucosal ROS levels originating from NOX1 and DUOX2 variants play also a role in susceptibility to VEOIBD and perhaps other severe IBD phenotypes.

Intestinal NADPH oxidases connect to antibacterial autophagy and endosomal pathways important for mucus secretion and may modulate the interplay between commensal bacteria and pathogens.\(^{12,13}\) Recent microbiome studies on a large pediatric cohort with new-onset Crohn’s disease assigned a unique role to changes in the rectal mucosal microbiota for disease classification.\(^{52}\) Changes in ROS generation at the mucosal surface will most likely result in dysbiosis, intestinal inflammation, and pathobiont development. Our functional studies provide strong support both for the pathogenic nature of the mutations identified in these VEOIBD patients and the role of epithelial ROS in protecting cells from bacterial attack.

Further phenotypic exploration of \( \text{NOX}/\text{DUOX} \) variants will be aided by studies in humans and improved animal models, as current IBD animal models seem often not to reflect human disease triggered by reduced ROS. For example, murine \( \text{Cybb} \) (Nox2) deficiency does not lead to spontaneous Crohn’s disease-like intestinal disease and gut inflammation, both observed in many CGD patients. Although \( \text{Cybb} \) knockout mice exhibit several hallmarks of CGD upon fungal or bacterial challenge, they were slightly protected in the dextran sodium sulfate-induced colitis mouse model.\(^{53}\) Similarly, \( \text{Nox1} \) deficiency in the murine mucosa did not alter dextran sodium sulfate–colitis patholog',\(^{34}\) although combined \( \text{Nox1} \) and \( \text{Il10} \) deficiency caused spontaneous colitis in mice.\(^{55}\) Mice harboring an inactivating \( \text{Duox2} \) variant or \( \text{Duoxa} \) deficiency showed severe hypothyroidism and increased colonization with \( \text{Helicobacter felis}.\)^\(^{11,56}\)

In conclusion, our findings demonstrate that novel \( \text{NOX1} \) and \( \text{DUOX2} \) NADPH oxidase variants resulting in attenuated ROS production and impaired mucosal defense occur in children with VEOIBD. This may influence IBD pathogenesis beyond childhood.

**Figure 4.** Bacteria-induced translocation of \( \text{DUOX2} \) and variants does not involve \( \text{NOD2} \) in colonic cells. HT29 cells stably expressing \( \text{DUOX2 WT} \), \( \text{DUOX2 R1211C} \), and \( \text{DUOX2 R1492C} \) were exposed to enteropathogenetic \( \text{Escherichia coli} \) (EPEC) for 5 hours. Immunofluorescence images of \( \text{DUOX2 (green)} \), \( \text{NOD2 (red)} \), and nuclei (blue). Scale bar: 15 \( \mu \text{m} \).
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