

# Genome-wide association study for Crohn's disease in the Quebec Founder Population identifies multiple validated disease loci

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**Genome-wide association (GWA) studies offer a powerful unbiased method for the identification of multiple susceptibility genes for complex diseases. Here we report the results of a GWA study for Crohn's disease (CD) using family trios from the Quebec Founder Population (QFP). Haplotype-based association analyses identified multiple regions associated with the disease that met the criteria for genome-wide significance, with many containing a gene whose function appears relevant to CD. A proportion of these were replicated in two independent German Caucasian samples, including the established CD loci *NOD2* and *IBD5*. The recently described *IL23R* locus was also identified and replicated. For this region, multiple individuals with all major haplotypes in the QFP were sequenced and extensive fine mapping performed to identify risk and protective alleles. Several additional loci, including a region on 3p21 containing several plausible candidate genes, a region near *JAKMIP1* on 4p16.1, and two larger regions on chromosome 17 were replicated. Together with previously published loci, the spectrum of CD genes identified to date involves biochemical networks that affect epithelial defense mechanisms, innate and adaptive immune response, and the repair or remodeling of tissue.**

haplotype | complex disease | *IL23R*

Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by transmural inflammatory lesions that can affect the entire gastrointestinal tract (1). The lifetime prevalence is 0.5–1% in Caucasian populations (2) and reflects the combined effects of genetic predisposition and environmental factors (3). Genetic linkage and candidate gene approaches (4–14) have contributed to the elucidation of loci influencing genetic susceptibility to CD. More recently, genome-wide association (GWA) studies (15–21) have provided further insight into the molecular pathogenesis of the disease. The top candidate genes or loci that consistently replicate include *NOD2*, *IL23R*, *ATG16L1*, the *IBD5* region on chromosome 5q31, and a region on 5p13.1 near the *PTGER4* gene. The nature of these genes suggests that the major genetic risk factors for CD are involved in the innate immune response and destruction of intracellular bacteria.

It is now clear that GWA studies provide a powerful and robust new tool for the identification of the multiple susceptibility alleles involved in complex diseases. Importantly, these types of studies have the ability to identify genes that impart only moderate increases in risk (21, 22). However, most studies performed to date have identified only a few top signals, and the validation of true association among signals with lower statistical significance remains a challenge. In addition, most of the GWA studies to date have been performed by using general populations, for which very large

sample sizes are required for success. They have also largely relied on single-marker analysis, with genome-wide haplotype-based association analyses receiving little attention.

In early 2004, we conducted a GWA study for CD using 382 trio samples from the Quebec Founder Population (QFP). This population descended in genetic isolation from several thousand founders who emigrated from France in the 17th century (23). The demographic history of the QFP, which is characterized by a population bottleneck, rapid population expansion, and little admixture, makes it a valuable resource for use in genetic studies (24). The population has been well characterized as having reduced genetic heterogeneity for Mendelian diseases (25).

We first used a haplotype-based GWA study to detect regions associated with CD. Several regions with association signals were then followed up by fine mapping at a greater marker density in a larger QFP sample of 477 trios. A selection of the most promising regions was then tested for replication in two independent German samples. In summary, this study identified multiple CD-associated loci, many of which were replicated in an independent Caucasian sample. We report details for several of these, including the previously reported *NOD2*, *IBD5*, and *IL23R* loci, along with four replicated regions on 3p21, 4p16.1, 17q11.1, and 17q22-q23.

## Results

**Genome-Wide Scan.** Disease association was systematically tested across the genome for both single markers and multimarker window haplotypes (three, five, seven, and nine contiguous markers). Sixteen regions with nominal *P* values  $<10^{-5}$  were identified (Table 1). The genome-wide significance of nominal *P* values was determined by permutation tests. The previously identified *NOD2*, *IBD5*,

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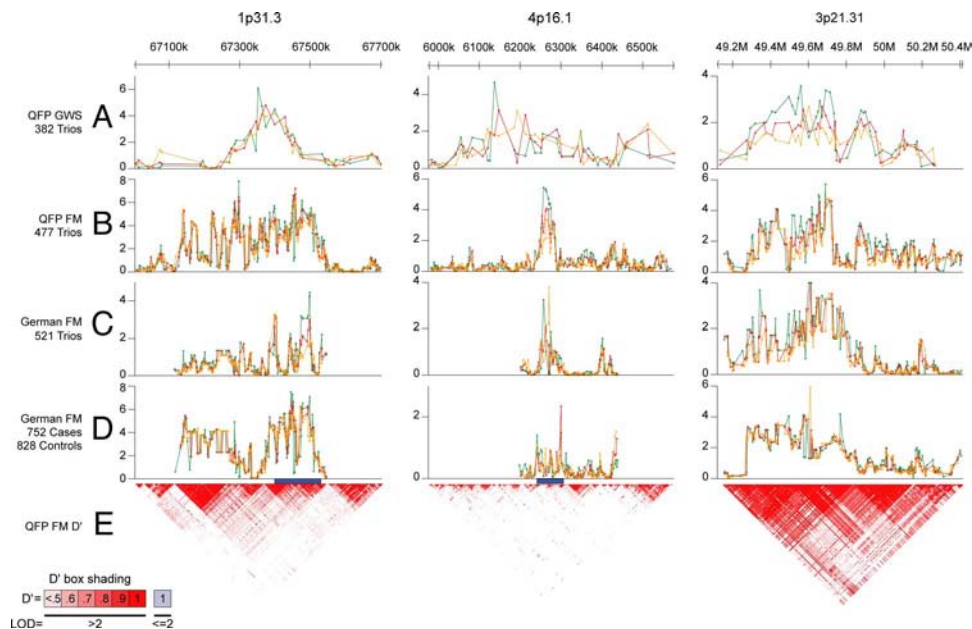
Abbreviations: GWA, genome-wide association; CD, Crohn's disease; QFP, Quebec Founder Population; FM, fine mapping; C.I., confidence interval; OR, odds ratio; LD, linkage disequilibrium.

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**Fig. 1.** Overview of GWA study, fine-mapping, and replication studies in regions on chromosomes 1p31.3, 4p16.1, and 3p21.31. *A* shows the results of the GWA analysis for three regions (1p31.3, 700 kb; 4p16.1, 600 kb; and 3p21.31, 1.2 Mb). Plots indicate the nominal  $-\log_{10} P$  values for haplotypes corresponding to five (green), seven (red), and nine (orange) consecutive marker windows. *B* shows the FM results for the QFP, *C* shows the FM results for the German trios, and *D* shows the FM results for the German cases/controls. Regions sequenced in the 1p31.3 and 4p16.1 regions are shown as a blue bar. *E* shows the LD structure in controls from the QFP based on the  $D'$  algorithm as implemented in Haploview 3.32 (36).

Although the lowest nominal  $P$  values occurred for haplotype associations, single SNPs within *IL23R* were associated with CD at nominal  $P$  values  $<10^{-5}$  in the QFP trios and German cases/controls (SI Fig. 7). Single-marker association results for 17 of the most significantly associated SNPs in all populations are presented in Table 3. Exact nominal  $P$  values, odds ratios (OR), and case/control allele frequencies for these markers are presented in SI Table 8A–F. Analyses of the combined QFP and German data sets resulted in substantially increased significance for many markers, with multiple SNPs associated at  $P$  values  $<10^{-7}$ . Although many of the most significant SNPs were in high LD, there appear to be

at least two distinct association signals in the 5' and 3' regions of the gene (SI Table 12A and B). Several two-marker haplotypes consisting of nonadjacent SNPs demonstrated an increase in significance by several orders of magnitude over either single marker alone. These included pairs of SNPs from the 5' and 3' region of the gene, suggesting the presence of an epistatic interaction between distinct risk alleles located in separate LD blocks.

Extended haplotypes spanning *IL23R* for all 17 SNPs in Table 3 were constructed (SI Table 11A and B and Table 4). Two risk and two protective haplotypes were significantly associated with CD in all population samples. These four haplotypes have a combined

**Table 3. Association of 17 Selected SNPs across *IL23R***

SNP#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RefSNP ID	rs11209003	rs11209008	rs2064689		rs1004819	rs2902440	rs11465802	rs2201841	rs11465804	rs11209026	rs1343151	rs10889676	rs10889677	rs998642	rs12567232	rs6669582	rs10789230
LD Block	2	2	4	4	4	4	5	6	6	6	6	6	6	6	6	Unique	Unique
Risk Allele	G	G	G	T	A	G	C	G	T	G	G	A	A	T	A	A	T
Type of SNP	Intergenic	Intergenic	Intronic	Intronic	Intronic	Intronic	Intronic	Intronic	Intronic	R381Q	Intronic	Intronic	3'UTR	Intergenic	Intergenic	Intergenic	Intergenic
p-values	QFP Trios, ultra-fine mapped																
p-values	German Trios, ultra-fine mapped																
p-values	German Case-Controls, fine mapped																
p-values	Combined QFP and German Trios, ultra-fine mapped																
p-values	Combined QFP Trios, German Trios and German Case-Controls, fine mapped																

Color scale indicates nominal  $P$  value for single marker association:

- $P < 10^{-7}$
- $P < 10^{-6}$
- $P < 10^{-5}$
- $P < 10^{-4}$
- $P < 10^{-3}$
- $P < 10^{-2}$

Single-marker association results for 17 representative SNPs that span the *IL23R* gene region and that were significantly associated in the QFP or replication samples. Each column corresponds to data for 1 of 17 SNPs. Color scale indicates nominal  $P$  value for single marker association. More detailed information for each SNP can be found in SI Table 11A.

**Table 4. Association of haplotypes formed by the 17 markers in *IL23R***

Population	Statistic	Risk		Protective	
		1	2	3	4
Qfp trios	<i>P</i>	1.86e-06	2.57e-03	0.04	0.05
	<i>F</i>	29.39   19.80	1.63   3.94	3.69   2.08	10.20   13.13
	OR	1.69 (1.36–2.09)	0.40 (0.22–0.74)	1.80 (1.02–3.19)	NS
German trios	<i>P</i>	0.01	1.15e-03	0.60	0.13
	<i>F</i>	26.48   21.28	0.91   3.09	3.65   3.20	9.13   11.33
	OR	1.33 (1.07–1.66)	0.29 (0.13–0.64)	NS	NS
Combined trios	<i>P</i>	1.95e-07	1.12e-05	0.07	0.01
	<i>F</i>	27.98   20.93	1.28   3.52	3.67   2.63	9.68   12.25
	OR	1.50T(1.29–1.75)	0.35 (0.22–0.57)	NS	0.77 (0.62–0.95)
German case controls	<i>P</i>	2.05e-03	6.31e-03	0.62	3.98e-03
	<i>F</i>	30.40   25.43	1.21   2.55	3.76   3.42	10.20   13.56
	OR	1.28 (1.09–1.50)	0.47 (0.27–0.82)	NS	0.72 (0.58–0.90)
All combined	<i>P</i>	1.00e-08	2.04e-07	0.05	5.75e-04
	<i>F</i>	29.53   23.34	1.25   3.11	4.17   3.26	10.40   13.12
	OR	1.38 (1.23–1.53)	0.39 (0.27–0.57)	NS	0.77 (0.66–0.89)

The four risk and protective haplotypes described in [SI Table 11B](#) are shown at the top. *P* values (*P*, significance of association of each haplotype tested against all other haplotypes in a 2 × 2 case control table using Pearson's  $\chi^2$ ), allele frequencies (*F*; cases/controls), and OR (95% C.I.) are shown for each of the analyses. NS, haplotype not significantly associated (C.I. for OR includes 1.0).

frequency of ≈45%. ORs for the significant risk and protective haplotypes were similar for the QFP and German samples. ORs for the most frequent risk haplotype ranged from 1.33 [confidence interval (C.I.) 1.07–1.66] in the German trios to 1.69 (C.I. 1.36–2.05) in the QFP sample. Risk haplotype 1 was significantly associated in all data sets, as was protective haplotype 2, which contains the opposite allele from risk haplotype 1 at each SNP position. Markers 1 and 3 (which are in high LD,  $r^2 = 0.84$ ) and Marker 6 (which is in moderate LD with Markers 1 and 3,  $r^2 = 0.30–0.31$ ) show consistent alternate alleles on both risk and both protective haplotypes, suggesting they are tagging disease mutations. Other SNPs, including the previously reported Marker 10 (R381Q) (15), may play functional roles in the disease, but their effect depends upon other alleles in the haplotype upon which they reside.

Immunohistochemical methods were used to examine the expression of *IL23R* in CD patients and controls. *IL23R* expression was detected on mononuclear cells within the colonic lamina propria of unaffected individuals but was significantly up-regulated in CD patients, primarily within epithelial cells ([SI Fig. 8](#)). The *IL23R* gene is known to give rise to multiple splice variants (26). Interestingly, the observed up-regulation seems to affect the shorter isoforms of the protein.

**Region 4p16.1.** The 30-kb replicated region located on chromosome 4p16.1 contains two genes, *JAKMIP1* and *LOC285484*. The maximum association signal for both the QFP and German trio fine-mapping (FM) data sets was located upstream of *JAKMIP1*, within *LOC285484* (Fig. 1). The entire region spanning *JAKMIP1* and *LOC285484* exhibits very low LD and contains several LD blocks, one of which spans the 5' ends of both *JAKMIP1* and *LOC285484*. Many of the SNPs within this block were associated at *P* values <0.001 (see [SI Fig. 9](#) for single-marker association plots).

The most significantly associated SNPs (all in nearly complete LD,  $r^2 = 0.996$ ) in the QFP (*P* values <10<sup>-5</sup>) were located within an intron of *LOC285484*. The most significant SNP within the *JAKMIP1* gene itself lies within the first intron (rs9991241, *P* = 4.17 × 10<sup>-4</sup>). The risk allele was present in 86.5% of cases and 80.3% of controls (OR of 1.56, 95% C.I. 1.22–2.01). In the German trios, the most significantly associated SNPs were located 3' to *LOC285484*. Four SNPs with *P* values <0.001 in the German trios lie within a 2-kb region and are in complete LD, with risk allele frequencies of 53% in the cases and 45% in the controls (OR 1.41,

95% C.I. 1.16–1.72). These SNPs were also associated in the QFP with similar *P* values. The risk and protective alleles, however, are reversed in the two trio samples. This phenomenon of association with opposite risk and protective alleles has been observed for other genes associated with complex disease in multiple populations. Although this lack of consistency can be the result of type 1 error, it has been argued that such replications may indeed be real, and that the “flip-flop” may be due to complex interactions with other disease loci (27).

**Region 3p21.3.** A replicated region is located on chromosome 3p21.3 (Fig. 1). Although this region was not among the most highly significant regions identified from the GWA study, it was selected for FM and replication studies based on the presence of strong functional candidates. The lowest nominal *P* value from the GWA study within this region (9.55 × 10<sup>-5</sup>) occurred at marker rs11718165, within an intron of the bassoon (*BSN*) gene. Two distinct association signals were observed in the FM analysis of QFP samples in two adjacent LD blocks spanning ≈800 kb, one centered at rs4855873 (*P* = 8.13 × 10<sup>-5</sup>), and one centered at rs1131095 (*P* = 1.99 × 10<sup>-6</sup>). These two associated regions were replicated in the German trios (*P* = 2.09 × 10<sup>-4</sup> and *P* = 1.0 × 10<sup>-4</sup> for five-marker haplotypes) and appear to be independent ( $r^2$  between haplotypes = 0.1830 in QFP trios,  $r^2 = 0.2972$  in German trios). Multiple single-marker associations (*P* values <10<sup>-5</sup>) were observed within both of these regions in the combined QFP and German trio analysis. In the full combined analysis, 17 single-marker associations at *P* values <10<sup>-5</sup> were observed in the first telomeric region (minimum *P* value = 3.16 × 10<sup>-7</sup> at rs6997). Five single-marker associations significant at *P* < 10<sup>-7</sup> were observed in the second centromeric region (minimum *P* value = 1.26 × 10<sup>-8</sup> at rs9822268, [SI Fig. 10](#)). This region, which exhibited only moderate statistical evidence in the GWA study (9.55 × 10<sup>-5</sup>), became the third-highest rank in the combined analyses with *P* values for haplotype association of 1.82 × 10<sup>-9</sup> (all trios) and 3.24 × 10<sup>-15</sup> (all three samples).

**Additional Regions.** Two additional regions demonstrated significant replication in the German case/control sample. Both of these regions are relatively large and may span multiple independent signals. The first, on 17q11, spans three relatively large blocks of LD and exhibits multiple signals with minimum *P* values of 5.13 × 10<sup>-5</sup> in the QFP and 1.29 × 10<sup>-7</sup> in the German case/control sample ([SI Fig. 5](#)). The second region, on chromosome 17q23, spans at least

three large LD blocks. Again, multiple signals were observed in this region, with minimum  $P$  values of  $5.39 \times 10^{-4}$  in the QFP and  $1.10 \times 10^{-5}$  in the German cases/controls (SI Fig. 6).

## Discussion

Recent GWA studies, including the one presented here, are beginning to reveal a clear picture of the major susceptibility genes and loci for CD (15–21). We identified three of the most replicated loci within our top seven GWA signals (*NOD2*, *IBD5*, and *IL23R*). Other loci that have been reported, such as the *ATG16L1* gene, the *IRGM* gene, a locus on 1q24, and an intergenic region near the *PTGER4* gene, are also observed in this study but exhibit lower statistical significance ( $\approx 0.001$ ). One of our most convincing replicated regions (3p21.3) was also observed in the most recent published GWA study (16, 21). The lack of complete overlap in association signals from the recent GWA studies may be due to genetic heterogeneity, differences in phenotype definition, sample sizes, and SNP distribution in the various scans.

We have also presented the most extensive analysis to date of the association of the *IL23R* region with CD. Our results suggest that it is highly unlikely that the nonsynonymous SNP rs11209026 (R381Q) fully explains the functional role of this gene in CD etiology. This SNP does not occur consistently in all risk and protective haplotypes. In addition, there is strong evidence for an epistatic interaction between polymorphisms located in the 5' and 3' end of the gene. It is possible that variants controlling isoform expression of *IL23R* are the major causative mutations. Interestingly, we have shown that a short isoform is up-regulated in CD patients.

The region on 4p16.1 contains two candidate genes (*JAKMIP1* and *LOC285484*). *JAKMIP1* is involved in IL23 signaling and binds to TYK2, a member of the Janus (tyrosine) kinase (Jak) family, which is associated with the IL12RB chain and mediates STAT-4 activation (28). The alternative disease gene at this locus, *LOC285484*, contains a characteristic high-mobility group (HMG)-box domain. The prototypic family member, *HMGB1*, is a DNA-binding molecule that binds single-stranded DNA and unwinds double-stranded DNA; however, *HMGB1* is also a secreted cytokine and may serve as a danger signal during acute and chronic inflammation after release by damaged cells and activated macrophages (29). The similarity of *LOC285484* to *HMGB1* suggests that it could be involved in aspects of intestinal inflammation and remodeling.

The region on 3p21.3 contains two adjacent but apparently independent replicated regions with strong candidate CD genes, including *GPX1*, *RHOA*, *DAG1 BSN*, *APEH*, and *MST1*. *GPX1*, located in the more telomeric region, encodes the powerful antioxidant, glutathione peroxidase isoform 1. Double-knockout mice lacking both *GPX1* and *GPX2* activity were found to have an inflammatory bowel disease phenotype (30). *RHOA*, also in the telomeric region, encodes a small GTPase involved in blockage of stress fiber formation in the innate cellular immune response in the presence of effector proteins released by pathogenic *Yersinia* species (31). The more centromeric region contains the *BSN* gene, which encodes a scaffolding protein expressed in axons. *MST1*, macrophage stimulatory protein 1, is involved in inflammation and tissue remodeling for wound healing. *APEH* (APH) encodes a serine peptidase with a reported functional role in the degradation of bacterial peptide breakdown products in the gut to prevent excessive immune response (32).

The results presented here illustrate the power of GWA studies to identify networks of disease susceptibility genes. We believe the success of this study is due in large part to the utilization of genome-wide haplotype association analyses, in addition to the use of unique population samples from the QFP. The biochemical pathways associated with CD susceptibility genes suggest a model of CD that appears to converge on pathophysiological pathways central to epithelial defense mechanisms, the interplay between

innate and adaptive immune response, and the repair or remodeling of tissue as part of a response to inflammatory and damage-induced stimuli. Such a model provides multiple potential targets for new therapies for CD.

## Methods

Details of the methods used in this study are available in SI Text.

**Population Samples.** The population sample used for the GWA study consisted of 382 CD patients collected within parent–parent–child trios sampled from the QFP. Recruitment into the study was subject to ethical review by various affiliated ethics review committees (SI Table 13), and all subjects gave written informed consent for participation in the study. Two additional samples were used for replication studies. These consisted of 521 affected child trios, 752 cases, and 828 independent controls, all from Northern Germany. German control individuals were obtained from the POPGEN biobank (33).

CD was diagnosed by colonoscopy, barium radiological examination during abdominal surgery, or exploratory biopsy, with patients showing signs of CD in either the colon or ileum or in both locations (SI Table 14). The expression of CD included fistulizing, stenosing, and inflammatory forms. Ulcerative colitis, acute infectious colitis, and indeterminate colitis were excluded.

**Genotyping.** The genome-wide scan SNP map was designed and genotyping performed by Perlegen Sciences, Mountain View, CA. Markers were chosen to maximize uniformity of coverage and were distributed approximately evenly throughout the genome. After removal of insufficiently polymorphic markers, genotypes for 164,279 markers were analyzed. For fine-mapping and replication studies, additional equally spaced SNPs to a density of one SNP per 2–5 kb within these regions were genotyped by using the Illumina GoldenGate platform.

**Statistical Analyses.** Genotype data were cleaned before analysis by removing markers or individuals that did not fit defined tolerances of genotype quality. These thresholds included a maximum of 1% missing values, conformity to Hardy–Weinberg equilibrium for markers, and conformity to Mendelian segregation patterns within trios.

Phase information was first deduced from the trio genotype information with the remaining unresolved phase assignments estimated using a modified version of the PL-EM algorithm (34). Haplotypes were estimated within 11-marker overlapping blocks, which advanced in one-marker increments across the chromosome. Estimated haplotypes were then ligated into chromosome long haplotypes by using trio information. Final phase assignment for any ambiguous marker was determined from consensus within the 21 sliding blocks within which it was contained. For the German case/control samples, phase was estimated directly by the PL-EM algorithm using 11 marker blocks.

Because  $\approx 25\%$  of the trios had affected parents, the data were not analyzed by using the transmission disequilibrium test. Instead, for the parent-affected trios, spousal chromosomes were used as controls. For child-affected trios, parental nontransmitted chromosomes were used as controls. Case-control analyses were performed by using a sliding window of one, three, five, seven, or nine markers. Case/control allele or haplotype frequency tables were constructed at each window position and size and, from these, a  $\chi^2$  value was calculated. A Pearson's  $\chi^2$  statistic with 1 degree or 2 degrees of freedom was calculated and nominal asymptotic  $P$  values determined for significance of association for single markers and for three-marker haplotypes, respectively. For five-, seven-, and nine-marker haplotypes, the expected values and variances of the square root of calculated  $\chi^2$  values were determined by the method of Smith (35) and used to construct a  $Z$  score. A nominal  $P$  value was calculated from this  $Z$  score.

Nominal  $P$  values were used to define candidate regions identified by the GWS. Peaks were identified as regions containing a maximum nominal  $-\log_{10} P$  value  $>3.0$  ( $P \leq 0.001$ ) and extending to the left and right until all haplotype or single marker  $-\log_{10} P$  values dropped below 1.5.  $P$  values for multimarker haplotypes were also calculated exclusively by permutation of case control status.

**Genome-Wide Significance.** The genome-wide significance of the observed nominal asymptotic  $P$  value at peaks was determined by performing random permutations of case and control status of haplogenotypes within trios, keeping haplotypes intact. For each permuted data set, a new GWA analysis was performed, and peaks were identified and ranked. Five hundred permutations were performed to construct the null distribution of the first, second, third, etc., order statistics for the  $-\log_{10} P$  values across the genome. The observed peaks in the original GWA study, ranked by their nominal  $-\log_{10} P$  values, were compared with the null distribution of their corresponding order statistics to determine genome-wide significance. A conservative upper bound and a range for the genome-wide significance of individual nominal  $P$  values were obtained by successively removing (peeling), one at a time, the highest remaining signal that was genome-wide significant ( $P \leq 0.05$ ) and measuring nominal  $P$  values of the remaining peaks against the distribution of order statistics shifted by one.

**Significance in the Replication Study.** Statistical significance of replication was determined by permutation studies in which case and control status of the German samples was randomly permuted to produce the null distribution of nominal  $P$  values within the German samples. Peak-wide replication significance in the German samples was estimated by first extracting the peak region from the QFP GWA analysis and then comparing it with peaks from the analysis of the German samples. An overlap of peaks would occur if a peak in the QFP study and a peak in the German sample both reside in the intersection of the two candidate peak regions. The peak-wide replication significance (based on 500 random permutations of case-control status) measures how often by chance alone an overlap occurs with a  $P$  value as significant as was observed in the replication sample. The permutation procedure corrects for all SNPs and SNP combinations considered to define a peak and its surrounding peak region and therefore represent a true peak-wide

replication probability. The permutations were performed without taking the phenotypic status of the parents into account.

**Sequencing.** Sixteen QFP samples were selected to represent at least two copies of all major haplotypes ( $\geq 5\%$  frequency) and one copy of minor haplotypes (1–5% frequency) observed. A region of 130 kb spanning the *IL23R* locus was sequenced and 339 SNPs, 60 microsatellites, and 18 insertions/deletions were identified (SI Table 8D). A region of 60 kb spanning the *JAKMIP1/LOC285484* association locus was sequenced, and 226 SNPs, 16 microsatellites, and 20 insertions/deletions were identified (SI Table 9D).

**Immunohistochemistry.** Paraformaldehyde-fixed paraffin-embedded biopsies were prepared from five normal controls and five patients with confirmed colonic CD. Two slides of each biopsy were stained with hematoxylin/eosin for routine histological evaluation. The other slides were subjected to a citrate-based antigen retrieval procedure, permeabilized by incubation with 0.1% Triton X-100 in 0.1 M PBS, washed three times in PBS, and blocked with 0.75% BSA in PBS for 20 min. Sections were subsequently incubated with the primary antibody (anti-IL23R, Abcam, Cambridge, MA) at a 1:200 dilution in 0.75% BSA for 1 h at room temperature. After washing in PBS, tissue-bound antibody was detected by using biotinylated goat anti-rabbit (Vector Laboratory, Burlingame, CA) and followed by HRP-conjugated avidin, both diluted at 1:100 in PBS. Controls were included by using irrelevant primary antibodies and omitted the primary antibodies by using only secondary antibodies and/or HRP-conjugated avidin. No significant staining was observed with any of these controls (data not shown).

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