CROHN’S DISEASE

Functional variants in the LRRK2 gene confer shared effects on risk for Crohn’s disease and Parkinson’s disease

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Crohn’s disease (CD), a form of inflammatory bowel disease, has a higher prevalence in Ashkenazi Jewish than in non-Jewish European populations. To define the role of nonsynonymous mutations, we performed exome sequencing of Ashkenazi Jewish patients with CD, followed by array-based genotyping and association analysis in 2066 CD cases and 3633 healthy controls. We detected association signals in the LRRK2 gene that conferred risk for CD (N2081K variant, tagging R1398H-associated haplotype, \( P = 9.5 \times 10^{-10} \)) or protection from CD (N551K variant, tagging R1398H-associated haplotype, \( P = 3.3 \times 10^{-4} \)). These variants affected CD age of onset, disease location, LRRK2 activity, and autophagy. Bayesian network analysis of CD patient intestinal tissue further implicated LRRK2 in CD pathogenesis. Analysis of the extended LRRK2 locus in 24,570 CD cases, patients with Parkinson’s disease (PD), and healthy controls revealed extensive pleiotropy, with shared genetic effects between CD and PD in both Ashkenazi Jewish and non-Jewish cohorts. The LRRK2 N2081D risk allele is located in the same kinase domain as G2019S, a mutation that is the major genetic cause of familial and sporadic PD. Like the G2019S mutation, the N2081D variant was associated with increased kinase activity, whereas neither N551K nor R1398H variants on the protective haplotype altered kinase activity. We also confirmed that R1398H, but not N551K, increased guanosine triphosphate binding and hydrolyzing enzyme (GTPase) activity, thereby deactivating LRRK2. The presence of shared LRRK2 alleles in CD and PD provides refined insight into disease mechanisms and may have major implications for the treatment of these seemingly unrelated diseases.

INTRODUCTION

The inflammatory bowel diseases (IBD) are composed of two major subtypes, Crohn’s disease (CD) and ulcerative colitis (UC), which are distinguished by the distribution of chronic inflammatory changes. In UC, the inflammation is relatively superficial and is confined to the colon. CD most commonly affects the terminal ileum (last part of the small intestine) and colon and is frequently associated with deep, transmural inflammation, often resulting in obstruction and abscess formation requiring resectional surgery.

Approved medical therapies for moderate to severe IBD are the same for CD and UC and include monoclonal antibodies against the proinflammatory tumor necrosis factor (TNF) cytokine and, more recently, antibodies against the αβ7 integrin, which blocks leukocyte trafficking to the intestine. However, present therapies provide prolonged deep remission in only a minority of IBD patients. Consequently, there is a substantial unmet need for more effective medical therapies, especially for CD patients. Genome-wide association studies (GWAS) have identified over 200 loci associated with IBD (1, 2), providing many new potential therapeutic targets. The large majority of these loci are common to CD and UC, implicating numerous pathways, notably the proinflammatory interleukin-23 (IL-23) pathway. In particular, R381Q within the IL-23 receptor (IL23R) is a loss-of-function allele that confers protection against developing IBD (3).

Monoclonal antibodies blocking the IL-23 pathway have demonstrated efficacy in IBD and a favorable safety profile (4). CD-predominant loci include nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and a number of autophagy genes [for example, ATG16L1—autophagy-related 16 like 1—and IRGM—immunity-related guanosine triphosphate (GTP)–binding and hydrolyzing enzyme (GTPase) family M protein]. NOD2 is an intracellular receptor for bacterial peptidoglycan and is expressed in a wide variety of cells including plasma cells, innate immune leukocytes (for example, monocytes, macrophages, and dendritic cells), and Paneth cells, which are located at the base of the small intestinal (but not typically colonic) crypts and produce potent antimicrobial peptides. Loss-of-function NOD2 risk alleles are associated with inflammation in the ileum rather than colon and a younger age of onset with an earlier need for resectional surgery. Among the autophagy-associated signals are the ATG16L1 T300A allele that results in ATG16L1 degradation through caspase-3 activation (5) and multiple polymorphisms in the 5q33.1 region that cause tissue-specific variation in IRGM expression (6, 7).

However, a fundamental limitation of common variant-predominant GWAS is the imprecise definition of genes, specific alleles, and mechanisms driving most association signals identified thus far, with the large majority of independent GWAS signals driven by common variants of modest statistical and functional effects. Furthermore, common
variation in composite is predicted to contribute only a modest fraction of expected heritability for many diseases. For these reasons, major sequencing efforts to identify rare variants of potentially higher statistical and functional effects are of importance for refining the pathways associated with disease pathogenesis and designing new therapies.

We hypothesized that uncommon CD susceptibility alleles with higher effects [that is, odds ratios (ORs)], which had eluded analysis in common variant-predominant GWAS, play an important role in genetic predisposition to CD and can elucidate new insights into CD pathogenesis. Here, we sought to identify the strongest functionally relevant associations and to characterize their biological implications. Given that a major epidemiological feature of IBD is its several-fold higher prevalence in Ashkenazi Jewish cohorts (8, 9) compared to that in non-Jewish Europeans, we performed exome sequencing of Ashkenazi Jewish CD cases, followed by custom array–based genotyping in a large case-control cohort. We identified independent coding CD risk and protective alleles in LRRK2, a large multifunctional gene that confers the greatest genetic effects reported thus far in Parkinson's disease (PD), a neurodegenerative movement disorder affecting the basal ganglia and characterized by resting tremor, bradykinesia, rigidity, and postural instability (10). The presence of shared alleles in CD and PD provides refined insight into disease mechanisms and may have major implications for the treatment of these two seemingly unrelated diseases.

RESULTS
Exome sequencing and HumanExome chip study design
We first performed exome sequencing of 50 Ashkenazi Jewish individuals with CD randomly selected from high-quality DNA samples and confirmed using prior chip data (11) to have 100% Ashkenazi Jewish ancestry to optimize cataloguing of new variants (fig. S1 and table S1). From these results, we selected 4277 putatively high-yield new mutations, adding these to the HumanExome BeadChip (fig. S2 and table S2). We next performed discovery-phase genotyping and association analyses in individuals with full genetic Ashkenazi Jewish ancestry (fig. S3 and table S3) (11).

Top coding-region associations in CD
In the discovery-phase cohort of 1477 unrelated CD cases and 2614 independent healthy controls, nonsynonymous variants at three loci on chromosomes 1, 12, and 16 demonstrated associations that reached a chip-wide significance (Table 1). In addition to the previously reported NOD2 and IL23R alleles, nonsynonymous variants [N2081D in LRRK2 and S6N in SLC2A13 (solute carrier family 2 member 13)], in strong linkage disequilibrium (LD) with each other ($r^2 = 0.91$), were identified to be associated with CD risk [minor allele frequency (MAF) in CD, 8.1%; OR, 1.73; $P = 2.56 \times 10^{-10}$; and MAFCD, 8.1%; OR, 1.73; $P = 2.68 \times 10^{-5}$, respectively]. The LRRK2 N551K variant was also associated with CD protection (MAFCD, 6.6%; OR, 0.65; $P = 7.06 \times 10^{-7}$; Table 1, Fig. 1A, and fig. S4). We then evaluated the evidence for CD association in an independent Ashkenazi Jewish cohort of 589 CD and 1019 controls (table S3). This replicated the association signals at LRRK2 N2081D (MAFCD, 7.4%; OR, 1.34; $P = 4.40 \times 10^{-5}$), at SLC2A13 S6N (MAFCD, 7.7%; OR, 1.46; $P = 9.58 \times 10^{-3}$), and at LRRK2 N551K (MAFCD, 7.0%; OR, 0.72; $P = 1.27 \times 10^{-2}$).

Meta-analysis revealed genome-wide significant CD risk at LRRK2 N2081D ($P = 9.51 \times 10^{-15}$) and at SLC2A13 S6N ($P = 1.39 \times 10^{-15}$) and protection at LRRK2 N551K ($P = 3.28 \times 10^{-7}$). A list of all coding variants with discovery-phase association $P < 2 \times 10^{-5}$ is provided in table S4. Notably, R1398H (MAFCD, 6.6%; OR, 0.71; $P = 7.33 \times 10^{-5}$) and K1423K (MAFCD, 5.9%; OR, 0.66; $P = 4.4 \times 10^{-6}$) in the LRRK2 gene, which previously have been reported to combine with N551K to form a protective haplotype in PD (12–15), were found to show weaker associations in CD (table S4).

Previous studies have implicated distinct common alleles in the LRRK2 region as being associated with CD (1, 16, 17). To further elucidate the genetic structure of the LRRK2 signal, we conducted a

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but not genome-wide significant, association with CD (unconditional analysis using the discovery cohort, which demonstrated that this broad association peak was entirely dependent on the coding mutation at N2081D in \textit{LRRK2} (Fig. 1B); SLC2A13 S6N, as well as the association signal from the previously reported GWAS hits, including nonsynonymous variant rs3761863 (M2397T) (16, 18), was substantially attenuated. Including N2081D genotypes as covariates verified the independence of the protective association signal at \textit{LRRK2} N551K linked to lower CD risk (OR, 0.67; \( P = 1.4 \times 10^{-15} \); Fig. 1B). Including N551K or R1398H genotypes from the protective haplotype as a covariate had minimal effect on the association signal. In phased haplotype association analysis (table S5), the N2081D risk variant occurred completely on the background of the protein-destabilizing allele M2397 (MAF\textsubscript{CD}, 45%; pairwise \( D' = 1.0; r^2 = 0.09 \) (18)), whereas the N551K-protective variant co-resided with the stabilizing 2397T (18) allele (pairwise \( D' = 0.94; r^2 = 0.06 \)). Adjusting for both N551K and N2081D variants together effectively eliminated the association signal at M2397T (conditioned, \( P = 0.015 \); unconditioned, \( P = 5.9 \times 10^{-4} \)).

The multifunctional kinase \textit{LRRK2} has attracted considerable attention, given that variants in this gene have been recognized as major risk factors for PD (19). Notably, the G2019S mutation in \textit{LRRK2} (the best known genetic cause of familial and sporadic PD worldwide and located in the same kinase domain as N2081D) showed suggestive, but not genome-wide significant, association with CD (unconditioned, OR, 1.9; \( P = 4.8 \times 10^{-3} \)) and no LD with N2081D (\( r^2 = 0.0 \)) in the Ashkenazi Jewish cohort.

Further replication and validation of the shared CD- and PD-associated alleles within the \textit{LRRK2} locus

To replicate our findings in the non-Jewish cohorts and to explore the pleiotropic effects of \textit{LRRK2} variation on CD and PD risk, we expanded our analysis to include a total of 8314 independent Ashkenazi Jewish and 16,401 independent non-Jewish participants comprising 6538 CD cases, 5570 PD cases, and 12,607 healthy controls genotyped in previous studies (table S3). After performing imputation and quality control measures, we conducted association testing on the set of \textit{LRRK2} variants in these data sets (see Supplementary Materials and Methods). As in the discovery cohort, in both Ashkenazi Jewish and non-Jewish validation cohorts, we observed a multimarker CD-associated signal within the \textit{LRRK2} gene (table S6) that was highly dependent on N2081D as a covariate (fig. S5, A and B). In addition, adjusting for N551K or R1398H as a covariate had minimal effect on the broad association peak. In the non-Jewish data set, association results showed similar marginal effects for N2081D \( [ \text{OR}_{\text{AJ}} , 1.7 \, (\text{CI}, 1.4 \text{ to } 2.0) \text{ versus OR}_{\text{NJ}} , 1.6 \, (\text{CI}, 1.3 \text{ to } 2.0)] \) and for N551K \( [ \text{OR}_{\text{AJ}} , 0.67 \, (\text{CI}, 0.57 \text{ to } 0.79) \text{ versus OR}_{\text{NJ}} , 0.89 \, (\text{CI}, 0.79 \text{ to } 1.0)] \), or R1398H \( [ \text{OR}_{\text{AJ}} , 0.71 \, (\text{CI}, 0.60 \text{ to } 0.84) \text{ versus OR}_{\text{NJ}} , 0.88 \, (\text{CI}, 0.78 \text{ to } 0.99)] \) but with substantially lower MAFs, especially for N2081D \( (\text{MAF}_{\text{AJ,CD}}, 8.0\% \text{ versus MAF}_{\text{NJ,CD}}, 2.9\%); \) Table 2). Notably, G2019S did not have nominally significant CD association \( (P = 0.12) \), likely because of subtle stochastic fluctuations in allele frequencies during imputation.

To examine the genetic link between CD and PD, we then assessed PD association with \textit{LRRK2} N2081D and N551K/R1398H in Ashkenazi Jewish and non-Jewish cohorts, observing association signals for all polymorphisms (Table 2). Specifically, the OR estimates of the protective variants, N551K and R1398H, were similar between CD and PD, with slight differences between Ashkenazi Jewish and non-Jewish cohorts \( [ \text{N551K: OR}_{\text{AJ,CD}} , 0.67 \, (\text{CI}, 0.57 \text{ to } 0.79) \text{ versus OR}_{\text{NJ}}, 0.77 \, (\text{CI}, 0.67 \text{ to } 0.90); \text{OR}_{\text{NJ,PD}}, 0.87 \, (\text{CI}, 0.77 \text{ to } 1.0); \text{R1398H: OR}_{\text{AJ,CD}}, 0.71 \, (\text{CI}, 0.60 \text{ to } 0.84) \text{ versus OR}_{\text{NJ}}, 0.84 \, (\text{CI}, 0.72 \text{ to } 0.98); \text{OR}_{\text{NJ,PD}}, 0.88 \, (\text{CI}, 0.78 \text{ to } 0.99); \text{OR}_{\text{NJ,PD}}, 0.88 \, (\text{CI}, 0.77 \text{ to } 1.0)] \). However, in both populations, the risk allele N2081D showed higher ORs in association with CD \( [ \text{OR}_{\text{AJ,CD}}, 1.7 \, (\text{CI}, 1.4 \text{ to } 2.0); \text{OR}_{\text{NJ,CD}}, 1.6 \, (\text{CI}, 1.3 \text{ to } 2.0)] \) than with PD \( [ \text{OR}_{\text{AJ,PD}}, 1.1 \, (\text{CI}, 1.0 \text{ to } 1.4); \text{OR}_{\text{NJ,PD}}, 1.3 \, (\text{CI}, 1.0 \text{ to } 1.6)] \). Adjusting for N2081D or N551K demonstrated no difference, with G2019S remaining the dominant PD signal (fig. S5, C and D).

To determine the degree of pleiotropy in the 5-megabase (Mb) genomic region centered on \textit{LRRK2}, we selected variants at least nominally \( (P < 0.05) \) associated with both CD and PD and assessed their direction and magnitude of effect across diseases. After LD pruning (that is, removal of correlated mutations with pairwise \( r^2 > 0.8 \)), thus
ensuring statistical independence among the remaining mutations), we detected a consistent pattern of correlated effect sizes, with 23 of 26 independent variants (88%) exhibiting effects in the same direction for both diseases in the Ashkenazi Jewish data set (binomial, \( P = 5.2 \times 10^{-5} \)) and, similarly, 25 of 29 variants (86%) in the non-Jewish data set (\( P = 7.6 \times 10^{-8} \); Fig. 2). Together, our findings suggest extended pleiotropy (shared genetic effects) between CD and PD throughout the LRRK2 locus.

**Network analysis of IBD patient tissues**

Given a strong LD within the LRRK2 locus containing several plausible candidate genes, including SLC2A13 and MUC19 (table S6), we conducted network analysis to explore which of these genes participates in biological pathways involved in CD pathogenesis. We constructed an IBD Bayesian network, using a previously described methodology (20), from gene expression data for 8382 genes. The expression data were collected in 203 intestinal biopsies that included ileum, ascending colon, descending colon and transverse colon, and inflamed and noninflamed sigmoid and rectum, all collected at baseline from 54 anti-TNFα–resistant CD patients enrolled in the Ustekinumab (anti–IL-12/IL-23) clinical trial (21, 22). Among the full set of genes, we defined a specific subset, located within IBD-associated loci previously defined in an immunochip-based large-scale genetic analysis (1), with the goal of projecting these genes onto the intestinal network and identifying coexpressed genes that act together. We then excluded genes previously associated with PD (23), including LRRK2 and genes within 1 Mb of LRRK2, to see whether either LRRK2 or other genes would be “recovered” by the network as being coexpressed with the IBD-associated genes. We found that the largest connected subnetwork of genes, which represents a set of coexpressed IBD-associated genes, contained LRRK2 but no other genes in the genomic neighborhood of LRRK2 (Fig. 3), thus implicating LRRK2 in IBD pathogenesis. Notably, of the 622 genes in this subnetwork, there were 102 (16.4%) IBD-associated genes, a 2.5-fold enrichment compared to the full intestinal network (hypergeometric, \( P = 7.6 \times 10^{-8} \)).

LRRK2 was closely connected to GPR65, a proton-sensing guanine nucleotide-binding protein–coupled receptor associated with IBD and altered lysosomal function (24), and to HLA-DPA1, an α subunit of the major histocompatibility complex protein/peptide-antigen receptor and a graft-versus-host disease antigen complex linked to both IBD (25) and PD (26).

**Effect of LRRK2 mutations on protein kinase activity and GTPase activity**

Prior studies in PD suggest a central role for increased LRRK2 kinase activity in disease risk resulting from gain-of-function mutations in the LRRK2 kinase domain. Given that both PD-risk G2019S and CD-risk N2081D are located in the kinase domain (Fig. 4A), we investigated the effect of CD-associated LRRK2 mutations on kinase activity. Specifically, we quantified phosphorylation of a newly identified LRRK2 substrate, Ras-related protein 10 (Rab10) (27), by wild-type LRRK2 protein and mutant LRRK2 proteins bearing G2019S, R1398H, N551K, N551K + R1398H, or N2081D mutations that were expressed and purified from human embryonic kidney (HEK) 293T cells (Fig. 4B). We demonstrated an ~30% increase in phosphorylated Rab10 (pRab10) in the presence of the LRRK2 N2081D mutation compared to wild-type LRRK2 (Fig. 4B) and also confirmed a previous report that the G2019S mutation increased pRab10 (27). In contrast, no change was observed in pRab10 in the R1398H-, N551K-, or N551K + R1398H–transfected HEK293T cells. ROC, a Ras/GTPase domain of complex proteins, is also a common site of PD-linked LRRK2 mutations, which presumably retain a higher fraction of LRRK2 in a GTP-bound “on” state, thereby promoting increased kinase activity and subsequent neurodegeneration (28, 29). The PD-protective R1398H variant, which...

is in strong LD with the CD-protective N551K variant, is located in the ROC domain (Fig. 4A). To determine the effects of LRRK2 variants on LRRK2 GTPase activity, we compared the ratio of guanosine di-phosphate (GDP)/GTP-bound LRRK2 in vitro across the variants (Fig. 4C). We found that the GTPase activity was increased in both LRRK2 R1398H- and N551K + R1398H–transfected HEK293T cells but not in HEK293T cells transfected with G2019S, N2081D, or N551K mutations (Fig. 4C).

Table 2. Allele frequencies and association statistics for LRRK2 nonsynonymous variants in imputed data sets. CI, confidence interval.

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<th>Variant</th>
<th>MAFCD (%)</th>
<th>MAFCPD (%)</th>
<th>MAFCtrl(%)</th>
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*Combined control MAF. Each healthy control was randomly assigned to only one disease association analysis to ensure independence. P values calculated using logistic regression.

Role of LRRK2 mutations in cytoskeletal and autophagic function in macrophages from CD patients

To further investigate the properties of the LRRK2 mutations (Fig. 4A), we characterized human monocyte-derived M1 macrophages collected from CD patients who carried LRRK2 N2081D (n = 4), N551K + R1398H (all samples selected for their N551K carrier status also carried R1398H; n = 5), or neither mutation (n = 4) in response to cellular serum-nutrient starvation (Fig. 5). No differences were detected in total LRRK2 expression by mutation status. Because LRRK2 has been reported to influence acetylation of α-tubulin, thus regulating cellular protein trafficking via the microtubule cytoskeleton, we determined the effect of the LRRK2 mutations on α-tubulin protein acetylation (Fig. 5A). Lower acetylation of α-tubulin was detected in macrophages from N2081D patient carriers under normal culture conditions and saline-induced starvation, suggesting impaired resting acetylation activity and a lack of response to cellular stress. In contrast, the highest basal acetylation of α-tubulin was detected in macrophages of noncarriers and carriers of the protective N551K + R1398H mutations and proportionally decreased after cellular stress induced...
pared lysosomal acidity, a key factor in autophagy, in response to stress, LRRK2 phages compared to N551K + R1398H macrophages, whereas all cells observed a smaller reduction in p62 expression in N2081D macro-
protein facilitating cargo recognition. After nutrient starvation, we (LC3B), and sequestosome-SQSTM1/p62 (p62), a ubiquitin-associated
bound form of the microtubule-associated protein 1 light chain 3
of the mutations on autophagy markers LC3-II, an autophagosome-
30 with autophagy (by nutrient starvation. Because -tubulin acetylation is associated.
Fig. 3. An LRRK2-focused subnetwork within the inflammatory bowel disease–associated gene network. A coexpression-based Bayesian network was constructed using gene expression data from intestinal biopsies in inflammatory bowel disease (IBD) patients to elucidate IBD-relevant genes within the chromosome 12q12 region. The full network comprised 8382 genes, of which 551 (6.6%) were IBD-associated. The largest connected subnetwork of genes defined within a distance that is two genes away from a known IBD-associated gene is shown. These IBD-associated genes were delineated in a previous immunochip-based study (1). Red nodes indicate established IBD-associated genes, and the cyan node represents LRRK2. Dark blue nodes indicate all other genes.
by nutrient starvation. Because -tubulin acetylation is associated 
with autophagy (30), one of the major pathophysiological processes involved in CD (and in PD) development, we next investigated the effect of the mutations on autophagy markers LC3-II, an autophagosome-bound form of the microtubule-associated protein 1 light chain 3β (LC3B), and sequestosome-SQSTM1/p62 (p62), a ubiquitin-associated protein facilitating cargo recognition. After nutrient starvation, we observed a smaller reduction in p62 expression in N2081D macrophages compared to N551K + R1398H macrophages, whereas all cells displayed a similar LC3-II ratio (stress/control) regardless of LRRK2 genotype (Fig. 5A). Despite little change in LC3-II, which is sometimes insensitive to autophagy alterations, a low response of p62 expression to stress suggested an impairment of cargo clearance. Finally, using a lysosome permeable fluorescent pH indicator (LysoSensor), we compared lysosomal acidity, a key factor in autophagy, in response to stress, between the LRRK2 N2081D and N551K mutant macrophages (Fig. 5B). We found that the relative change in mean fluorescence intensity after starvation, although varying among individuals, was decreased (alkaline) in risk N2081D carriers and increased (acidic) in carriers of the protective N551K + R1398H variants (Fig. 5B). These data suggest that N2081D and N551K + R1398H mutations in CD patient macrophages have opposing effects on LRRK2 protein function that, in turn, can alter the autophagy-lysosome response to cellular stress.

Additive effects and phenotypic impact of LRRK2 variants
In contrast to the dominant effect of the G2019S mutation on PD risk, we observed an additive effect of N2081D mutations on CD risk because testing for dominant and recessive disease models did not show any increase in association statistical significance (table S4). To assess the strength of the combined effect across the LRRK2 variants, we calculated additive burden scores (defined as the log sum of the number of risk-conferring alleles carried by each individual, weighted by the CD OR, which is highly correlated with PD OR, as shown in Fig. 2) based on their genotypes. The additive effects of the LRRK2 risk alleles strongly correlated with both CD and PD risk (fig. S6), indicating an overall similar genetic architecture throughout the LRRK2 locus underlying both diseases. There was no evidence of interaction effects between any of the nominally associated variants.

Moreover, because of a recent study implicating essential roles for both NOD2 and LRRK2 in proper lysozyme sorting in Paneth cells (31), a group of secretory cells in the ileum with a vital role in maintaining the function of the epithelial barrier, we next examined the effect of LRRK2 N2081D risk alleles on CD disease location. Whereas 80.5% of CD patients homozygous for the wild-type LRRK2 allele had ileal involvement, heterozygous and homozygous carriers of the N2018D variant demonstrated ileal involvement in 86.1 and 90.9% of individuals, respectively (P = 0.01, χ² test; Table 3). In addition, carrying the risk allele at N2081D was significantly associated with a younger age of onset (26.5 years for noncarriers, 24.6 years for heterozygous carriers, and 20.8 years for homozygous carriers; P = 0.002, linear regression). Neither LRRK2 N551K nor R1398H showed any meaningful correlation with age of onset or ileal involvement in CD (Table 3).

DISCUSSION
Here, we performed exome sequencing, followed by array-based exome chip genotyping, in several independent cohorts of Ashkenazi Jewish CD cases and controls. Among protein-coding variants, in addition to the well-established NOD2 and IL23R associations, we observed genome-wide significant associations for chromosome 12q12 risk alleles.S6N in SLC2A13 and N2081D in LRRK2 (P < 5 × 10⁻⁸), in high LD with each other (r² = 0.91), and an independent protective CD-association signal at LRRK2 N551K. All previous GWAS association signals in or near LRRK2 were significantly attenuated when including N2081D as a covariate. This included the common coding variant M2397T (16) reported in one study to lower posttranscriptional LRRK2 protein (18). Given the high LD between S6N in SLC2A13 and N2081D in LRRK2, we applied coexpression approaches to define the likely contributing gene. In our Bayesian network analysis of IBD intestinal tissue, we observed a highly connected subnetwork containing IBD-associated genes and LRRK2, but not containing any other genes within the chromosome 12q12 region, including SLC2A13. SLC2A13 is a glucose transporter that is not expressed in the gut or the immune system and has not been previously linked to IBD, further suggesting that
lysosomal function (24), and the observed 12q12 signal is driven by the LRRK2 gene. In this gene expression network, LRRK2 was connected to GPR65, a gene with an IBD-associated risk allele at I231L that has been implicated in impaired LRRK2 expression network, the observed 12q12 signal is driven by the LRRK2 domain structure and the respective locations of the N551K, R1398H, and N2081D amino acid substitutions relative to the previously reported PD-associated G2019S mutation and CD-associated M2397T mutation. (B) Representative immunoblot (left) and quantification (right) of Rab10 phosphorylation (pRab10) by wild type (WT) and LRRK2 variants in patient macrophages in vitro. (C) Guanosine triphosphate binding and hydrolyzing enzyme (GTPase) activity of WT and LRRK2 variants. Representative guanosine triphosphate (GTP) hydrolysis assay (left) and the fraction of hydrolyzed GTP (guanosine diphosphate, GDP) over bound GTP (right). All values represent the mean of three independent experiments ± SE, and significance was calculated by analysis of variance (ANOVA). *P ≤ 0.05 and **P ≤ 0.01. ARM, armadillo; ANK, ankyrin repeat region; LRR, leucine-rich repeat; ROC, Ras of complex proteins; COR, C terminal of ROC; MAPKK, mitogen-activated protein (MAP) kinase kinase; WD40, WD40 protein-protein interaction domain.

Notably, both LRRK2 N2081D and N551K variants were also associated with PD in both Ashkenazi Jewish and non-Jewish cohorts (Table 2). Whereas previous reports have documented that LRRK2 N2081D confers PD risk and that the N551K-R1398H-K1423K haplotype confers protection (12–15), we now demonstrate that these specific nonsynonymous variants in LRRK2 genetically link CD to PD. Despite the same direction of the effect, the effect size for the risk variant N2081D was substantially higher for CD compared to PD (Table 2). G2019S, the maximally associated risk allele in PD (32, 33) occurring in the same domain as N2081D (Fig. 4A), although not in LD with it, showed suggestive association with CD in the Ashkenazi Jewish discovery cohort only. Further association analysis of independent common variants in >24,500 PD and CD cases and controls suggested additional extensive genetic pleiotropy between CD and PD within the extended LRRK2 locus, with a consistent pattern of correlated effect sizes (Fig. 2) in both Ashkenazi Jewish and non-Jewish data sets. A recent independent report has suggested that PD is associated with an increased risk of IBD (34). Together, these results point toward potential shared genetic and epidemiological links between these two diseases and can help identify a subgroup of patients with CD who are at a higher risk for developing PD.

Numerous functional roles for LRRK2 have been reported, including vesicular trafficking and endocytosis, protein synthesis, immune response regulation, inflammation, and cytoskeleton homeostasis, among others (35). In addition to their association with PD and CD risk, variations in the LRRK2 locus have been also independently linked to excessive inflammatory responses in patients with leprosy (36) and risk of particular types of cancer (37). In the gastrointestinal tract of CD patients, LRRK2 expression is restricted to lamina propria macrophages, dendritic cells, and B lymphocytes and is induced by interferon-γ, which is consistent with its role in IBD (38). A recent study has found high expression of LRRK2 in Paneth cells in the ileum, demonstrating that both NOD2 and LRRK2 are required for proper lysozyme sorting in Paneth cells (31). Our correlations of N2081D in LRRK2 to an earlier age of CD onset and an ileal location mirror previously reported NOD2 risk allele phenotypic correlations. Specifically, we showed that carriers of two copies of the N2081D risk allele had almost a 6-year earlier age of onset compared to noncarriers and predominantly ileal disease involvement, which may be consistent with the recent report of the effects of LRRK2 in Paneth cells (39) that are exclusively located in the small intestine. These findings are of clinical importance because a large recent phenotype-genotype analysis of all IBD-associated loci identified only a handful of mutations, including in NOD2, that had considerable effects on age of onset and disease location in CD; in that study, the LRRK2 N2081D variant was not specifically tested (40). Defining altered Paneth cell function stratified according to various LRRK2 and NOD2 genotype combinations should be a focus of future studies.

Most of the PD-causing mutations fall within the kinase, Ras of complex proteins (ROC) and a C-terminal of ROC (COR) domains, resulting in increased kinase activity or GTP binding, leading to neurodegeneration. Our findings showed that both kinase domain disease-associated mutations, G2019S (PD) and N2081D (CD), increased the phosphorylation of the LRRK2 substrate Rab10. Previous studies have reported that the G2019S mutation increases the phosphorylation of
Moreover, we also showed the link between the protective ROC domain R1398H mutation and an increase in GTPase activity (42). Although our statistical analysis prioritized the N551K mutation as significantly associated with a reduced risk of CD, in our biochemical analysis, N551K alone did not yield any detectable effect. On the basis of a high LD between N551K and R1398H mutations and the fact that all N551K human carriers that were analyzed also carried R1398H, we tested the combined effect of N551K + R1398H on GTPase activity and concluded that the actual physiological protective effect is driven by R1398H and not by N551K. Notably, human macrophages from N551K + R1398H carriers also demonstrated an enhanced autophagy response to cellular stress.

We speculate that the precise nature of the lysosomal alterations likely differs between these two diseases. Autosomal recessive mutations in the GBA (glucosylceramidase β) gene, which cause Gaucher’s disease and are highly associated with PD (with most cases involving dominant transmission), are also prevalent in Ashkenazi Jewish populations. Here, we did not find GBA mutations to be associated with CD. This would suggest that PD and CD pathophysiologies differ in the cell-specific properties of the lysosomes in neurons or glia versus inflammatory or Paneth cells, respectively, or with respect to distinct hydrolytic targets, namely, glycolipids versus bacterial peptidoglycans, respectively. Nevertheless, naturally occurring protective alleles, such as the R1398H variant in LRRK2, are of particular importance because they define a desired functional effect for therapeutic development.

Just as the loss-of-function, protective R381Q variant in IL23R would predict that blocking the IL-23 pathway would be safe and effective, our present findings suggest that targeting LRRK2-mediated signaling may be beneficial in the treatment of both CD and PD.

**Table 3. Subphenotypic values by LRRK2 N2081D and R1398H genotype in pooled Ashkenazi Jewish and non-Jewish CD cohorts.** Similar results were found for the N551K variant (in strong LD with R1398H; r² = 0.81). P values were calculated using simple linear regression. SD, standard deviation; N, group sample size; ns, not significant.

<table>
<thead>
<tr>
<th>N2081D genotype</th>
<th>Age of CD onset (SD) [N]</th>
<th>Disease location in ileum [N]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>26.5 (14.0) [5601]</td>
<td>80.5% [5311]</td>
</tr>
<tr>
<td>GA</td>
<td>24.6 (13.1) [482]</td>
<td>86.1% [453]</td>
</tr>
<tr>
<td>GG</td>
<td>20.8 (9.0) [12]</td>
<td>90.9% [11]</td>
</tr>
</tbody>
</table>

P = 0.002

<table>
<thead>
<tr>
<th>R1398H genotype</th>
<th>Age of CD onset (SD) [N]</th>
<th>Disease location in ileum [N]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>26.3 (13.9) [5365]</td>
<td>81.1% [5095]</td>
</tr>
<tr>
<td>GA</td>
<td>26.4 (14.1) [701]</td>
<td>80.7% [652]</td>
</tr>
<tr>
<td>AA</td>
<td>27.2 (19.4) [29]</td>
<td>71.4% [28]</td>
</tr>
</tbody>
</table>

P = 0.01

ns

Several Rab family members, leading to an abnormal cytosol-membrane Rab protein distribution, which could result in the disruption of autophagy (27). Consistent with this report, our studies in human monocyte-derived macrophages from CD patients carrying the N2081D mutation demonstrated faulty stress responses directly related to autophagy, including impaired autophagic cargo clearance, lysosomal acidification, and tubulin acetylation defects also found in PD models (41).
Among the limitations to our study is the fact that our CD cohorts were not explicitly screened for PD and vice versa, potentially allowing for the inclusion of individuals with both diseases in one disease category (either CD or PD). However, both CD and PD are relatively rare in the general population (~0.2 and ~1%, respectively), and misclassification of such patients would be expected to have minimal impact on any analyses. In addition, we studied the Ashkenazi Jewish population, given its higher CD prevalence, but this focus limited our cohort size and thus the power to identify new, rarer contributing alleles. Because the exome-sequencing phase of our study involved only 50 individuals, there are certainly many rare Ashkenazi Jewish–specific variants that were not tested in the association phases, and some of these likely play a role in CD pathogenesis. Finally, our Bayesian network analysis, while offering a method to examine gene function in an unbiased manner apart from disease association, did so indirectly and with only gene expression data from whole tissue used to construct our network.

Our study strongly implicates the contribution of LRRK2 in CD risk, as shown through multiple complementary approaches, including genome-wide screening, Bayesian network analysis, genotype-phenotype correlations, and functional studies. The LRRK2 N2081D risk allele and the N551K/R1398H protective alleles, as well as numerous other variants within the LRRK2 locus, revealed shared genetic effects between CD and PD risk, providing a potential biological basis for clinical co-occurrence. Our findings suggest that LRRK2 may be a useful target for developing drugs to treat CD.

**MATERIALS AND METHODS**

**Study design**

We first performed exome sequencing of 50 Ashkenazi Jewish individuals with CD (44 independent individuals and three full-sibling pairs) having sufficient power to detect new variants with an MAF of >0.015 to catalog variation in the Ashkenazi Jewish population that may confer risk for CD (43). Because little genetic variation in Ashkenazi Jewish data sets was available from prior public genome sequencing, we sought to extend the coverage of available commercial genotyping platforms by adding new variants detected in our exome sequencing analyses. In particular, we favored polymorphic sites that were less likely to be tagged in a previous well-powered GWAS of CD in the Ashkenazi Jewish population, given its higher CD prevalence, but this focus limited our cohort size and thus the power to identify new, rarer contributing alleles. Because the exome-sequencing phase of our study involved only 50 individuals, there are certainly many rare Ashkenazi Jewish–specific variants that were not tested in the association phases, and some of these likely play a role in CD pathogenesis. Finally, our Bayesian network analysis, while offering a method to examine gene function in an unbiased manner apart from disease association, did so indirectly and with only gene expression data from whole tissue used to construct our network.

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**Discovery and replication of new variants associated with CD**

We performed $\chi^2$-based association testing on all variants genotyped by the Exome chip. We tabulated all nonsynonymous variants with $P$ values suggestive of CD association ($P < 2 \times 10^{-8}$), a threshold we estimated using Bonferroni correction with the approximate number of polymorphic variants genotyped using our platform. This enabled strong and widespread correlations among exomic variants (that is, “chip-wide significance”). We collected genotypes at these markers in independent case and control cohorts with full Ashkenazi Jewish ancestry. These replication data were combined with those generated by Exome chip genotyping for a meta-analysis using the METAL program with default parameters (48); coding variation with genome-wide significant $P$ values ($P < 5 \times 10^{-8}$) is presented as positive association signals (Table 1).

**Imputation-based comparative analysis of CD and PD**

Additional non-Jewish CD and PD and Ashkenazi Jewish PD data sets were added to the Ashkenazi Jewish CD data (imputation cohorts; table S3), and reference-free imputation using MACH was performed to facilitate direct comparisons across groups at specific variants (49). Both unconditioned and conditional analyses were conducted using logistic regression on pooled empiric (directly genotyped) and probabilistic (imputed) genotypes.

**Network analysis**

We constructed an adult IBD Bayesian network, using previously described methodology (20), from gene expression data generated on 203 intestinal biopsies that included ileum, ascending, descending, and transverse colon, inflamed and noninflamed sigmoid, and rectum, all collected at baseline from 54 anti-TNFα–resistant CD patients enrolled in the Ustekinumab (anti–IL-12/IL-23) clinical trial (21) with the goal of projecting these genes onto the intestinal network and identifying coexpressed genes that act together. This type of probabilistic causal network structure has previously been demonstrated to represent biologically functional pathways across a broad range of diseases including obesity and diabetes (20, 50–52), asthma and chronic obstructive pulmonary disease (53, 54), and Alzheimer’s disease (55). We next excluded genes previously associated with PD (23), including LRRK2 and genes within 1 Mb of LRRK2, to see whether either LRRK2 or other genes could be “recovered” by the network as being coexpressed with the IBD-associated genes. We then identified the largest connected subgraph from the set of IBD-associated genes projected onto the network. To focus on pathways potentially relevant to CD pathogenesis, we removed, from our analysis, all genes more than two edge lengths away from any of these IBD-associated genes.

**Rab10 in vitro kinase assay**

LRRK2 was incubated with Rab10 or inhibitor for 30 min incubation on ice in 30 μl of kinase buffer [20 mM tris (pH 7.5), 1 mM diithiothreitol (DTT), 15 mM MnCl₂, and 20 mM β-glycerophosphate], Reactions were initiated by adding 50 μM cold adenosine triphosphate (ATP). After
30 min at 37°C, reactions were stopped by addition of Laemmli buffer and boiling at 95°C for 10 min. Samples were resolved on 4 to 12% precast SDS–polyacrylamide gel electrophoresis (PAGE) gels (Invitrogen). Samples were then subjected to Western blot using anti-Rab10 (ab2462, Cell Signaling Technology) and anti-pT173 Rab10 (University of Dundee). LI-COR imaging was used to detect phospho- and total Rab10 on the same membrane, and Image Studio Lite was used for quantification.

**GTP hydrolysis assay**

GTPase activity of LRRK2 was measured in 30 μl of GTPase buffer [20 mM tris (pH 7.5), 150 mM NaCl, 1 mM DTT, 5 mM MgCl2, and 1 mM EDTA] at 30°C for 90 min, where the reaction rate is still in a linear phase, as previously established, allowing for quantification by densitometry (29). Reactions were initiated with the addition of 50 μM cold GTP and [α-32P]GTP (3000 Ci/mmol; PerkinElmer Life Sciences). Reactions were terminated by adding 0.5 M EDTA. Two microliters of the reaction mixture was dotted onto thin-layer chromatography (TLC) plates (EMD Millipore), and GDP and GTP were separated by TLC using 0.5 M KH2PO4 (pH 3.5) for 60 min. The TLC plate was dried for 15 min, and radioactive signal was captured using a phosphor screen (GE Healthcare Life Sciences) and a Typhoon scanner. ImageQuant densitometry was used to quantify the phosphor signal.

**Autophagy studies in human samples**

M1 macrophages from CD patients were derived from whole peripheral blood monocytes according to the manufacturer’s instructions (PromoCell). Monocytes were polarized to mature M1 macrophages in the DXF M1 macrophage generation medium (M1 medium, resting condition; PromoCell) for 12 days and then incubated in phosphate-buffered saline (PBS) and M1 medium for 45 min. Cells were then lysed, and 10 μg of total protein was loaded onto 4 to 12% Bis–Tris Plus precast SDS–polyacrylamide gels, transferred onto a polyvinylidene fluoride (PVDF) membrane, and probed with primary rabbit anti-LRRK2 antibody (ab133474, Abcam), mouse anti-acetylated α-tubulin (T7451, Sigma-Aldrich), rabbit anti-α-tubulin (ab4074, Abcam), mouse anti-SQSTM1 (sc-28359, Santa Cruz Biotechnology), and rabbit anti-LC3B (NB100-2220, Novus Biologicals). The corresponding horseradish peroxidase (HRP)–conjugated secondary antibody was applied for detection. Total α-tubulin was used as a loading control for normalization, and protein densitometry was performed using ImageJ software. LRRK2 degradation was assessed as the ratio of degraded LRRK2 to total LRRK2 (full length + degraded) protein. α-Tubulin acetylation was assessed as the ratio of acetylated to total α-tubulin.

Next, M1 macrophages (1 × 10^5 cells per experiment), in M1 medium and PBS, were pulsed with LysoSensor Green DND-189 (L-7535, Life Sciences). Reactions were terminated by adding 0.5 M EDTA. Two microliters of the reaction mixture was dotted onto thin-layer chromatography (TLC) plates (EMD Millipore), and GDP and GTP were separated by TLC using 0.5 M KH2PO4 (pH 3.5) for 60 min. The TLC plate was dried for 15 min, and radioactive signal was captured using a phosphor screen (GE Healthcare Life Sciences) and a Typhoon scanner. ImageQuant densitometry was used to quantify the phosphor signal.

**Statistical analysis**

Genotyping quality control was performed following guidelines produced by the Cohorts for Heart and Aging Research in Genome Ep-
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Y.P.); Edwin and Caroline Levy and Joseph and Carol Reich (to S.B.); Sinai Ulcerative Colitis: Experimental and Systems Studies (SUCCESS) grant (to J.H.C. and I. Peter); the Sanford J. Grossman Charitable Trust (to J.H.C.); the Cedars-Sinai F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute Research Funds (European Union grant no. IBD-BIOM 305479); the Crohn's and Colitis Foundation and the Joshua L. and Lisa Z. Greer Chair in IBD Genetics (to D.P.B.M.); The Leona M. and Harry B. Helmsley Charitable Trust (to D.P.B.M and I. Peter); the Parkinson's Disease Foundation (to L.N.C.); the Meyerhoff Inflammatory Bowel Disease Center and the Atran Foundation (to S.R.B.); the University of Pittsburgh Inflammatory Bowel Disease Genetic Research Chair (to R.H.D.); the Robert P. & Judith N. Goldberg Foundation, the Bumpus Foundation, and the Harvard NeuroDiscovery Center (to T.F.); and The Leona M. and Harry B. Helmsley Charitable Trust (to D.P.B.M and I. Peter). Genotyping services for selected PD cohorts were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the NIH to Johns Hopkins University (contract no. HHSN268200782096C).

Functional variants in the LRRK2 gene confer shared effects on risk for Crohn's disease and Parkinson's disease


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A shared history

Crohn's disease (CD), an inflammatory bowel disease, has a relatively high prevalence in Ashkenazi Jewish populations. Hui et al. conducted genome-wide association analysis in 2066 CD patients and 3633 healthy control individuals of Ashkenazi Jewish ancestry and identified two functional variants in the LRRK2 gene. The LRRK2 gene has been previously linked to the development of Parkinson's disease (PD). The new LRRK2 variants conferred risk for CD (N2081D) or protection from CD (N551K/R1398H). Analysis of other variants within the LRRK2 locus in 24,570 individuals revealed similar genetic effects between CD and PD in both Ashkenazi Jewish and non-Jewish cohorts. The presence of shared LRRK2 alleles in CD and PD provides insight into disease mechanisms and potential treatments.