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Common variants at five new loci associated with early-onset inflammatory bowel disease

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Abstract

The inflammatory bowel diseases (IBD) Crohn's disease and ulcerative colitis are common causes of morbidity in children and young adults in the western world. Here we report the results of a genome-wide association study in early-onset IBD involving 3,426 affected individuals and 11,963 genetically matched controls recruited through international collaborations in Europe and North America, thereby extending the results from a previous study of 1,011 individuals with early-onset IBD[1]. We have identified five new regions associated with early-onset IBD susceptibility, including 16p11 near the cytokine gene *IL27* (rs8049439, $P = 2.41 \times 10^{-9}$), 22q12 (rs2412973, $P = 1.55 \times 10^{-9}$), 10q22 (rs1250550, $P = 5.63 \times 10^{-9}$), 2q37 (rs4676410, $P = 3.64 \times 10^{-8}$) and 19q13.11 (rs10500264, $P = 4.26 \times 10^{-10}$). Our scan also detected associations at 23 of 32 loci previously implicated in adult-onset Crohn's disease and at 8 of 17 loci implicated in adult-onset ulcerative colitis, highlighting the close pathogenetic relationship between early- and adult-onset IBD.

Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the gastrointestinal tract that most commonly arise during the second and third decades of life. Incidence, family, twin and phenotype concordance studies suggest that IBD is highly heritable, albeit complex, spurring an ongoing search for genetic factors that confer susceptibility to this disease[2,3]. Genome-wide association studies (GWASs) applying high-density SNP array technology have greatly expanded the number of genetic factors implicated in IBD pathogenesis to include 32 loci associated with Crohn's disease and 17 associated with ulcerative colitis, spanning pathways involved in adaptive (*IL23R, IL10, IL12B, STAT3*) and innate (*CARD15, ATG16L1,IRGM*) immunity[4-7].

Most genetic analyses in IBD have been performed in adult-onset disease[2,3]. Early-onset IBD, however, has unique characteristics of phenotype, severity and familiality[8,9], features that provide support for the search for loci that may be specific to early-onset disease. In addition, because early-onset IBD has a stronger familial component than the adult disease, studies targeting this subgroup potentially provide additional power to identify genes that contribute modest effects, as illustrated by the success of our previous scan in identifying 20ql3 and 21q22 as IBD loci[1].

We now report results from the largest GWAS conducted so far in early-onset IBD (Fig. 1). Our IBD discovery cohort (DC-IBD) comprised 2,413 individuals of European ancestry with IBD (cases), including 1,636 with Crohn's disease (DC-CD), 724 with ulcerative colitis (DC-UC) and 53 with IBD of unclassified type (IBD-U), and 6,158 genetically matched controls, and was genotyped on the Illumina HumanHap550 platform. Affected individuals were recruited from multiple centers from four geographically discrete countries and diagnosed before their nineteenth birthday according to standard IBD diagnostic criteria (Supplementary Table 1). Our study extends a previous IBD GWAS that was based on a subset of these cases (1,011 IBD cases, including 647 with Crohn's disease, 317 with ulcerative colitis and 47 with IBD-U; Supplementary Table 2)[1]. An independent replication cohort (RC1) of 482 early-onset IBD cases (289 with Crohn's disease, 120 with ulcerative colitis and 73 with IBD-U) and 1,696 genetically matched controls was gathered from the Children's Hospital of Philadelphia (CHOP) health system and collaborating centers. We refer to Crohn's disease and ulcerative colitis subanalyses of data set RC1 as RC1-CD and RCl-UC, respectively. A second replication cohort (RC2-CD) of 531 Crohn's disease cases diagnosed in childhood and 4,109 controls was assembled by the International IBD Genetics Consortium (IIBDGC). This cohort is based on subsets of data from genome-wide scans generated by the US National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)[10], the Wellcome Trust Case Control Consortium (WTCCC)[11] and a Belgian-French[12] collaboration, which have been combined previously in a large-scale meta-analysis[5]. We computed P values in each cohort by comparing single-marker allele frequencies using χ^2 statistics on SNPs that passed quality-control criteria. We conducted meta-analysis across multiple studies using a Z-score transformation. See Online Methods for more detailed descriptions of cohorts and methods used in this study.

We first searched for previously unreported signals, including loci that met stringent genome-wide significant ($P < 5 \times 10^{-8}$) and suggestive ($P < 1 \times 10^{-6}$) thresholds in our three discovery scans (DC-CD, DC-UC and DC-IBD). For confirmation of these loci, we sought evidence for replication in two independent early-onset cohorts (RC1 and RC2-CD). Lastly, we combined our discovery and replication cohorts for Crohn's disease (Table 1), ulcerative colitis (Table 2) and IBD (Table 3) in a meta-analysis.

Analysis of DC-CD identified a region on 16p11 as the single new genome-wide significant locus; the most significant SNP in the block of linkage disequilibrium (LD) containing this locus, rsl968752, yielded a value of $P = 2.09 \times 10^{-8}$, with the minor allele (A) conferring risk (odds ratio (OR) = 1.25 (1.16-1.36)). We observed nominal replication of rsl968752 in the RC2-CD data set ($P = 0.036$, OR = 1.09 (0.94-1.27)) and a trend for association in the combined analysis of the replication cohorts (RC1-CD and RC2-CD), using two-sided P values (P = 0.059; Supplementary Table 3).

***Figure 1** Overview of genome-wide association results, (a-c) Chromosomal plots of-logP against genomic location for discovery scans in the early-onset DC-CD (a), DC-UC (b) and DC-IBD (c) cohorts.* Red and blue dotted lines represent thresholds for genome-wide significant ($P = 1 \times 10^{-7}$) and suggestive ($P = 1 \times 10^{-6}$) signals, respectively. Loci on 16p11, 22q12 and 2q37 emerged from these analyses as suggestive signals and were validated in the follow-up cohorts RC1 and RC2-CD. The following signals did not replicate or show significance in the meta-analysis: lq22 (CD), 10q25 (UC), 16q21 (UC), 18ql2 (UC), 8q24 (IBD) and 15q22 (IBD). The remaining loci have been previously identified in majority adult-onset genome scans of Crohn's disease and ulcerative colitis. Loci on 22ql2 and 20ql3 were identified in a previous early-onset IBD scan involving a subset of the cohort analyzed in this study[1].

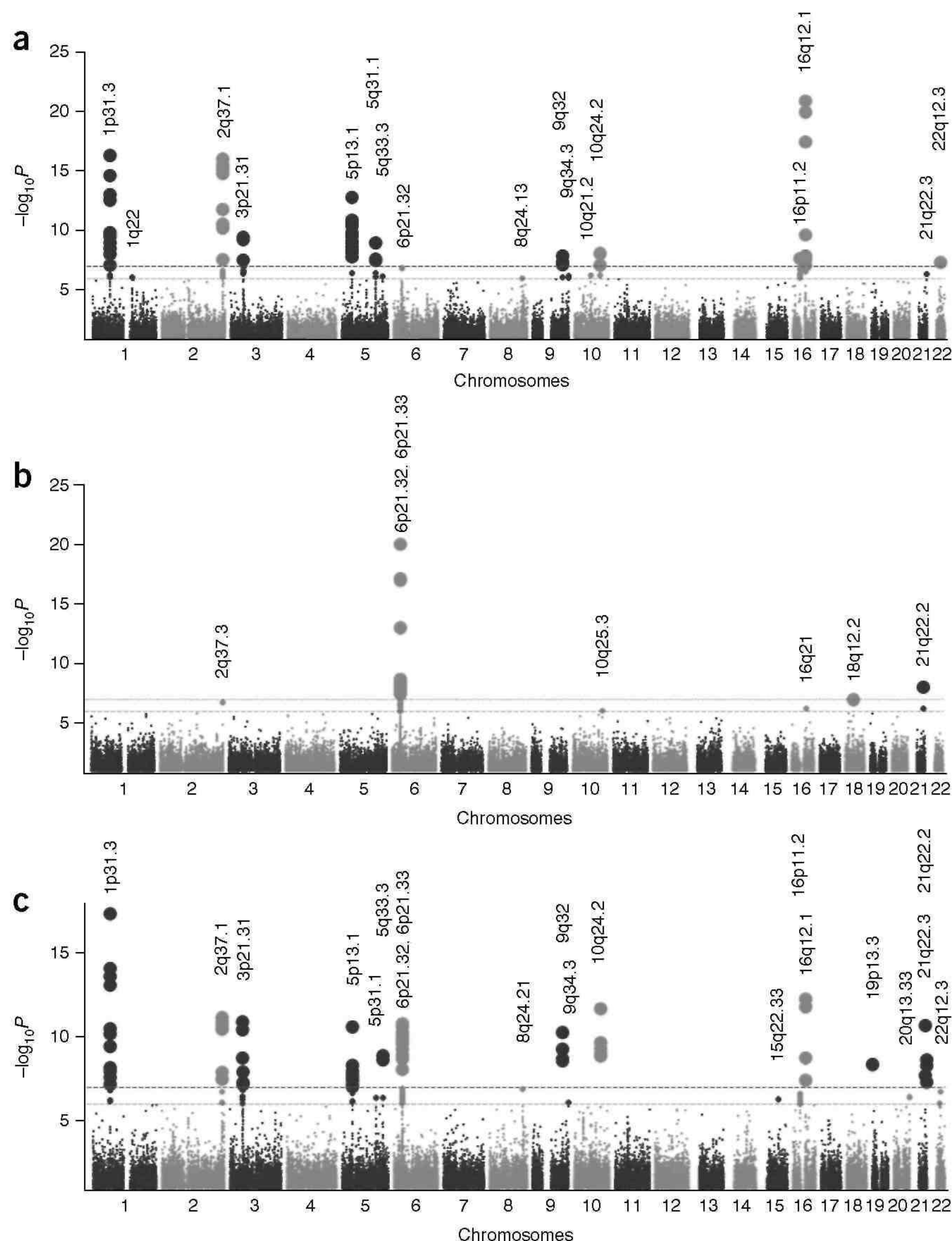

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***Table 1** Newly discovered loci significant in the GWAS of the DC-CD, RC1-CD and RC2-CD cohorts*

				CD discovery (DC-CD) (n = 1,636/6,158)				CHOP CD replication (RC1-CD) (n = 289/ 1,696)				IIBDGC replication (RC2-CD) (n = 531 /4,109)				Combined (n = 2,456/11,963)	
Band (Mb)	Genes	SNP	Allele	P	MAF_A	MAF_U	OR [95% CI]	P	MAF_A	MAF_U	OR [95% CI]	P	MAF_A	MAF_U	OR [95% CI]	P	Z
10q22.3 (80.71-80.73)	*ZMIZ1*	rS1250550	A/T	1.59E-05	0.28	0.32	0.83 [0.76-0.90]	0.001	0.28	0.35	0.73 [0.60-0.88]	0.005	0.20	0.24	0.82 [0.70-0.96]	4.41E-10	-6.24
16p11.2 (28.45-28.81)	*IL27, SULT1A1, SULT1A2, EIF3C*	rS8049439	C/G	2.72E-07	0.42	0.37	1.23 [1.13-1.32]	0.28	0.40	0.38	1.10 [0.92-1.32]	0.0014	0.42	0.39	1.14 [1.00-1.30]	2.87E-09	5.94
22q12.2 (28.75-28.86)	*H0RMAD2, MTMR3, LIF*	rS2412973	A/T	6.16E-06	0.50	0.46	1.19 [1.10-1.29]	0.0095	0.52	0.46	1.26 [1.06-1.51]	0.0160	0.50	0.46	1.15 [1.01-1.31]	3.77E-08	5.50

MAF, minor allele frequency.

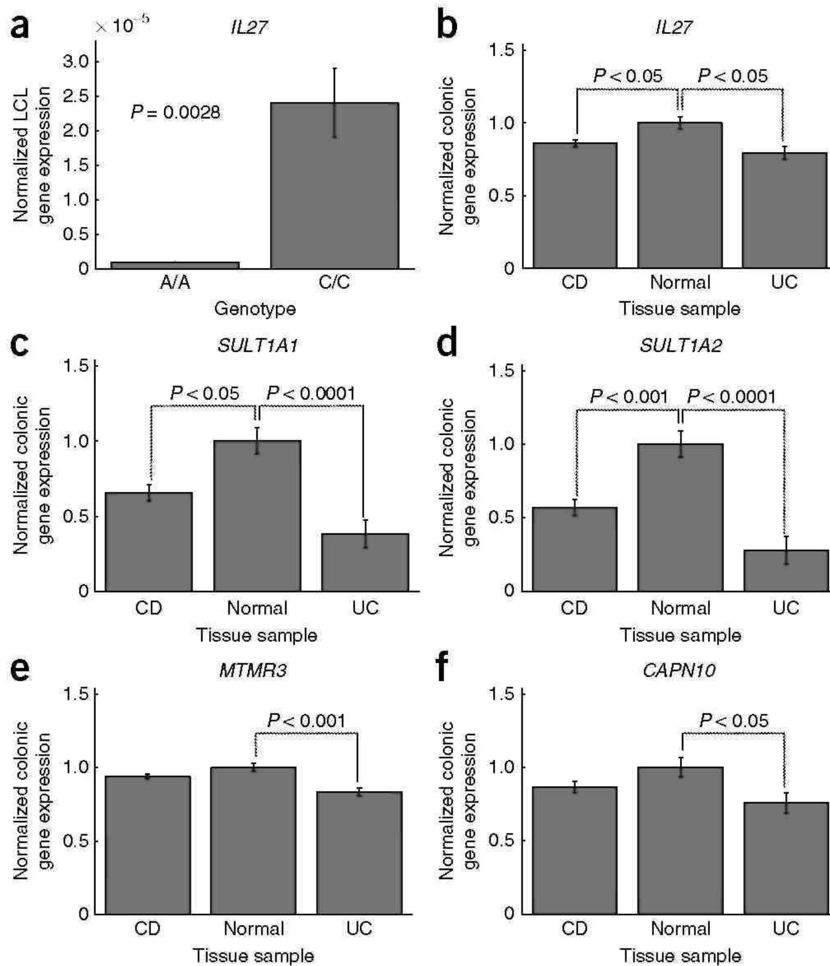
***Table 2** Newly discovered loci significant in the GWAS of the DC-UC and RC1-UC cohorts*

				UC discovery (DC-UC) (n = 724/6,158)				CHOP UC replication (RC1-UC) (n = 120/1,696)				Combined (n = 844 / 7,854)	
Band (Mb)	Genes	SNP	Allele	P	MAF_A	MAF_U	OR [95% CI]	P	MAF_A	MAF_U	OR [95% CI]	P	Z
2q37.3 (241.21-241.42)	*CAPN10, GPR35, KIF1A, RNPEPL1*	rs4676410	A/T	1.70×10^{-7}	0.24	0.18	1.41 [1.24-1.61]	0.0611	0.25	0.20	1.34 [0.99-1.82]	3.64×10^{-8}	5.51

Table 3 *Newly discovered loci significant in the GWAS of the DC-IBD, RC1 and RC2-CD cohorts*

				IBD discovery (DC-IBD) (*n* =2,413/6,158)				CHOP IBD replication (RC1-IBD) (*n* = 482/1,696)				IIBDGC replication (RC2-CD) (*n* = 531 /4,109)				Combined (*n* = 3,426/11,963)	
Band (Mb)	Genes	SNP	Allele	*P*	MAF_A	MAF_U	OR [95% CI]	*P*	MAF_A	MAF_U	OR [95% CI]	*P*	MAF_A	MAF_U	OR [95% CI]	*P*	Z
19ql3.11 (38.44-38.45)		rsl0500264	A/T	1.18E-05	0.21	0.18	1.21 [1.11-1.31]	0.0034	0.22	0.18	1.30 [1.07-1.58]	0.00064	0.12	0.12	1.08 [0.89-1.31]	4.26E-10	6.24
22ql2.2 (28.75-28.86)	*H0RMAD2, MTMR3, LIP*	rs2412973	A/T	9.14E-07	0.50	0.46	1.18 [1.11-1.26]	0.0052	0.51	0.46	1.23 [1.05-1.43]	0.016	0.50	0.46	1.15 [1.01-1.31]	1.55E-09	6.04
16p11.2 (28.45-28.81)	*IL27, SULT1A1, SULT1A2, EIF3C*	rs8049439	C/G	2.38E-07	0.41	0.37	1.20 [1.12-1.28]	0.34	0.40	0.38	1.08 [0.92-1.26]	0.0014	0.42	0.39	1.14 [1.00-1.30]	2.41 E-09	5.97
10q22.3 (80.71-80.73)	*ZMIZ1*	rsl250550	A/T	3.55E-05	0.29	0.32	0.86 [0.80-0.92]	0.0016	0.29	0.35	0.78 [0.66-0.92]	0.00461	0.20	0.24	0.82 [0.70-0.96]	5.63 E-09	-5.83

Figure 2 *Colonic and LCL mRNA expression of genes in significantly associated loci.* The A allele of rsl968752 confers risk in our early-onset Crohn's disease and IBD cohorts (OR = 1.23 (1.12-1.40)). rsl968752 lies in a block of LD containing the *IL27gene.* (a) Cell lines from individuals (n = 5) with the A/A genotype at rsl968752 have over 90% decrease in *IL27*gene expression as compared with those with the C/C genotype. (b) *IL27*colonic expression was significantly lower in samples obtained from individuals with early-onset Crohn's disease and ulcerative colitis cases than in normal tissue. (c,d) Expression of both *SULT1A1* (c) and *SULT1A2* (d) genes (also in the *IL27* locus) was also significantly lower in Crohn's disease and ulcerative colitis tissue than in normal tissue. (e,f) Expression of putative IBD gene *MTMR3* at 22q12 (e) and the putative ulcerative colitis gene *CAPN10* at 2q37 (f) was significantly lower in ulcerative colitis tissue than in normal tissue. All significant expression effects were found to be independent of treatment and histological inflammation score (data not shown). Error bars represent s.e.m.



Analysis of our combined IBD discovery scan yielded suggestive association at the 16pll locus (rs8049439, $P = 2.38 \times 10^{-7}$, OR = 1.20 (1.12-1.28)). rs8049439 is located ~200 kb upstream of rsl968752, and the two loci are in strong LD ($r^2 = 0.796$). rs8049439 showed a statistically significant association ($P = 0.00144$, OR= 1.14 (1.00-1.30)) in the RC2-CD data set, whereas meta-analysis of rs8049439 in the discovery data set and the two replication cohorts (RC1, RC2-CD) reached genome-wide significance in both IBD ($P = 2.41 \times 10^{-9}$) and Crohn's disease ($P = 2.87 \times 10^{-9}$; Tables 1 and 3). In addition, analysis of the available data in a meta-analysis data set for adult-onset Crohn's disease[5] demonstrated that an LD proxy for these two SNPs, rs4788084 (r^2 = 0.83 to rsl968752, and $r^2 = 0.86$ to rs8049439), was also associated with Crohn's disease (P = 0.0035; Supplementary Table 4). Of note, we found that the risk-conferring minor allele (G) at rs8049439 shares haplotypes with the risk-conferring ancestral allele (A) of the type 1 diabetes SNP rs4788084 (HapMap CEU, $r^2 = 0.864$)[13].

The LD block incorporating rsl968752, rs8049439 and rs4788084 contains multiple genes, including *IL27, CCDC101, CLN3, EIF3C, NUPR1, SULT1A1* and *SULT1A2.* Of these, we considered *IL*27, which encodes an immunomodulatory cytokine that regulates adaptive immunity responses, to be the most plausible candidate

gene for susceptibility to IBD. Analysis of *IL27* gene expression in lym-phoblastoid cell lines (LCLs) obtained from ten healthy individuals homozygous for rsl968752 (that is, either A/A or C/C) showed that those individuals with two copies of the risk allele (A/A) had a several-fold decrease in *IL27* expression relative to those with two copies of the nonrisk allele (C/C; P = 0.0031; Fig. 2a), suggesting that this SNP may exert a regulatory effect on *IL27* gene expression. In addition, colonic expression of *IL27* was significantly lower in 30 individuals with early-onset Crohn's disease than in 11 healthy controls ($P < 0.05$; Fig. 2b). These effects remained significant after correction for medication use and histological inflammation score.

When examining the colonic expression of other genes at this locus, we also detected significantly lower expression of *SULT1A1* and *SULT1A2* in both early-onset Crohn's disease ($P < 0.05$, $P < 0.001$) and ulcerative colitis ($P < 0.0001$, $P < 0.0001$) as compared with healthy controls (Fig.2c,d).*SULT1A1*and *SULT1A2* encode sulfotransferases that catalyze sulfate conjugation of catecholamines, phenolic drugs and neurotransmitters. These biological functions make *SULT1A1* and *SULT1A2* less attractive as IBD candidate genes. We also observed a strongly expressed quantitative trait locus (eQTL) for *EIF3C* expression in LCLs at this locus (lod score = 5-8) on the basis of publicly available data[14]; however, we did not observe altered *EIF3C* expression in Crohn's disease or ulcerative colitis cases relative to healthy controls, nor did we detect an eQTL for *EIF3C* in colonic tissue at SNPs in this region (Supplementary Figs. 1 and 2). *EIF3C* encodes a eukaryotic translation initiation factor that forms part of the basic translational machinery, which also makes *EIF*3C less likely to be an IBD candidate gene. Additional allele-specific expression effects were not observed for the other genes at this locus either in our LCL and colonic expression data sets (Supplementary Fig. 1) or in the public database[14]. Taken together, these results point most strongly to *IL*27 as a candidate gene associated with early-onset IBD; however, further functional and fine-mapping studies are warranted to confirm this and rule out the involvement of other genes at this locus.

The first of the two suggestive early-onset disease associations was identified in the DC-IBD cohort at 22ql2 (rs2412973, $P = 9.14 \times 10^{-7}$, OR= 1.18 (1.10-1.26)). This SNP replicated in RC1 ($P = 0.0052$, OR = 1.23 (1.05-1.43)) andRC2-CD ($P = 0.016$,OR = 1.15 (1.01-1.31)), yielding $P = 1.55 \times 10^{-9}$ in a meta-analysis across all three early-onset IBD cohorts (Table 3). Meta-analysis of rs2412973 across the data sets DC-CD, RC1-CD and RC2-CD also reached genome-wide significance ($P = 3.77 \times 10^{-8}$). rs2412973 also showed association in the CD meta-analysis data set with an age of onset primarily in adulthood[5] ($P = 0.000953$; Supplementary Table 4). rs2412973 is located within *HORMAD2,* an open reading frame whose putative functions include mitotic checkpoints, chromosome synapsis and DNA repair. The LD block incorporating rs2412973 also contains *MTMR3,* encoding myo-tubularin-related protein-3, and *LIF,* encoding leukemia inhibitory factor, a cytokine that stimulates differentiation in leukocytes. We observed a significant difference in colonic *MTMR3* expression in biopsies from individuals with ulcerative colitis as compared with controls ($P < 0.001$), but not in those from individuals with Crohn's disease (Fig. 2e), and we did not detect colonic eQTL for *MTMR3* near rs2412973. Other genes in the LD block did not exhibit significant, expression effects (Supplementary Fig. 3).

The second suggestive association was identified only in DC-UC at 2q37 (rs4676410, $P = 1.70 \times 10^{-7}$, OR 1.41 (1.24-1.61)). This SNP showed only a trend for replication in the small RC1-UC cohort ($P = 0.0611$, OR = 1.38 (1.08-1.77)), but yielded genome-wide significance in the combined analysis ($P = 3.64 \times 10^{-8}$; Table 2). rs4676410 lies within *GPR35,* which encodes an orphan receptor primarily expressed in the intestine of humans and rats. Other genes in the LD block of rs4676410 include *CAPN10, KIF1A* and *RNPEPL1. CAPN10* colonic gene expression was significantly lower in ulcerative colitis cases than in controls ($P < 0.05$; Fig. 2f). *CAPN10* encodes a Ca^{2+}-regulatedthiol-protease involved in cytoskeletal remodeling and signal transduction. We did not observe significant expression effects in the remaining genes (Supplementary Figs. 1 and 2).

We next combined our discovery DC-IBD and replication cohorts (RC1, RC2-CD) for a genome-wide meta-analyses of early-onset Crohn's disease, ulcerative colitis and IBD. These analyses yielded two new loci achieving genome-wide significance. The first new signal is associated with IBD (rsl0500264, $P = 4.26 \times 10^{-10}$) and is located at 19ql3 in a small block of LD devoid of known genes lying within 50 kb of *SLC7A10* and *CEBPA.* Notably, rsl0500264 showed only nominal association in the adult-onset Crohn's disease meta-analysis ($P = 0.0217$), suggesting that this locus may be weighted more toward early-onset disease. The second new signal, rsl250550, lies on 10q22 inside the *ZMIZ1* gene and is associated with both Crohn's disease ($P = 4.41 \times 10^{-10}$) and IBD combined ($P = 5.63 \times 10^{-9}$; Tables 1 and 3). In addition to showing significance in the early-onset meta-analysis, rsl250550 robustly associates in the majority adult-onset Crohn's disease meta-analysis[5] ($P = 3.27 \times 10^{-5}$). *ZMIZ1* encodes a PIAS-like protein that interacts with Smad4 to regulate Smad3 transcription and modulate transforming growth factor-β signaling[15]. Despite achieving robust significance in our meta-analysis, these loci merit replication in an independent cohort.

We conducted a meta-analysis of our discovery and replication cohorts to determine association with the 49 previously reported IBD loci implicated in adult-onset disease, determining nominal ($P < 0.05$) and Bonferroni-corrected ($P < 0.001$, correcting for 49 hypotheses; Supplementary Table 5) significance. Of 32 previously confirmed loci associated with adult-onset Crohn's disease, 29 were nominally significant and 21 were significant after Bonferroni correction in meta-analysis of DC-CD and RC1-UC data sets. Of eight additional Crohn's disease loci that attained nominal significance ($P < 0.05$) in the previously reported majority adult-onset metaanalysis[5], two showed significant association with early-onset Crohn's disease, namely the *IL18R1-IL18RAP* locus on 2ql2 (rs917997, $P = 6.84 \times 10^{-5}$, $Z= 3.98$) and the C-C motif chemokine (*CCL*) gene cluster on 17q12 (rs991804, $P = 2.31 \times 10^{-4}$, $Z = -3.68$; Table 4). We found that 13 of 17 previously identified adult-onset ulcerative colitis loci showed nominal significance, and 8 were significant after Bonferroni-corrected meta-analysis of DC-CD and RC1-UC data sets, including *IL23R* on lp31 and *IL26* on 12ql5 (Supplementary Table 5). Our data also supported the association of loci on 20ql3 and 21q22 with early-onset IBD, as previously reported[1] on the basis of analyses of a subset of our discovery cohort (Supplementary Note).

We also evaluated previously reported loci associated with adult-onset Crohn's disease for association with early-onset ulcerative colitis, and vice versa. Examining 32 known Crohn's disease signals in our ulcerative colitis cohort implicated two loci that had not previously been associated with adult-onset ulcerative colitis susceptibility in early-onset ulcerative colitis: *ICOSLG* on 2lq22 (rs762421, $P = 2.54 \times 10^{-5}$, $Z = 4.21$) and *ORMDL3* on 17q12 (rs2872507, $P = 7.62 \times 10^{-4}$, $Z = 3.37$; Supplementary Table 5). When examining the association of early-onset Crohn's disease with 17 previously reported adult-onset ulcerative colitis signals, we detected association only with the ulcerative colitis gene *IL10* on lq32.1 (rs3024505, $P = 0.00048$, $Z = 3.49$), suggesting that this locus may also play a role in early-onset Crohn's disease susceptibility.

Our study adds insight into the pathogenic mechanisms mediating early-onset IBD and its close relationship with adult-onset disease In particular, identification of *IL27* as a candidate gene for Crohn's disease susceptibility lends further support to the involvement of th(T-helper 17 pathway[16,17] in pathogenesis of Crohn's disease, complementing gene discoveries in other genome scans *(IL23R, STAT3 JAK2, IL12B)*[2,3,5]. In addition, our discovery of five new IBD susceptibility loci through analysis of a genetically enriched early-onse disease cohort underscores the validity of this approach in the study of complex disease.

Table 4 *Newly discovered effects of previously known or suggested adult IBD risk loci in early-onset IBD*[a]

								CD (DC-CD, RC1-CD) (n= 1,925/ 7,854)		UC (DC-UC, RC1-UC) (n= 844/ 7,854)		IBD (DC-IBD, RC1) (n= 2,895/ 7,854)	
CDk[b]	CDp[b]	UCk[b]	UCp[b]	Band	Mb	Genes	SNP	*P*	*Z*	*P*	*Z*	*P*	*Z*
		•		lq32.1	205.01	*IL10, IL19, IL20*	rs3024505	***4.84 x 10^{-4}***	**3.49**	**6.20 × 10^{-4}**	**3.42**	**6.84 × 10^{-6}**	**4.50**
	•			2ql2.1	102.44	*IL18R1, IL18RAP*	rs917997	***6.84 x 10^{-5}***	**3.98**	0.17	1.38	**5.98 × 10^{-5}**	**4.01**
	•			17ql2	29.61	*CCL11, CCL2, CCL7*	rs991804	***2.31 x 10^{-4}***	**-3.68**	2.78 × 10^{-2}	-2.20	**6.64 × 10^{-5}**	**-3.99**
•				17ql2	35.29	*ORUDIS*	rs2872507	3.65 × 10^{-4}	3.56	***7.62 x 10^{-4}***	**3.37**	**3.13 × 10^{-6}**	**4.66**
•				21q22.3	44.44	*ICOSLG1*	rs762421	**3.32 × 10^{-7}**	**5.10**	***2.54 x10^{-5}***	**4.21**	**3.19 × 10^{-10}**	**6.29**

[a]Listed are 5 of 49 previously identified adult-onset IBD loci that were either (1) previously nominal adult IBD signals that are verified by our data or (2) validated adult signals previously shown to have an effect on early-onset UC (CD) and found in our study to have an effect on early-onset CD (UC). [b]Filled circles in the first four columns of the table specify whether the given row represents a known CD locus (CDk), putative/nominal CD locus (CDp), known UC locus (UCk) and/or putative/nominal UC locus (UCp). Loci replicating at a Bonferroni-corrected $P < 0.05$ (corrected for 49 hypotheses) are denoted in bold; newly discovered significant effects are denoted in bold italics.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. Gene Expression Omnibus (GEO): colonic gent expression data set, GSE10616. Entrez Gene: *IL*27, 246779; *SULT1A1* 171150; *SULT1A2,* 601292; *EIF3C,* 8663; *HORMAD2,* 150280 *MTMR3,* 8897; *LIF,*

159540; *ZMIZ1,* 57178; *CAPN10,*11132; *GPR35* 2859; *KIF1A,* 547; *RNPEPL1,* 57140.

Note: *Supplementary information is available on the Nature Genetics website.*

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AUTHOR CONTRIBUTIONS

M.I., R.N.B., A.G., R.K.R., V.A., M. Dubinsky, S.K., S.F.A.G., M.S.S., J. Satsangi and H.H. participated in study conception and design. R.N.B., A.G., R.K.R., V.A., M. Dubinsky, S.K., T.D.W., S.S., R.G., M.C., A. Latiano, B.D., J. Stempack, D.J.A., K.T., B.K., J.L., J.E., R.G., M. Stephens, A. Levine, D.P., J.V.L., S.C., S.L.G., C.E.K., G.D.F., E.C.F., C.H., G.O., R.M.C., A.M., L.D., D.C.W, M.S.S., S.F.A.G., J. Satsangi, H.H., D.S.M., D.M. and M.B.H. recruited patients and directed sample collection. C.E.K., E.C.F., G.O., R.M.C. and J.T.G. performed genotyping and quality-control measures on all data sets and H.H. supervised all sample organization and genotyping. L.D., R.G. and A.M. supervised gene expression experiments. M.I. performed statistical analysis with supervision from H.H. M.I., J.P.B., P.S., K.W, H.Z, R.G., J.H.F. and M. Daly provided bio informatics, database and statistical support. M.I. and H.H. wrote the manuscript. M.I., R.N.B., A.G., R.K.R., V.A., M. Dubinsky, S.K., S.F.A.G., M.S.S., J. Satsangi and H.H. participated in drafting and critical revision of the manuscript. All authors contributed to the final paper, with M.I., R.N.B., A.G., R.K.R., V.A., M. Dubinsky, S.K., M.S.S., J. Satsangi and H.H. playing key roles.

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ONLINE METHODS

Participants. The early-onset IBD discovery case cohort (DC-IBD; Supplementary Table 1) consisted of 2,413 Europeans with ancestry cases of IBD (1,636 with Crohn's disease, 724 with ulcerative colitis and 53 with IBD-U) recruited from multiple centers from four geographically discrete countries that met the study's quality-control criteria and were successfully matched with disease-free control subjects from the United States (see **Supplementary Note** for additional details). The Research Ethics Boards of the respective hospitals and other participating centers approved this study, and written informed consent was obtained from all subjects (or their legal guardians).

Genotyping. We performed high-throughput genome-wide SNP genotyping using Illumina Infinium II HumanHap550 BeadChip technology at the Center for Applied Genomics at CHOP as described[18]. After genotyping, we excluded 270 individuals with IBD for whom >2% of genotypes were missing. Control subjects used for this study were also filtered to include those with a genotyping frequency of >98%.

Population stratification. We applied the program STRUCTURE to our quality-controlled data set to exclude 316 individuals with <95% European ancestry on the basis of ancestry informative markers[19]. After these exclusions, our cohort consisted of 2,784 IBD cases, including 1,887 Crohn's disease-only, 835 ulcerative colitis-only and 54 IBD-U cases. Controls were filtered for 95% European ancestry, as determined by STRUCTURE, yielding 7,315 total subjects.

Because of the differing geographical origins between our cases and controls, we performed PCA-based genetic matching (eigenmatching) to minimize intra-European population stratification. Eigenmatching uses singular value decomposition of genotypic data to match cases to their closest controls in the space of k principal components. This approach is a variant of a published method[20]; however in contrast to that method, we employed matching as a filtering criterion. Unlike EIGENSTRAT, a common approach to correct for the effects of stratification by adjusting genotype values, eigenmatching removes samples from both cases and controls that are responsible for stratification. We eigenmatched IBD cases and controls through a multistep procedure. First, we computed principal components for our data set by running smartpca in the EIGENSTRAT[21] package on 100,000 random autosomal SNPs. Second, we applied a matching algorithm implemented in MATLAB to the principal components results[21]. This algorithm determines a distance for each case-control pair after mapping each sample to coordinates on the basis of the top k eigenvalue-scaled principal components. The algorithm then matches each case to up to p nearby controls, keeping only cases that match between p and q controls within a distance d (where p, q, d and k are user-specified parameters).

We genetically matched cases in the discovery cohort by using the top seven principal components, matching cases to three genetically related controls, and keeping cases with between one and three controls within a distance of 0.1. We chose $k = 7$ on the basis of the decay plot of the eigenvalues corresponding to the top principal components. We chose 0.1 as a distance threshold d after manual optimization minimizing the genomic inflation factor (λ) while maximizing power (that is, number of cases). After matching, we obtained λ values of 1.13, 1.09 and 1.16 for the early-onset IBD, Crohn's disease and ulcerative colitis cohorts, respectively. Our final discovery cohort after matching consisted of 2,413 cases and 6,158 controls, including 1,636 Crohn's disease and 724 ulcerative colitis cases. A summary of the number of subjects who met quality-control and genetic matching criteria for study inclusion is shown in Supplementary Table 1. We also performed genetic matching in the replication cohort. Given the smaller number of cases, we employed $k = 7$ principal components to match each case to ten genetically related controls, keeping cases with between one and ten controls within a distance of 0.05. This yielded λ values of 1.16,1.14 and 1.08 for the IBD, Crohn's disease and ulcerative colitis replication cohorts, respectively. Our final replication cohort RC1 consisted of 482 early-onset IBD cases and 1,696 controls, including 289 Crohn's disease, 120 ulcerative colitis and 73 IBD-U cases.

Replication experiments. We assembled an early-onset IBD replication cohort (RC1) that included cases that were not genetically matched to controls during creation of the discovery cohort and additional cases from the CHOP health system that were obtained through an ongoing collection effort. Additional cases were also genotyped by Illumina Infinium II HumanHap550 BeadChip technology using standard approaches for quality-control filtering (see above). Cases in RC1 were genetically matched to an independent set of European ancestry controls gathered from the CHOP internal collection. After genetic matching, this data set consisted of 482 early-onset IBD cases (289 Crohn's disease, 120 ulcerative colitis and 73 IBD-U) and 1,696 controls. Subsets of data set RC1 corresponding to Crohn's disease and ulcerative colitis subtypes are designated as RC1-CD (n = 289) and RC2-UC (n = 120).

We used a second early-onset IBD replication cohort (RC2-CD) obtained from the IIBDGC. The IIBDGC replication experiment was based on data from genome-wide scans by the NIDDK[10], WTCCC[11] and a Belgian-French[12] collaboration that have previously been combined to undertake a large-scale meta-analysis[5]. Imputed genotype data (using HapMap II B35 r21 (CEU) as the reference population) was used when directly genotyped data were unavailable for a given cohort (details of the genotyping platforms used in each GWAS have been previously described[5]). In total, 531 early-onset cases (17.1% of the total IIBDGC Crohn's disease cases) and 4,109 population controls were included in the replication effort. Mean age at onset in the IIBDGC early-onset cases was 14.6 yr (s.d. = 3.27 yr). Details of the ascertainment and characterization of the IIBDGC cohort, as well as the quality-control procedures applied to the GWAS data sets, are provided in the original scan and replication publications[10-12,22,23]. Recruitment of study subjects was approved by local and national institutional review boards, and informed consent was obtained from all participants.

Lastly, we followed up select early-onset IBD signals from a meta-analysis of adult-onset Crohn's disease[5], which combined data from three scans totaling 3,230 cases and 4,829 controls.

Association analysis. To detect significantly associated susceptibility alleles, we compared single-marker allele frequencies using χ^2 statistics on SNPs with a minor allele frequency of >1% and with Hardy-Weinberg equilibrium $P > 10^{-5}$. All tests of association were carried out using PLINK[24] and MATLAB with standard criteria for SNP quality-control filtering (see Supplementary Note). Given a conservative estimate of less than -500,000 independent hypotheses, we determined genome-wide significance with a Bonferroni-corrected *P*-value threshold of 1.0×10^{-7}. We also examined nominal signals below a *P*-value threshold of 1×10^{-6}. We excluded 'loner' signals whose significance level was discordant with that of adjacent SNPs in their LD or genomic neighborhood. SNP coordinates were obtained from the National Center for Biotechnology Information (NCBI)

Build 36 and LD information was obtained using HapMap II B36 r27 (CEU). We used PLINK and MATLAB to determine nominal *P* values, ORs and confidence intervals for ORs for all SNPs tested. We compared our association results with SNPs in published data sets by using either the exact SNP or the best LD surrogate ($r^2 >$ 0.2) found on our scan.

We combined data from multiple scans for meta-analysis by computing a *Z* score at each SNP. In brief, *P* values in each scan *i* were transformed via the inverse normal cumulative distribution function into a *Z* score z_i, with signs of the score indicating direction of effect relative to the minor allele, that is, positive *Z* scores for risk-conferring variants (OR > 1) and negative *Z* scores for protective variants (OR < 1). *Z* scores z_i from individual studies were summed into a z_{meta} value using weights w_i for each scan computed as sqrt(*Ni/N*) where N_i is the number of individuals in study *i* and *N* is the total number of individuals across all studies. The combined *Z* score z_{meta} was transformed into a *P* value via the normal cumulative distribution function. Z scores were computed for 299,238,489,951 and 299,238 SNPs in the Crohn's disease, ulcerative colitis and IBD meta-analyses, respectively. All meta-analysis computations were implemented and performed using MATLAB.

Gene expression analysis. We examined allele-specific effects on gene expression for significantly associating loci by assaying total RNA in genotyped LCLs. We also compared gene expression between colonic biopsy specimens obtained from early-onset IBD cases and normal controls to detect disease-specific gene expression differences. We evaluated allele-specific effects on the expression of genes *1127* and *EIF3C* for the rsl968752 variant on 16pl 1 and genes *HORMAD2* and *LIF* for the rs2412973 variant on 21q22 (see

Supplementary Note). In brief, RNA was isolated from Hap Map- CEU population samples using Trizol (Invitrogen). Real-time RT PCR was performed on a Bio-Rad iCycler System using SYBR Green detection (Bio-Rad). cDNA template was made from 2 µg of total RNA by using the Invitrogen cDNA Synthesis kit. Primer sequences were designed using Integrated DNA Technologies (IDT). β-Actin was used as a control. Each reaction was carried out in triplicate wells on one plate. Fold change between the A/A and C/C genotype was calculated with the comparative C_T method. Results were normalized to β-actin for cDNA quantification. Data were analyzed by analysis of variance (AN OVA). We also examined allele-specific changes on gene expression in a publicly available LCL database[14].

We examined colonic expression of selected candidate genes located in the LD blocks of our most significant signals. Gene expression was assayed in individual colonic biopsy specimens from subjects with early-onset Crohn's disease (n = 30) and early-onset ulcerative colitis (n = 10), and from healthy controls (n = 11). Individuals were aged from 5 to 18 years at time of biopsy with a median age of 13 years. Inflammation was quantified in colon biopsies by using the Crohn's Disease Histological Index of Severity, and ranged from grade 1 to 12 with a median of 3.5. Of the 30 individuals with Crohn's disease, 16 were receiving one or more of the

following medications at time of biopsy: 5-ASA, Cytoxan, 6-mercaptopurine and methotrexate. After informed consent, colonic biopsies were obtained from subjects with Crohn's disease and ulcerative colitis, and healthy controls. All of the biopsies for IBD cases and healthy controls were obtained from the ascending colon, with the exception of one subject with ulcerative colitis whose biopsy was obtained from the rectum. Colon biopsies were immediately placed in RNAlater stabilization reagent (Qiagen, Germany) at 4 °C. Total RNA was isolated by an RNeasy Plus Mini Kit (Qiagen) and stored at -80 °C. Samples were then submitted to the CCHMC Digestive Health Center Microarray Core where the quality and concentration of RNA were measured by the Agilent Bioanalyser 2100 (Hewlett Packard) using an RNA 6000 Nano Assay to confirm a 28S/18S ratio of 1.6-2.0. We amplified 100 ng of total RNA by using a Target 1-round Aminoallyl-aRNA Amplification Kit 101 (Epicentre, WI). The biotinylated cRNA was hybridized to Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 arrays, containing probes for 22,634 genes. The images were captured by an Affymetrix Genechip Scanner 3000. The complete data set is available at the NCBI Gene Expression Omnibus. Of note, this data set includes two additional expression profiles for two individuals with early-onset Crohn's disease who were biopsied both pre- and posttherapy The posttherapy samples for these individuals were not used in analyses for this paper (that is, we used gene expression values only from pretherapy samples). Significance of gene expression changes was assessed by one-way ANOVA with Tukey-Kramer multiple comparison correction and P-value thresholds of $P < 0.05$, $P < 0.001$ and $P < 0.0001$. Genes demonstrating significant expression differences were further evaluated by one-way analysis of covariance (ANCOVA) with Tukey-Kramer multiple comparison correction and the same P-value thresholds, applying histological inflammation score and number of concurrent medication as covariates to assess the impact of these clinicopathological factors on the expression results. We also determined allele-specific colonic gene expression in colonic tissue for our most significantly associated SNPs. For this, we used a subset of IBD cases ($n = 13$) for which both gene expression was measured and genome-wide genotyping was done (as part of the current study). We determined allele-specific expression by one-way ANOVA with a significance threshold of 0.05. All gene expression analyses and statistical tests were implemented and performed in MATLAB.

URL. GEO, http://www.ncbi.nlm.nih.gov/geo.