NOX1 loss-of-function genetic variants in patients with inflammatory bowel disease

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Abstract

Genetic defects that affect intestinal epithelial barrier function can present with very early onset inflammatory bowel disease (VEOIBD). Using whole genome sequencing, a novel hemizygous defect in *NOX1* encoding NAPDH oxidase 1 was identified in a patient with ulcerative colitis-like VEOIBD. Exome screening of 1,878 paediatric patients identified further seven male IBD patients with rare *NOX1* mutations. Loss-of-function was validated in p.N122H and p.T497A, and to a lesser degree in p.Y470H, p.R287Q, p.I67M, p.Q293R as well as the previously described p.P330S and the common NOX1 SNP p.D360N (rs34688635) variant. The missense mutation p.N122H abrogated reactive oxygen species (ROS) production in cell lines, *ex-vivo* colonic explants and patient-derived colonic organoid cultures. Within colonic crypts, NOX1 constitutively generates a high level of ROS in the crypt lumen. Analysis of 9,513 controls and 11,140 IBD patients of non-Jewish European ancestry did not reveal an association between p.D360N and IBD. Our data suggest that loss-of-function variants in *NOX1* do not cause a Mendelian disorder of high penetrance but are a context specific modifier. Our results implicate that variants in NOX1

change brush border ROS within colonic crypts at the interface between the epithelium and luminal microbes.

Introduction

In the gastrointestinal tract, the epithelium is the primary barrier separating the high density of microbes in the intestinal lumen from the immune cells in the lamina propria. Defects in epithelial barrier function predispose to inflammatory bowel disease (IBD) in humans and cause intestinal inflammation in animal models.[1, 2]

Genetic risk variants for IBD in loci linked to genes relevant for epithelial integrity have been identified by genome wide association studies (GWAS) studies (e.g. *GNA12*, *HNF4A*, *MUC19* or *XPB1*).[3, 4] In addition, there is an expanding group of monogenic defects associated with significant functional impact on intestinal epithelial barrier function.[5, 6] Defects in intestinal epithelial guanylcyclase gene *GUCY2C* or the solute carrier family 9 member 3 (*SLC9A3*) encoding the sodium hydrogen exchanger NHE3 cause congenital diarrhea and Crohn's disease-like intestinal inflammation in approximately 30% of cases.[7, 8] Likewise, defects in *IKBKG* causing impaired NF-κB signaling result in epithelial apoptosis and intestinal inflammation.[9] In patients with TTC7A-deficiency, defects in epithelial PI4K and Rho signaling cause disturbed epithelial polarization leading to multiple intestinal atresia or very early onset intestinal inflammation.[10–12]

Recently, we reported a common *NOX1* missense mutation associated with ulcerative colitis (UC) in males of Ashkenazi Jewish ancestry, in addition to a rare *NOX1* mutation in a patient with early onset of intestinal inflammation.[13] NOX1 is the catalytic subunit of superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex 1, and is comprised of NOX1, p22^{phox}, NOXA1, NOXO1 and Rac1-GTP.[14–16] NOX1 is a close structural homologue of NOX2, the catalytic subunit of the multimeric NADPH oxidase complex 2 (NOX2, p22^{phox}, p67^{phox}, p47^{phox}, p40^{phox} and Rac2-GTP), the enzyme responsible for the microbicidal 'respiratory burst' in phagocytes. However, in contrast to NOX2, NOX1 is located in the membrane of the intestinal epithelial cells[17–21] and, may have additional functions in endothelial cells and in non-gastrointestinal epithelium.[22]

Here, we investigate several loss-of-function missense variants in *NOX1* that were identified by whole genome and whole exome sequencing in patients with VEOIBD. We identified eight males with six hemizygous non-synonymous *NOX1* variants, with variable functional impact upon reactive oxygen species (ROS) production as compared to the previously described p.P330S and the common p.D360N (rs34688635) variants. We identified two variants with nearly complete loss-of-function and a further four variants with reduced activity. Primary patient-derived intestinal colonic organoids of the loss-of-function variant p.N122H were used to validate those findings.

Results

Index patient

We studied a male patient of Caucasian origin who presented with bloody stools and failure to thrive at the age of two years (Table 1). He was diagnosed with IBD at the age of five years. His disease progressed from proctitis to pancolitis at the age of eight years without upper gastrointestinal involvement (Figure 1a). Histological examination of colonic specimens showed crypt architectural distortion, cryptitis and crypt abscess formation (Figure 1a). Histologic assessment of epithelial cell proliferation or goblet cell formation did not reveal marked differences compared to inflammatory controls (Supplementary Figure S1). The patient required oral corticosteroid therapy at diagnosis, followed by thiopurine and then methotrexate therapy. After years of poorly controlled inflammation, mucosal healing was achieved with anti-TNF therapy (adalimumab). Nine years after diagnosis, his disease was complicated by Epstein Barr virus-associated haemophagocytic lymphohistiocytosis (HLH) whilst on azathioprine therapy, requiring intensive care therapy. Mutations in *XIAP* and *SAP* were excluded at that time. There was no family history of IBD.

Whole genome sequencing of the patient led to the identification of a novel hemizygous variant in the NADPH oxidase 1 *NOX1*; NM_007052; c.A364C; p.N122H (Supplementary Figure S2). The mutation was validated with Sanger sequencing and inherited in an X-linked manner from the patient's mother (Figure 1b). The mutation is not present in 1,678 CD, 1,089 UC patients or 1,862 non-IBD controls, or in any public exome database covering an excess of 120,000 human alleles (e.g. 1000 Genomes, Exome Aggregation Consortium ExAC), thus supporting the view that this is an extremely rare genetic variant (Supplementary Table 1). The amino acid N122 is located in the 3rd transmembrane domain and is evolutionary highly conserved across species (Figure 1c). *In silico* analysis by several tools including SIFT, PolyPhen-2 and CADD,[23] predicted that the mutation causes a loss-of-function (Supplementary Table 2). Furthermore, the p.N122H variant affects all known isoforms of *NOX1* (Figure 1d). Other hemizygous, homozygous or compound heterozygous variants in the index patient were considered unlikely pathogenic (Supplementary Table 3). None of these variants have been described as causative for IBD or EBV-induced hemophagocytosis.

NOX1 expression and epithelial-derived ROS

To understand the relative distribution of ROS-generating NADPH oxidases in the human gastrointestinal tract and to compare non-inflamed homeostasis with inflammatory conditions, we determined the mRNA expression pattern of several NADPH oxidases and their subunits. Consistent with previous literature,[17–20] *NOX1* is highly expressed in the colonic epithelium with a gradient from ileum to the distal colon (Figure 1e). The subunits *NOXA1* and *NOXO1* are constitutively expressed along the human gastrointestinal tract (Figure 1e). Whereas phagocytic *NOX2* can be detected even in the non-inflamed mucosa, we observed no expression of *NOX4* and very low expression of *NOX5* (Supplementary Figure S3a). In parallel with the *NOX1* expression gradient, we found increased ROS generation in *ex vivo* biopsies obtained from lower compared to the upper gastrointestinal

tract suggesting that NOX1 is the primary source of superoxide in the non-inflamed colonic epithelium (Supplementary Figure S3b,c).

We visualized superoxide production of colonic crypts *ex vivo* using nitroblue tetrazolium chloride (NBT). Reaction of NBT with superoxide results in formation of water-insoluble blue formazan precipitates which can be observed by light microscopy (Figure 1f). Following 60 minutes incubation, we observed the deposition of formazan at the outer margin of colonic crypts indicating the constitutive release of superoxide from the colonic epithelium into the crypt lumen (Figure 1f). Co-incubation with ML171[24] or DPI, both NADPH-oxidase inhibitors, suggested a reduction of superoxide production (Figure 1g). This finding was confirmed by the luminescence assay (Figure 1h).

Ex-vivo NOX1 biopsy and primary organoids

To assess the functional consequences of p.N122H mutation on colonic epithelial ROS production, we first assessed superoxide release in *ex vivo* colonic biopsies from the index patient and compared those to biopsies from patients with IBD and non-inflamed controls. Although biopsies from the NOX1 p.N122H variant patient generated low level of superoxide, this was within the range seen in other patients with quiescent IBD and non-inflamed controls (Figure 2a). However, since phagocytes that express *NOX2* are present in non-inflamed biopsies (Supplementary Figure S3a) and lamina propria infiltrating phagocytes likely contribute to ROS measurements in IBD patient biopsies, the assay specificity is limited and an epithelial NOX1 defect is potentially masked.

NOX1 p.N122H reduces ROS production in primary organoids

To validate the functional impact of the genetic defect in epithelial cells, we generated primary epithelial organoids from colonic biopsy specimens of the NOX1 p.N122H patient, a male IBD control patient and a healthy female control without IBD (Figure 2b). Both IBD patients were in clinical remission at the time of the endoscopy. Colonic organoids express *NOX1* mRNA, along with its subunits *NOXA1* and *NOXO1* and this expression did not differ between the index p.N122H patient and the IBD control patient (Figure 2c). We found high *DUOX2* mRNA expression but only minimal *NOX2* expression in the colonic epithelial organoids reflecting the epithelial origin and the absence of phagocytes (Supplementary Figure S3a).

Colonic epithelial organoid cells carrying NOX1 p.N122H mutation produced significantly less constitutive superoxide than control organoid cells derived from the IBD patient or a patient without inflammatory disease (Figure 2d). Of note, whereas PMA stimulation increased superoxide generation by control organoids, PMA stimulation of NOX1 p.N122H organoids did not. Even after stimulation with PMA, superoxide production by NOX1 p.N122H organoids was significantly less than baseline ROS of IBD control organoids (Figure 2d).

Functional characterization of multiple NOX1 hemizygous variants in VEOIBD

To find additional *NOX1* variants in patients with VEOIBD we screened exomes of 1,878 pediatric IBD patients. We identified 5 additional non-synonymous variants in *NOX1* in

separate patients in whom no other known or plausible genetic monogenic diagnosis was established (Table 1). The missense variants were predicted to be damaging by SIFT, PolyPhen-2 or CADD scores and were rare (MAF<1%) genetic variants (Supplementary Tables 1 and 2). All variants changed conserved amino acids (Supplementary Figure S4) and were found in regions with significant homology between *NOX1* and *NOX2* (Supplementary Figure S5).

To characterize the functional impact of those variants we transfected the multimeric NADPH oxidase complex 1 in the colonic epithelial cell line HCT116. Alongside the rare *NOX1* variants p.N122H, p.I67M, p.R287Q, p.Q293R, p.Y470H and p.T497A that we identified in this study, we tested the previously described p.P330S variant[13] and the common polymorphism p.D360N (allele frequency European-non-Finnish, 2.4%). Three variants in patients P14, P15 and P16 affected potential splice sites which we could not assess functionally (Supplementary Tables 1, 2 and 4). We used HCT116 cells since these cells express only low levels of *NOX1* subunits (data not shown) and endogenous ROS generation cannot be detected (Figure 3a). Co-expression of wild-type *NOX1* together with the subunits *NOXA1* and *NOXO1* resulted in superoxide generation in unstimulated and PMA-stimulated condition (Figure 3a,b). In contrast, co-expression with *NOX1* variants variably diminished ROS generation. Whereas NOX1 variants p.N122H (index patient) and p.T497A completely abrogated both spontaneous and PMA-induced ROS production, the NOX1 variants p.I67M, p.R287Q, p.Q293R, p.P330S, p.D360N (common polymorphism) or p.Y470H were observed to either decrease spontaneous ROS and/or diminish PMA-stimulated ROS (Figure 3a,b). Expression levels of wild-type and variant *NOX1* constructs were comparable (data not shown). This suggests that the rare genetic variants affected the ROS production to a variable degree between complete loss-of-function and moderate reduction comparable to polymorphisms.

Characteristics of patients with loss-of-function NOX1 variants

We analyzed the IBD phenotype in all male patients with confirmed loss-of-function *NOX1* variants (Table 1 and Supplementary Table 4). Out of the 10 male patients with hemizygous *NOX1* mutations, four presented with CD, three with UC and three with IBDU. In congruency with *NOX1* expression, disease location was always colonic, with two patients also having perianal disease. Median age of onset was 7.9 years (range 1.8 to 10.5 years). Medical treatment included corticosteroids, aminosalicylates, azathioprine, methotrexate or anti-TNF (infliximab or adalimumab in 4 of 8 patients) and one patient underwent ileocecal resection for stricture. No infections with intestinal pathogens were recorded. With exception of the index patient, no other severe HLH/EBV-driven pathologies were recorded.

Population based characteristics of genetic loss of function NOX1 variants

In addition to those rare variants in *NOX1*, common polymorphisms might similarly contribute to IBD susceptibility. Of note, the common NOX1 polymorphism p.D360N has recently been associated with UC in Ashkenazi Jewish males.[13] To determine whether this is Ashkenazi Jewish-specific or an association across several populations, we performed a genetic analysis of individuals of non-Jewish European ancestry. Although we observed a trend in the US European ancestry cohort (Table 2 and Supplementary Tables 5 and 6),

neither analysis of allele counts in UK cohorts nor analysis of summed allele counts from a total of 9,513 healthy controls and 11,140 IBD patients revealed an association (Table 2). This suggests that the association of NOX1 p.D360N with UC in males is not present across different populations. Variation is also reflected by a variable distribution of p.D360N allele frequency across populations (Figure 4a).

To further understand the importance of *NOX1* variation, we compared the frequency of hemizygous *NOX1* missense and *NOX1* loss-of-function variants (STOP codon, frameshift, predicted splice site), with those in *NOX2*, the causative gene of most patients with chronic granulomatous disease. The frequency of genetic missense variation in *NOX1* is higher as compared to the homologous *NOX2* (Figure 4b). Similarly, the constraint metric based on the ExAC dataset indicates that loss-of-function variants in *NOX1* are tolerated as indicated by a probability of being loss-of-function intolerant[25] (pLI) of 0 (Figure 4c). In contrast *NOX2* is extremely intolerant to any loss-of-function variation (pLI 0.998)[25] as are many other genes causing X-linked primary immunodeficiencies (Figure 4c). This suggests that *NOX1* underwent less evolutionary pressure as compared to *NOX2* or other X-linked immunodeficiency genes.

In summary, our data suggest that NOX1 is the key NADPH oxidase for baseline ROS in human colonic crypt epithelium. Complete loss-of-function variants in *NOX1* are rare and not associated with a distinct IBD sub-phenotype. Population statistics suggest that loss-of-function variants in *NOX1* are rare but likely tolerated.

Discussion

In this study we investigated rare X-linked variants in *NOX1* in eight male IBD patients. Functional analysis suggests a variant-dependent loss of NOX1-mediated superoxide generation. The clinical phenotype of affected patients was early onset of IBD with progressive and severe colonic disease, refractory to conventional therapy. The affected cohort demonstrates that NOX1-dependent ROS production is essential for superoxide production in colonic crypts.

Our characterization of six rare variants in NOX1 (p.I67M, p.N122H, p.R287Q, p.Q293R, p.Y470H and p.T497A) expands the recent description of the rare NOX1 variant p.P330S and the common NOX1 polymorphism p.D360N in two male VEOIBD patients.[13] Furthermore, the NOX1 variant p.R287Q, which is functionally characterized in this study, has also been described but not characterized in an independent cohort of children with VEOIBD, adding further weight to the significance of epithelial NOX1.[26]

Diverse mechanisms maintain intestinal epithelial integrity, which serves as a critical barrier to luminal bacteria, viruses and fungi.[1, 2] Amongst these, NOX1 is likely to play a relevant role.[27, 28] The generation of superoxide by NOX1 at the colonic epithelial border may have an anti-adhesive or anti-invasive effect on bacteria, contributing to the barrier function of the inner mucus layer within colonic crypts. Indeed, our data confirm previous findings that the gene expression of *NOX1* follows the density of bacterial colonization in the gastrointestinal tract.[17–20] Reduced generation of superoxide due to genetic variants in *NOX1* may affect

crypt homeostasis allowing subsequent crypt colonization by luminal microbiota. NOX1-derived superoxide also influences intestinal epithelial differentiation and goblet cell function.[29–31] Colonic mucus produced by goblet cells provides an important barrier between the intestinal microbiota and the colonic epithelium, maintaining sterility at the epithelial border.[32]

The importance of NADPH oxidases for barrier function is also exemplified by DUOX2, an NADPH oxidase family member, which is expressed throughout the GI tract. Notably, defects in *DUOX2* have been recently described as a potential susceptibility factor in patients with VEOIBD.[13, 33] In animal models, defects in the Drosophila *duox2* homolog or in Zebrafish increased susceptibility against enteropathogens.[34, 35] Whereas *Duoxa−/−* mice on thyroid hormone replacement showed normal growth and no evident phenotype,[36] recent studies suggested that colonization of segmented filamentous bacteria in the ileum, bacterial translocation into mesenteric lymph nodes, and *Salmonella typhimurium* systemic dissemination, is dependent on DUOX activity.[37] Although *DUOX2* expression is inducible and is increased in patients with intestinal inflammation[33, 38] thereby potentially contributing to epithelial stress and intestinal tissue damage, these data indicate that DUOX activity may a functional mechanism that restricts bacterial colonization at the mucosal interface.

Interestingly, NOX1 activity regulates *DUOX2* expression in the intestinal epithelium during *C. rodentium* infection.[39] Although loss of epithelial *Cyba* protected against *C. rodentium* and *L. monocytogenes* infection, this is likely an indirect effect due to the resulting overgrowth of H_2O_2-producing *lactobacilli*.[39] This suggests an unexpected level of functional hierarchy and complex collaboration between NOX1 and DUOX2.[39]

Despite the complete loss of brush border ROS that has been observed in two *NOX1* variants (p.N122H and p.T497A), we have not observed an increased susceptibility to intestinal infections such as *Salmonella* or *Yersinia*. This is in agreement with recent data suggesting that lack of Nox1 does not affect infection susceptibility in a mouse model of *Salmonella typhimurium*-induced typhlitis.[40] Nonetheless, larger patient numbers with complete loss-of-function *NOX1* variants are required to more definitively determine the impact of these mutations on susceptibility to infection. Interestingly, the patient with the p.N122H mutation developed severe HLH in the context of EBV infection whilst on azathioprine therapy.[41] Viral spreading has been shown to be increased in Nox1-deficient mice infected with influenza virus,[42] implicating a role for NOX1 in viral control. Again, larger patient numbers are required to fully understand the penetrance of the *NOX1* variant phenotype.

Given the relatively high load of non-synonymous mutations of *NOX1* compared to *NOX2* and the large number of *DUOX2* mutations in humans, it is likely that NOX/DUOX enzymes have partially redundant functions. It is therefore likely that both NOX1 and DUOX2 play a role in susceptibility to intestinal inflammation, and loss-of-function variants alone cause not highly penetrant Mendelian type disorders. In keeping with a second hit hypothesis, Nox1-deficiency in Il10-deficient mice resulted in earlier and more severe intestinal pathology, but loss of Nox1 alone did not cause intestinal inflammation.[30] Similarly, patients with congenital secretory diarrhea due to activating mutations in the

guanylate cyclase C (*GC-C*)[7, 43] or inactivating mutations in *SLC9A3* encoding for the sodium/proton exchanger NHE3,[8] a downstream target of *GC-C*, may also present with an IBD phenotype. In these patients the decreased epithelial uptake of sodium results in changes of mucosal ion content and pH, and Nhe3-deficiency in mice leads to an altered composition of the intestinal microbiota.[44, 45] This dysbiosis drives intestinal inflammation in a T cell-dependent model of intestinal inflammation.[46] Our studies suggest that loss of ROS at the colonic crypt may have a similar effect on barrier function.

Altogether these data suggest a highly context specific role of epithelial NADPH oxidases in intestinal homeostasis. Whilst in some animal models, a lack of Il10 and Nox1 increases epithelial stress and susceptibility to colitis[30], in other instances, lack of NADPH oxidase activity is protective. Indeed a protective role of Nox1 and Duox2 is suggested in a mouse model of ileocolitis caused by lack of protective glutathione peroxidase factors.[47–49] In this model GPx1- and GPx2-knockout leads to oxidative stress caused by Nox1 and Duox2 with a dominant role of Nox1 for crypt epithelial apoptosis.[48, 49] This oxidative stress is dependent on the presence of the intestinal microbiota since germ free mice are protected and colitis develops within one week of bacterial colonization.[50] Given the role of NOX1 as a modifier of intestinal microbiota, we can only speculate whether IBD patients with loss-of-function *NOX1* variants would benefit from therapeutic microbiome-altering strategies (e.g. antibiotics, nutrition, probiotics or fecal transplant). Further studies characterizing the human gut microbiota in the setting of NOX1 deficiency are required.

It is possible that the human population genetics reflect this context specific role of *NOX1*. Whereas in populations like Ashkenazi Jews, the hemizygous NOX1 p.D360N variant is associated with UC, in European descent populations it is not. Substantially stronger genetic variation of *NOX1* compared to *NOX2* suggests that the overall impact on disease susceptibility of these genes is significantly different despite the high degree of protein conservation and similarity. It is even possible, that the degree of genetic variation in *NOX1* is caused by environmental factors such as infections where reduced ROS is protective.

In conclusion, we report six novel variants in the *NOX1* gene in patients with IBD, two of which are associated with complete loss-of-function of the gene product and with loss of ROS production. Our study illustrates the complexity of epithelial barrier function in the pathogenesis of IBD and the need better to understand the functional impact of ROS production at the colonic crypt epithelium as a cause of colonic inflammation.

Methods

Human subjects

The recruitment of participants was approved by relevant ethics committees or institutional review boards at the individual contributing institution. Written informed consent was obtained from all patients/legal guardians and healthy controls.

Patients with IBD (including the index patient P1) and controls without intestinal inflammation were prospectively recruited as a part of the Oxford IBD cohort study or the Oxford Gastrointestinal Illness Biobank (REC 09/H1204/30 & REC 11/YH/0020).

Replication genotyping of the p.N122H variant was performed in 2,767 IBD patients and 1,862 non-IBD controls. These individuals were enrolled by the UK IBD Genetics Consortium (http://www.ibdresearch.co.uk/). Exomes of 1,878 pediatric onset IBD patients without genetic diagnosis were screened for additional rare *NOX1* variants. Samples were previously genotyped as part of the COLORS in IBD study (n = 150; Oxford, UK), the NEOPICS consortium (n = 545; Toronto, Canada, www.neopics.org) or the RISK cohort[51] (n = 1,183; Atlanta, USA). Genetic association testing of NOX1 p.D360N (rs34688635) was conducted on genotype data from IBD patients of non-Jewish European ancestry from the following cohorts: the European ancestry exome chip dataset[52] (n = 10,523), the Oxford IBD cohort study (n = 513) and the COLORS in IBD study (n = 104). Sequence data from healthy individuals were obtained from the European ancestry exome chip dataset[52] (n = 5,726) and the INTERVAL study (n = 4,436; www.intervalstudy.org.uk). Population controls were also obtained from the UK10K project (www.uk10k.org).

Genotyping

Whole genome sequencing and NOX1 variant p.N122H identification—The index mutation in *NOX1* was identified by whole genome sequencing as part of the WGS500 project.[53] DNA was analyzed from peripheral blood mononuclear cells. In summary, whole genome sequencing was performed on 3.5–7.5 ng DNA on either the Illumina HiSeq2000 or the HiSeq2500 run in standard mode using v2.5 or v3 sequencing chemistry. The genomic DNA was fragmented, end-paired, A-tailed and adapter-ligated before size selection and amplification for a multiplexed library preparation as described in Taylor et al.[53] The libraries were paired end sequenced and sequenced to an average coverage between 27 and 40 quality reads per base. Resulting 100 base pair reads were mapped to the GRCh37d5/hg19 human reference sequence using Stampy and the SNVs and short InDels were called with Platypus.[54] Variant files were analyzed using QIAGEN's Ingenuity® Variant Analysis™ software (www.qiagen.com/Ingenuity, QIAGEN Redwood City). High quality calls were analyzed. For candidate analysis, we investigated a gene list of known variants associated with monogenic forms of IBD.[5] Filters for variant frequency (<1%; 1000 Genomes Project) and predicted deleterious filters (SIFT and PolyPhen) were applied to prioritize high quality calls for non-synonymous and likely pathogenic variants.

Replication genotyping of the p.N122H variant—Replication genotyping on the Sequenom plex was carried out at the Sanger Institute.

Whole exome sequencing—Regeneron performed whole exome sequencing on a cohort of 1,183 probands with pediatric onset IBD (ages 0–18.5 years), including their affected and unaffected parents and siblings, where available (total samples = 2,704). Sample preparation, whole exome sequencing, and sequence data production were performed as previously described.[55] The exomes of RISK cohort study participants (n = 545) were captured using SureSelect Human All Exon kit v2 (Agilent Technologies), using 3 μg of genomic DNA. Subsequently, captured libraries were sequenced on Illumina HiSeq2000 by 100-base pair paired-end (PE) sequencing. Image processing and base calling was performed using standard Illumina software. Raw sequence reads were mapped relative to the human genome

reference sequence (GRCh38/hg38) using the PEMapper software tool (Cutler DJ et al, submitted) to identify single nucleotide variants (SNVs).

Sanger sequencing—*NOX1* variants were validated using Sanger sequencing according to standard techniques.

Histology

Historic routine haematoxylin and eosin histology sections from the patient identified to harbor the NOX1 p.N122H mutation and IBD controls were analyzed. Staining of goblet cells or the proliferation marker Ki67 was performed on archived paraffin-embedded colonic epithelial biopsies. Specimens were stained using an Alcian blue and periodic acid-Schiff's reagent staining kit (Clin-Tech Limited, UK) or rabbit monoclonal antibody to Ki67 (clone SP66, AbCam, Cambridge, USA), respectively. Nuclear fast red and haematoxylin (Sigma-Aldrich, Gillingham, UK) were used to counterstain cell nuclei.

Protein sequence alignment

Multiple sequences were aligned using ClustalW2.[56] Data were obtained from the National Center for Biotechnology Information (NCBI). Sequence alignment is based on the following accession numbers: *Danio rerio,* NP_001095857.1; *Xenopus tropicalis,* XP_002939249.2; *Gallus gallus*, NP_001095300.1; *Mus musculus*, NP_757340.1; *Homo sapiens,* NOX1 isoform 1–3, NP_008983.2, NP_039249.1 and NP_001258744.1; *Homo sapiens,* NOX2, NP_000388.2.

Expression analysis

Colonic biopsies were stored in RNAlater (Qiagen) prior to analysis. RNA was extracted using RNAeasy kit (Qiagen), transcribed in cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and expression data were obtained using TaqMan primers (Life Technologies) for *NOX 1–5*, *NOXO1*, *NOXA1*, *DUOX1* and *DUOX2*. Results were normalized to *GAPDH* expression. Relative gene expression was calculated using the Ct method.

Colonic epithelial organoid cultures

Human colon crypts were isolated and organoids were cultured as previously described.[57] Briefly, crypts were embedded in Cultrex® Reduced Growth Factor Basement Membrane Extract, (BME) Type 2 RGF PathClear (Amsbio) and seeded in 48-well plates. The BME was polymerized for 10 minutes at 37°C, and 250 μL/well of organoid culture media was added. Organoid culture media consisted of advanced Dulbecco's modified eagle medium/F12 supplemented with penicillin/streptomycin (10 mmol/L), HEPES (1 M), Glutamax (10 mmol/L) and the following growth factors: Wnt conditioned media (50%) B27 (1×), EGF (50 ng/mL), Noggin (10%), R spondin (20%), gastrin (10 nM), Nicotinamide (10 mM), A83-01 (500 nM) and SB202190 (10 μM). Y-27632 (10 μmol/L; Sigma) was included in the medium for the first five days in culture. Once organoid growth was established, the culture media was refreshed every three days.

Transfection of HCT116 cells

The colonic epithelial cell line HCT116 was maintained in McCoy's 5A Modified (Gibco) supplemented with 10% fetal bovine serum. Cell systems were based on reconstitution of the multimeric NOX1 complex containing NOX1, NOXO1 and NOXA1 in HCT116 cells. PcDNA3.1-hNox1, pcDNA3.1-hNoxa1 and pcDNA3.1-hNoxo1 were kind gifts from Botond Banfi & Karl-Heinz Krause (Addgene plasmid # 58344, # 58531 and # 58530).[19, 58] Mutations were introduced using site-directed mutagenesis and were verified by sequencing (Eurofins Genomics). HCT116 cells were transfected with Lipofectamine 2000 (Invitrogen) following manufacturer's instruction. Expression of wild-type and *NOX1* variants was confirmed by quantitative PCR. ROS production was analyzed by L-012 enhanced chemiluminescence 48 hours post-transfection.

ROS assays

ROS generation by colonic epithelial biopsies, organoid cells and transiently transfected HCT116 cells was measured using L-012 enhanced chemiluminescence (Wako laboratories). After washing with PBS, cells or biopsies were pre-incubated in L-012 (100 μM) for 10 minutes, followed by stimulation with Phorbol 12-myristate 13-acetate (PMA, 5 μg/mL, Sigma) or addition of diphenyleneiodonium (DPI, 10 μM, Sigma) and 2-acetylphenothiazine (ML171, 10 μM, Tocris) in indicated conditions. Emission of light was recorded at baseline and thereafter as a kinetic assay every 1–10 minutes in opaque white 96-well plates (FLUOstar OPTIMA, BMG labtech).

For standardization, all intestinal epithelial biopsies were collected using Olympus Endojaw needle forceps with a cup-opening size of 7.2 mm and analyzed within 90 minutes of collection with minimal handling. Organoids were trypsinised into single cell suspension using TrypLETM express (Gibco), counted and equal cell numbers dispensed into white 96-well plates.

For microscopy of ROS-producing epithelial cells, *ex vivo* colonic biopsies were incubated with 100 μg/mL nitroblue tetrazolium chloride (NBT, Sigma) in PBS-glucose (1 g/L) with or without NADPH-oxidase inhibitors. After 60 minutes incubation, formation of formazan was analyzed by light microscopy (Carl Zeiss Axioskop2, 200× magnification) on glass slides without cover slips. Images were photographed using Q-Imaging software. Microscopy images were cropped and analyzed using ImageJ software.[59]

Disease activity amongst IBD patients was characterized as active or quiescent on the basis of clinical symptoms (diarrhea, rectal bleeding, and abdominal pain), endoscopic features (mucosal vascular pattern, bleeding, or ulceration), histological features (cryptitis and crypt abscesses, lamina propria polymorphonuclear cell infiltrate), and serum biomarkers (C-reactive protein).

Data and statistical analysis

All the data analyses were performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, California, USA) or R (The Foundation for Statistical Computing, R version 3.2.2). Unpaired data were compared using Mann-Whitney U test and paired data

were compared with Wilcoxon signed-rank test. Significance of differences between multiple groups was performed with ANOVA and post-test correction with Dunnet. Genetic association testing in cases and controls was conducted with the ALLELIC test.[60] Pooled analyses were performed on the summed allele counts of different cohorts. P-values of less than 0.05 after correction for multiple testing were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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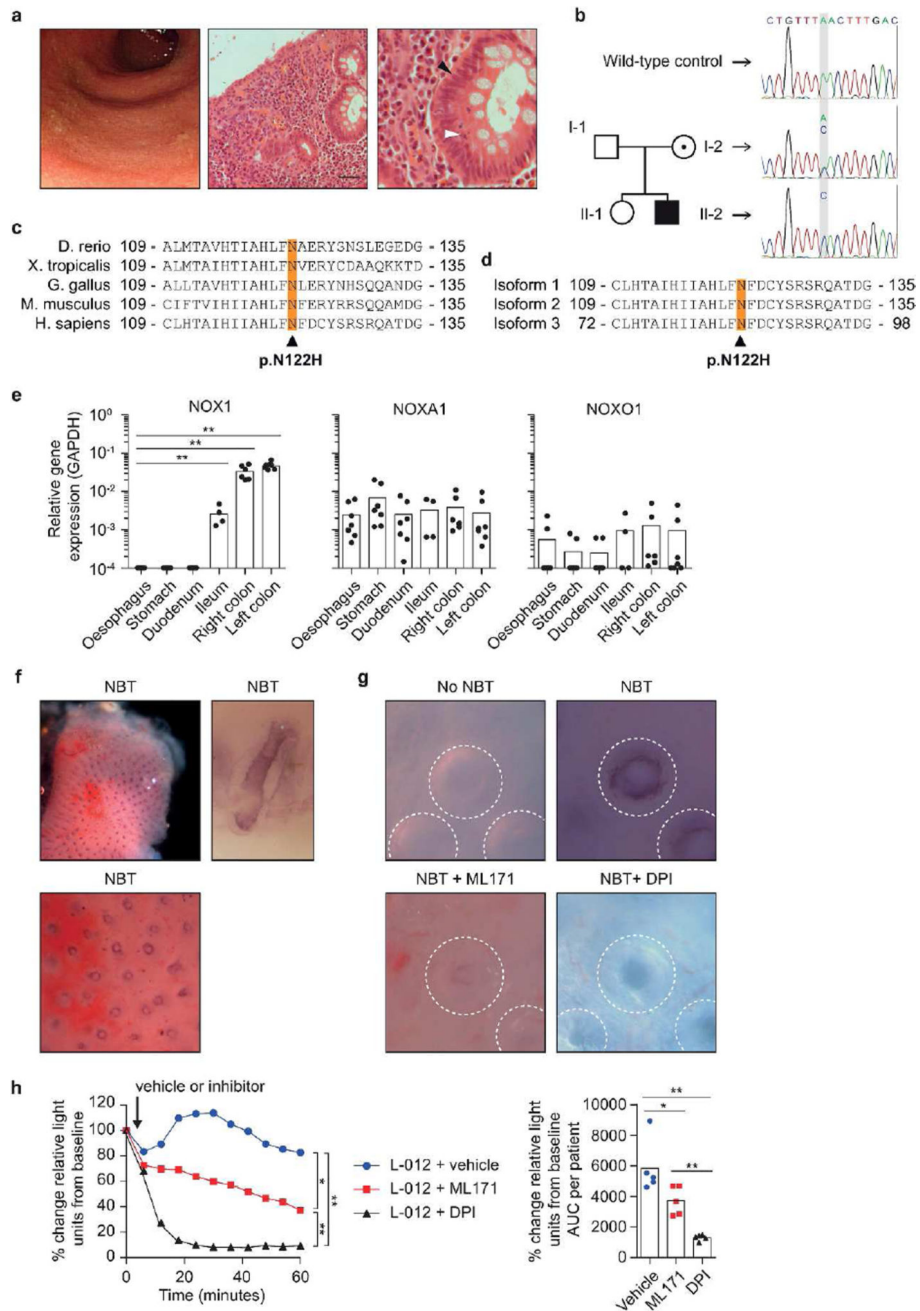
Figure 1. Clinical phenotype of the NOX1 p.N122H patient and NOX1 contribution to baseline ROS within colonic crypts

(a) Endoscopy and histology from NOX1 mutant patient. Colonoscopy shows loss of vascular pattern, ulceration and luminal bleeding (left). H&E stain of colonic biopsies shows crypt architectural distortion and dense inflammatory cell infiltrate in the lamina propria (scale bar 50 μm, middle). Magnification of NOX1 mutant epithelial crypt demonstrates elongated nuclei (black arrow head) and crypt abscesses (white arrow head, right). **(b)** Family pedigree and Sanger sequencing of *NOX1* gene around the mutation site in a wild-type control and family members. **(c)** NOX1 protein sequence alignment of different genera

showing the evolutionary conserved asparagine at position 122. **(d)** The NOX1 variant p.N122H affects all three isoforms of NOX1. Amino acid sequence was aligned using ClustalW. **(e)** Analysis of *NOX1, NOXA1* and *NOXO1* expression by quantitative PCR in biopsies of the gastrointestinal tract. Single biopsies per segment were obtained from 4–7 non-inflamed individuals. Bars represent mean. Statistical significance was determined by Mann-Whitney U test. **(f)** *Ex-vivo* colonic biopsies (within 90 minutes of collection) were incubated with nitroblue tetrazolium (NBT) in PBS-Glucose at room temperature. Biopsy specimens were analyzed using light microscopy immediately after 60 minutes NBT incubation (top left, biopsy overview showing dark crypts containing reduced formazan; bottom, ring-like deposition of formazan at epithelial margin of crypts; top right, single colonic epithelial crypt captured longitudinally at high magnification). **(g)** Microscopy of ROS-producing epithelial cells in colonic crypts of *ex-vivo* biopsies detected by NBT dye (200× magnification). After 60 minutes of incubation a ring-like deposition of formazan at the epithelial margin of crypts was evident. In indicated conditions biopsies were co-incubated with either a NOX1 inhibitor (ML171) or a non-specific NAPDH oxidase inhibitor (DPI), to verify the signal. **(h, left)** DPI or ML171 inhibit superoxide generation and L-012-enhanced chemiluminescence on colonic biopsy specimens *ex-vivo*. Following the detection of superoxide at baseline, DPI, ML171 or vehicle were added (black arrow) and chemiluminescence was recorded over 60 minutes. For each biopsy light emission was normalized to baseline reading. Data represent mean of 9 biopsies per condition obtained from 5 non-inflamed controls. **(h, right)** Results of kinetic assay calculated as AUC per patient. Statistical significance was determined by Mann-Whitney U test.
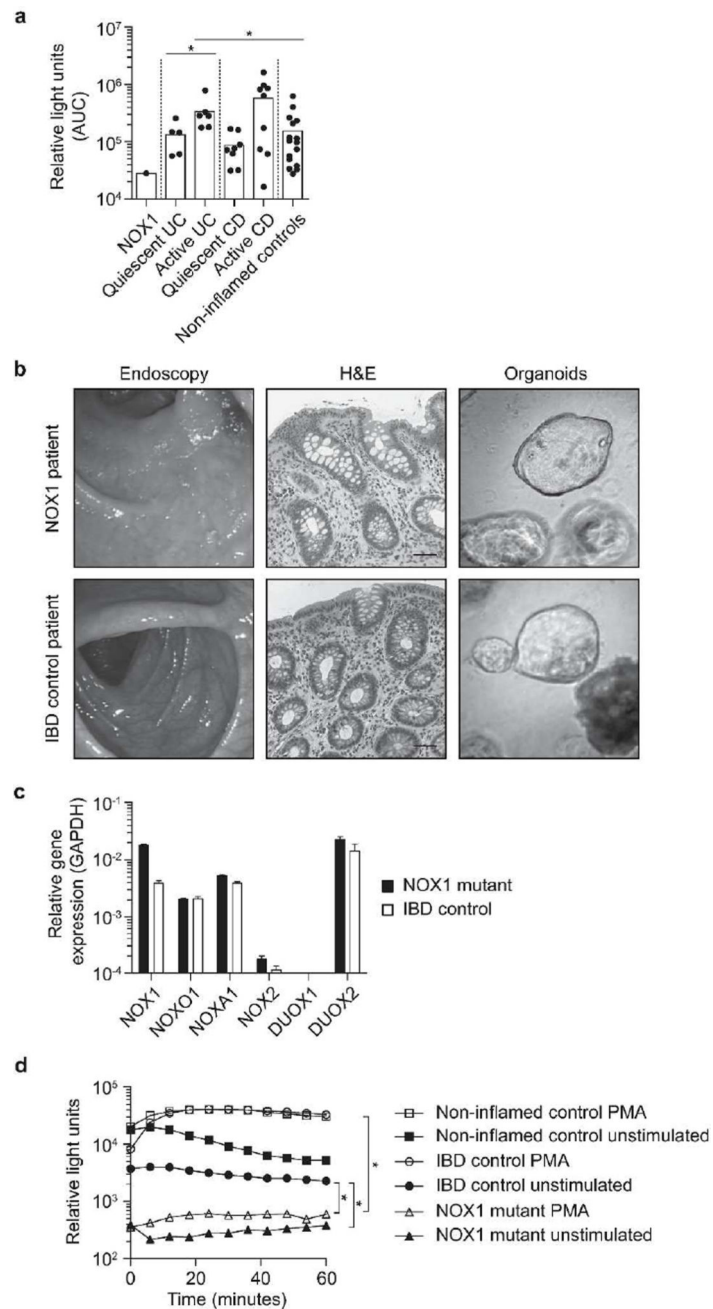
Figure 2. NOX1 p.N122H diminishes epithelial ROS in primary organoids

(a) Superoxide generation of colonic epithelial biopsies measured over 60 minutes by L-012-enhanced chemiluminescence and total light emission quantified by area under the curve (AUC). Results are derived from 17 patients with colonic Crohn's disease (active disease n = 10), 11 patients with ulcerative colitis (active disease n = 6), 16 non-inflamed controls and the patient with NOX1 p.N122H mutation. One symbol represents the mean of 1–3 pan-colonic biopsy specimens (both right and left sided) per patient. Active versus quiescent disease dichotomised according to composite assessment including endoscopic appearance (mucosal ulceration, loss of vascular pattern, bleeding), biomarkers (CRP), and

histology (density of inflammatory infiltrate, cryptitis, crypt abscesses). Statistical significance was determined by Mann-Whitney U test. **(b)** Organoids from patient with *NOX1* mutation and IBD control patient were generated from colonic biopsies obtained during quiescent disease state. Representative images of endoscopy, histology (H&E staining, scale bar 50 μm), and organoids are displayed. **(c)** Analysis of NADPH oxidases and certain subunits by quantitative PCR in NOX1 patient and IBD control organoids. **(d)** Kinetic assay of PMA-activated L-012-enhanced chemiluminescence on NOX1 mutant, IBD control and non-inflamed control organoid cells. Relative light units (RLU) were measured over 60 minutes. Results are representative of 2 independent experiments measured in replicates. Statistical significance was determined by Wilcoxon signed-rank test comparing the AUC.
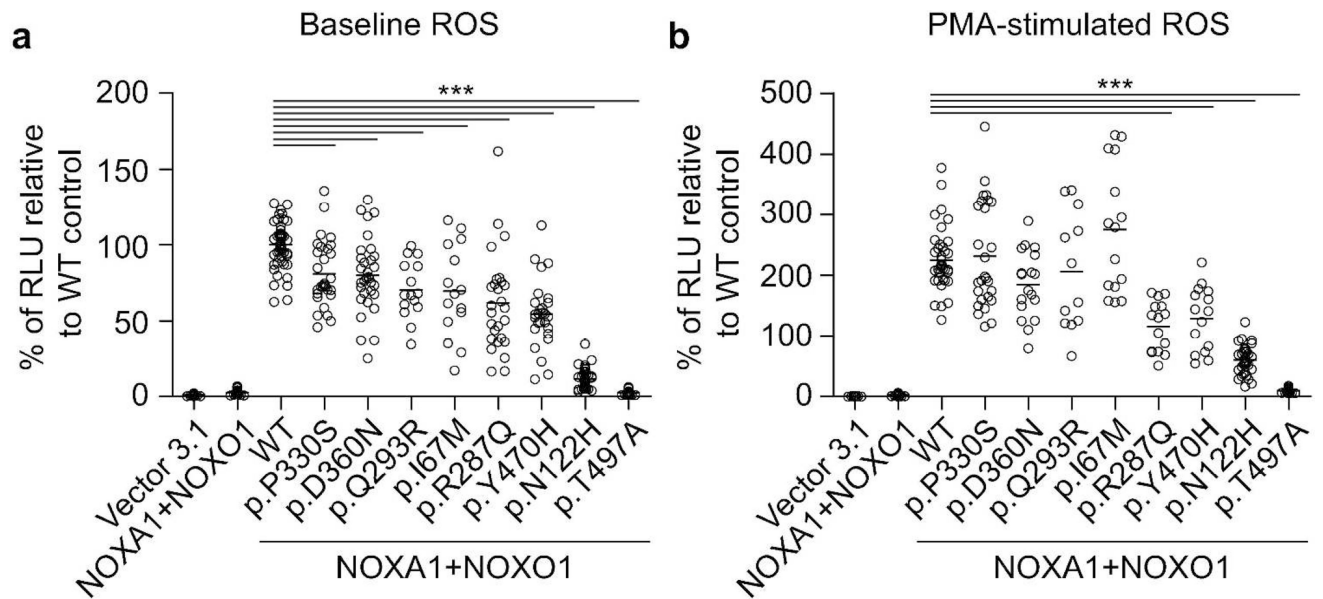
Figure 3. Comparative analysis of *NOX1* variants
(**a, b**) Generation of superoxide in colonic epithelial cell line HCT116 reconstituted with multimeric NADPH oxidase complex and expressing wild-type or variant *NOX1*. ROS was analyzed by L-012-enhanced chemiluminescence in the absence (**a**) or presence of PMA (**b**). Chemiluminescence was measured 48 hours post-transfection and recorded over 60 minutes. Results represent eight independent experiments with 3–4 replicates. Each dot represents one replicate. Experiments were normalized to mean relative light units (RLU) without PMA. Line represents mean. Statistical significance was determined with ANOVA and post-test correction performed with Dunnett (multiple comparisons to one single control).
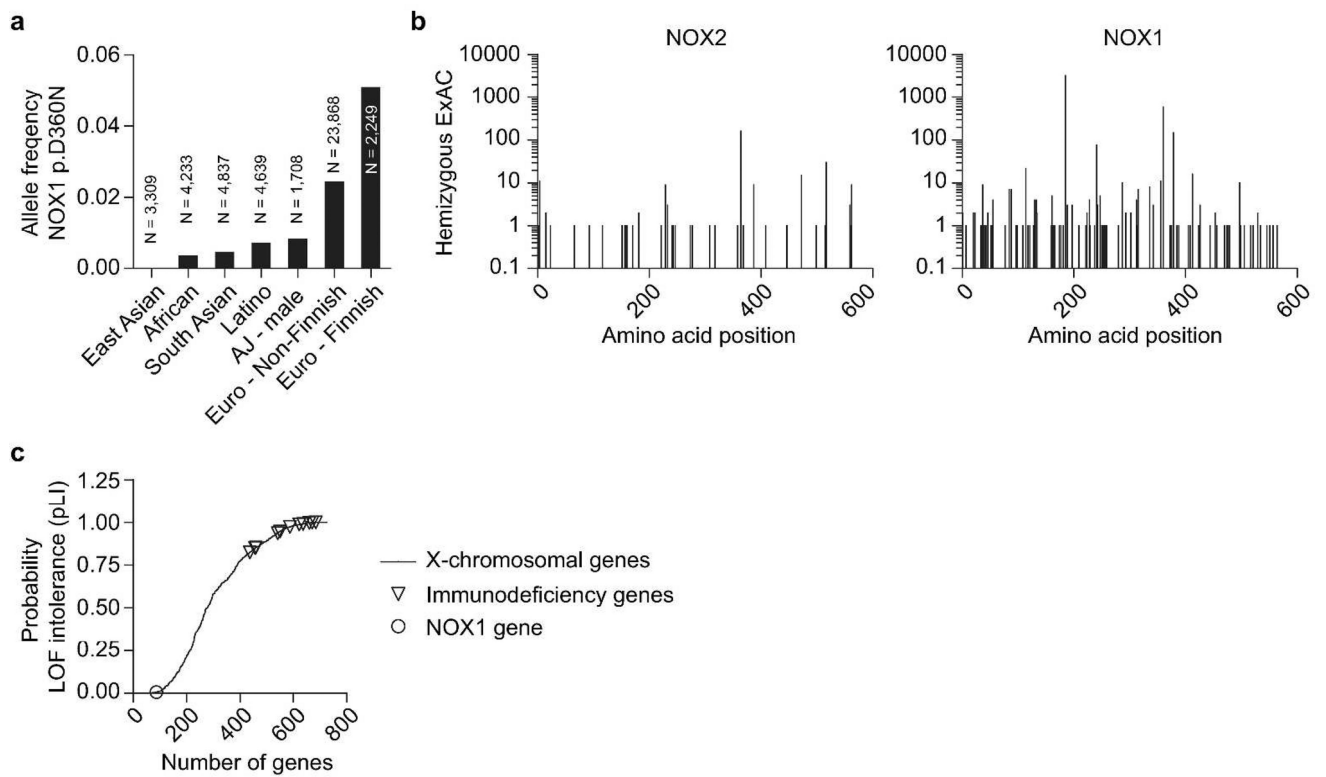
Figure 4. NOX1 population frequencies and genetic variation
(a) Allele frequency of NOX1 p.D360N in different populations according to Exome Aggregation Consortium (ExAC) data and published literature.[13] **(b)** Number of hemizygotes found for a given variant (missense, nonsense, frameshift) in NOX2 and NOX1 is blotted according to amino acid position (X-axis). Frequency is based on ExAC data. **(c)** Probability of loss of-function intolerance (pLI) of 727 human X-chromosomal genes based on ExAC data. X-linked primary immunodeficieny genes and *NOX1* gene are highlighted.

Table 1

Clinical characterization of male patients with non-synonymous NOX1 hemizygous variants.

Patient ID	NOX1 variants	Gen der	Age of diagnosis (of symptoms) in years	Diagnosis Paris class.[62]	Family history for IBD	Intestinal and extra-intestinal symptoms/findings on examination (age in years)	Histology	Treatment for IBD
P1	c.A364C p.N122H	M	5 (2)	IBDU; E4	Negative	Presented with bloody stools and failure to thrive (2), progression from proctitis (5) to pancolitis (19), terminal ileum normal, perianal skin tags; EBV-associated HLH (14) whilst on AZA	Cryptitis, crypt architectural distortion, crypt abscesses, focal Paneth cell metaplasia, single micro-granuloma.	Oral CS, ASA, AZA, MTX, ADA
P2	c.T1408C p.Y470H	M	9.7	CD; L3L4a, B1	Negative	Initially presented with weight loss and rectal bleeding	Granulomata, neutrophilic infiltration into crypts and surface epithelium	Oral CS, IFX, MTX
P3	c.C201G p.I67M	M	8.6 (7.8)	UC; E4	Mother (UC)	Presented with abdominal pain and alternating formed stool/ diarrhea with blood/mucous (7.8). At diagnosis microscopic pancolitis (8.6), later macroscopic disease in rectosigmoid (17.5); No EIMs	Neutrophil infiltrate, (peri-) cryptitis, branching and focal dropout of glands, regeneration. No granuloma.	5-ASA
P4	c.G860A p.R287Q	M	9.9 (9.5)	CD; L3L4a, B1p	Brother and aunt (CD)	Presented with abdominal pain and loose stools (9.9); right colon and ileal disease distribution (13); Perianal skin tags and fistula (15.8)	Granulomata, focal branching, (peri-) cryptitis, intraepithelial lymphocytes, lymphoplasmacytic infiltrate, basal cell hyperplasia	5-ASA, SALZ, AB
P5	c.G860A p.R287Q	M	10.5 (8.5)	CD; L3L4ab; B2p	Brother and aunt (CD)	Presented with long standing abdominal pain, variably loose stools/constipation (8.5), and more recent periodic blood PR (10.3). Diagnostic scope primarily small bowel disease and minor colonic involvement; perianal fistula (10.5).	Cryptitis, pericryptitis, crypt distortion, cell infiltrate, giant cells. No granulomas.	5-ASA, AB, IFX, CS, NUT, ADA. Ileocecal resection for stricture (12.8); second ileocecal resection (15.6)
P6	c.G860A p.R287Q	M	5.7 (5.3)	UC; E2	cousins (CD or UC)	Presented with rectal bleeding (5.3). At scope, diagnosed with left sided colitis (5.7). No GI Sx since age 9yo. At 15yo, diagnosed with Stage IVB non-bulk nodular sclerosing	Chronic inflammatory infiltrate	Previously SALZ. Currently on no therapy.

Patient ID	NOX1 variants	Gender	Age of diagnosis (of symptoms) in years	Diagnosis Paris class.[62]	Family history for IBD	Intestinal and extra-intestinal symptoms/findings on examination (age in years)	Histology	Treatment for IBD
						Hodgkin's disease. In remission following therapy.		
P7	c. A878G p.Q293R	M	9.7 (9.4)	CD: L2L4a	Negative	Presented with bloody diarrhea, arthralgia, lethargy, and weight loss (9.4). Pancolitis at diagnosis (9.7), upper GI inflammation (17.5); arthralgias	Cryptitis, crypt abscesses, Paneth cell metaplasia, apoptotic epithelial cells involving most colonic crypts, transepithelial inflammation. No granulomas.	AB, IV and oral CS, SALZ, MTX, IFX
P8	c.A1489G p.T497A	M	7.1 (7.0)	UC; E4	Negative	Bloody diarrhea (7.0); diagnosed with pancolitis (7.1). Single episode of pancreatitis (13.3). In clinical remission since age 13.3.	Focal cryptitis, crypt abscesses, moderate architectural distortion, paneth cell metaplasia,	5-ASA, SALZ, CS.

Abbreviations: AB, oral or intravenous antibiotics given for treatment of colitis, bowel decontamination, and fistula treatment; ADA, adalimumab; 5-ASA, 5-aminosalicylic acid; AZA, azathioprine; CD, Crohn's disease; CS, corticosteroids; EBV, Epstein–Barr virus; EIMs, extra-intestinal manifestations; F, female; HLH, hemophagocytic lymphohistiocytosis; IBDU, IBD unclassified; IFX, infliximab; M, male; MTX, methotrexate; NUT, polymeric/elemental diet; PR, per rectum; SALZ, sulfasalazine; UC, ulcerative colitis.

Table 2

Population genetics of p.D360N variant

Gender	Ancestry	Group	N	MAF (%)	p-value Cases vs. controls	OR
Male		Control	4744	2.66		
	European	IBD	5549	2.74	0.843	1.03
		UC	2462	3.45	0.068	1.31
		CD	2895	2.18	0.217	0.82
	Ashkenazi Jewish[13]	Control	1708	0.82		
		UC	297	3.37	1.25×10^{-3}	4.22
Female		Control	4769	2.44		
	European	IBD	5587	2.77	0.149	1.14
		UC	2263	3.05	0.041	1.26
		CD	3138	2.50	0.857	1.02
	Ashkenazi Jewish[13]	Control	906	0.99		
		UC	262	1.53	0.343	1.55

MAF, minor allele frequency; OR, odds ratio. P-values were determined by the ALLELIC test or Fisher's exact method[13].