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Characterization of the enhancer and promoter landscape of inflammatory bowel disease from human colon biopsies

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Inflammatory bowel disease (IBD) is a chronic intestinal disorder, with two main types: Crohn’s disease (CD) and ulcerative colitis (UC), whose molecular pathology is not well understood. The majority of IBD-associated SNPs are located in non-coding regions and are hard to characterize since regulatory regions in IBD are not known. Here we profile transcription start sites (TSSs) and enhancers in the descending colon of 94 IBD patients and controls. IBD-upregulated promoters and enhancers are highly enriched for IBD-associated SNPs and are bound by the same transcription factors. IBD-specific TSSs are associated to genes with roles in both inflammatory cascades and gut epithelia while TSSs distinguishing UC and CD are associated to gut epithelia functions. We find that as few as 35 TSSs can distinguish active CD, UC, and controls with 85% accuracy in an independent cohort. Our data constitute a foundation for understanding the molecular pathology, gene regulation, and genetics of IBD.

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Inflammatory bowel disease (IBD) is an umbrella term for a range of chronic idiopathic disorders, of which Crohn’s disease (CD) and ulcerative colitis (UC) constitute the two major entities, both with an increasing incidence and prevalence worldwide, with an estimated 2.5–3 × 10⁶ patients in Europe alone. UC is characterized by mucosal inflammation of the colon, whereas CD may affect all layers of the intestine throughout the gastrointestinal tract (Fig. 1a). The distinction between CD and UC is critical for correct medication and especially surgery, yet the diagnosis is challenging. In a previous meta-analysis of 22,038 IBD patients, it was impossible to distinguish UC or CD in 13% of cases. This is a major problem for patients who have failed medical therapy and are facing colectomy. Genome-wide association studies (GWAS) have identified >200 loci containing IBD-associated variants, but IBD-associated single-nucleotide polymorphisms (SNPs) can only explain 15–20% of the disease phenotype. Moreover, ~70% of IBD-associated SNPs are non-coding and might affect gene regulation. However, a comprehensive map of active promoters and enhancers is lacking for IBD patients.

Here we present CAGE analysis on biopsies from the descending colon of 94 subjects (cohort 1): 25 active UC, 17 UC patients in remission (UCi), 3 CD patients in remission (CDi), and 29 control subjects (Ctrl) (Fig. 1a). Surprisingly, UCi and CDi were not readily distinguishable from Ctrl (Supplementary Note 1). Hence, we excluded UCi and CDi from the systematic analysis below.

In order to identify differentially expressed TSSs, we used the generalized linear model framework in edgeR. As observed above, CDa and UCa samples were characterized by a shared response compared to Ctrl (PC1 in Fig. 2a.), and secondly by differences between CDa and UCa (PC2 in Fig. 2a). To capture this, we defined sets of significantly up- or downregulated TSSs identified in both CDa and UCa vs. Ctrl (IBDup and IBDdown). To identify TSSs distinguishing CDa and UCa, we identified TSSs significantly upregulated in UCa vs. CDa, defined as CDspec, and TSSs significantly upregulated in UCa vs. CDa, defined as UCspec. The differential expression analysis recapitulated the PCA results: the number of TSSs within IBDup (4376) and IBDdown (2536) was much larger than in CDspec (337) and UCspec (71) (Fig. 2b, Supplementary Data 4). The inclusion of additional patient data where one or more TSSs responded differently compared to the other TSSs inside the same gene (as exemplified in Fig. 1c): 2068 genes showed this pattern in IBDup/IBDdown and 82 in CDspec/UCspec (Supplementary Data 5).

Because most functional annotations are on gene rather than TSS level, we defined differentially expressed genes by summing the contribution of each TSS within each GENCODE gene model and repeating the differential expression analysis above (Supplementary Data 6, Supplementary Fig. 2b). Gene Ontology (GO) analysis (Fig. 2c, Supplementary Data 7) of IBDup genes showed a strong overrepresentation of GO terms related to inflammatory response and cytokines. IBDup genes were also enriched for colon-specific processes previously related to IBD pathogenesis, for example remodeling of the extracellular matrix (FDR = 2.66e−09) and antibacterial peptide secretion (FDR = 1.02e−14). Because small networks of biologically linked genes are challenging to identify through GO analysis, we supplemented this analysis with STRING network analysis. This identified smaller sets of upregulated genes with known functions in gut epithelia barrier integrity, including gap junctions (connexins AQP5, GJA1,5) and tight junctions (claudins CLDN1, 2, 10, 14, and 18) (Supplementary Fig. 2c). Interestingly, the full set of CLDN genes could distinguish IBD from Ctrl subjects, because a subset was part of the IBDdown set (Supplementary Fig. 2d–e). More generally, the IBDdown set was enriched for terms related to xenobiotic response and drug/steroid processing (Fig. 2c): STRING analysis showed that proteins corresponding to IBDdown genes were involved in cell cycle and growth, solute and membrane transport and maintenance of fluid balance in the...
Fig. 1 Defining the TSS landscape of IBD. a Overview of data set. Pinch biopsies from the descending colon were taken from 94 human subjects, classified into active ulcerative colitis (UCa), active Crohn’s disease (CDa), UC and CD patients in remission (UCi, CDi) and controls (Ctrl: subjects screened for IBD where all subsequent investigations turned out normal). For each biopsy, a CAGE library was produced, resulting in the detection of TSSs and enhancer regions. Schematics show the typical inflammatory patterns in the intestinal system, the approximate location of biopsy sampling and number of subjects in each group. b Detection and annotation of gene TSSs. Top panel shows an example gene with CAGE-defined TSSs, which are annotated as main, alternative or novel TSSs defined by their overlap with GENCODE gene annotation as indicated in callouts. CAGE-defined TSSs not falling into any of the categories were defined as novel intergenic TSSs. Left bottom panel shows the number of detected TSSs in each category (colors correspond to callouts in top panel), split by CAGE expression strength measured as tags per million (TPM). Right bottom panel shows the expression distribution of each category of TSSs as boxplots. c Genome-browser example of the detection of annotated and novel TSSs in the ST6GAL1 gene. From top to bottom, the browser plot shows the genomic location investigated, RefSeq gene annotation (exons are denoted as boxes, green indicate forward strand transcription). Below, CAGE TPM expression on the forward strand is shown as average across subjects (green bars) and for individuals (pink heat map, each row is one subject, columns are widened 5× for readability), split by subject group. Annotated and novel TSSs, annotated as in b are highlighted. Note that the first novel alternative TSS is upregulated in CDa and UCa vs. remaining groups, while the last novel alternative TSS has the opposite pattern (block arrows indicate TSSs and their overall strength in each subject group for these two TSSs). Conversely, the annotated TSSs are detected but not substantially changing between groups.
intestine (Supplementary Fig. 2f), with a strong sub-network of genes involved in steroid hormone biosynthesis.

The CDspec set was enriched for terms related to steroid, lipid, and lipoprotein metabolism (Fig. 2c). Because of the limited number of genes in the UCspec set, only a handful of GO terms and lipoprotein metabolism (Fig. 2c). Because of the limited number of genes in the UCspec set, only a handful of GO terms were overrepresented, including several terms linked to gap junctions. These results largely mirrored those identified by RNAseq in ileal pediatric CD20. This study identified a CD-vs-UC upregulation of apolipoprotein genes, which was also present in our set (Supplementary Fig. 2g). Thus, it is clear that while CDa and UCa share a strong inflammatory component, we show that genes that differentiate CDa and UCa are not primarily related to...
immune response but rather to cellular functions associated to gut and gut epithelia.

Differential expression analysis will favor genes showing low variance within groups. However, gene expression variance across patients with the same diagnosis is relevant for patient stratification and precision medicine. We found that five antibacterial peptide genes, defensins DEFA5 and DEFA6 (normally expressed in small intestine²¹ and Paneth cells) and c-lectins REG1A, REG1B, and REG3A (normally expressed in pancreas and small intestine²²) were highly upregulated in IBD and at the same time showed extreme variance across UC and CD subjects (Fig. 2d). The upregulation of these genes was highly correlated to cytokine induction (Supplementary Fig. 2h). The large variance might indicate patient-specific responses to inflammatory factors, disease chronicity or cell metaplasia, and again highlights the role of epithelia-specific genes IBD response.

Colonic biopsies consist of multiple cell types, whose composition may change in IBD. We assessed this in two ways. First, we took advantage of the FANTOM5 TSS atlas, where individual TSSs were annotated as being preferentially expressed in one or more cell types sampled across the human body based on CAGE expression²¹. With these data, we could estimate overall cell type enrichments for each differentially expressed TSS group across all FANTOM5 primary cell types (Fig. 2e, Supplementary Data 8). IBDup, TSSs were enriched in immune-related cells, while IBDonw TSSs were enriched in epithelial cells. Cell-type enrichment patterns of CDpec and UCpec TSSs were different: CDpec TSSs were mostly enriched for immune-related cells (including basophils, monocytes, and eosinophils) and intestinal epithelial cells, while the UCpec TSSs were mostly enriched in epithelial cells and mesenchymal cells. Although THelper cells have previously suggested to define the difference between CDa and UCa (reviewed in ref. ²²), they were not enriched in our analysis, consistent with previous results²³.

Second, because FANTOM5 data only included cells from healthy tissue, we wanted to also assess whether induction of an inflammatory response induced IBD signature genes in immune cells and/or epithelial cells. To address this, we used primary cultures of colonic epithelia organoids and monocytes, which were stimulated with TNF (also known as TNFα, one of the most prominent inflammatory molecules associated with flaring disease in IBD patients²⁵). Cells were stimulated with TNF for 4 and 24 h (Fig. 2f, Supplementary Fig. 3a, Supplementary Data 9) and the expression of 35 TSSs (established in the classification section below) was measured by qPCR. The same TSSs were measured by qPCR in gut biopsies from an independent cohort with 18 CDa, 37 UCa and 46 Ctrl subjects (cohort 2). We found that out of a total of 21 TSSs that were upregulated in UCa or CDa vs. control, 66% (14/21) and 81% (17/21) were also upregulated in TNF-stimulated organoids and monocytes, respectively, at any time point; 47% (10) of TSS were upregulated in both stimulated organoids and monocytes (Fig. 2f and Supplementary Fig. 3b, c). It is thus unlikely that the IBD expression change is solely attributed to immune cells; epithelial cells are likely part of shaping the transcriptional changes associated with UC and CD.

Thus, in agreement with the GO analysis, these cell type enrichment results indicated that the difference between Ctrl, CDa, and UCa does not only lie in immune cells and their associated general immune response pathways, but also in cells and genes associated with gut epithelia.

The enhancer landscape of inflammatory bowel disease. Gene and TSS-focused analysis as above can give insights into gene expression but not their regulation by distal enhancers. As established previously, bidirectional CAGE peaks accurately predict enhancer locations and activity³⁵. Using the same method as in ref. ¹³, we predicted a permissive set of 37,231 enhancers, and a strict subset of 10,670 enhancers where ≥8 samples had detectable enhancer expression (the latter set was used in the rest of the analyses; Supplementary Data 10, 11 and Supplementary Fig. 4a). The strict enhancer set overlapped ENCODE DNase hypersensitive sites (DHSs)²⁶ in 90% of cases (P < 2e−16, by sampling), and these enhancers were significantly more conserved across mammals than non-transcribed DHSs from gut tissue²⁷ and randomly selected genomic regions (P < 5.2e−4, two-sided Mann–Whitney U test; Fig. 3a). These enhancers were also strongly enriched for ENCODE transcription factor (TF) ChIP-seq peaks from diverse cells (Fig. 3b). Similarly, they were highly enriched for H3K27ac and H3K4me1 ChIP-seq signals from rectal and colonic mucosa, and to a lesser degree other intestinal regions (Fig. 3c and Supplementary Fig. 4b). FAIRE-seq signals from colonic CD and control patients²⁸,²⁹ were also enriched (Supplementary Fig. 4c). However, the regions had less H3K27ac and H3K4me1 ChIP-seq enrichment in immune cells and non-gut tissues (Fig. 3c and Supplementary Fig. 4b). Thus, the enhancer candidates had the hallmarks of enhancer regions in general, but seemed most strongly used in gut tissue.

PCA of enhancer expression showed separation between inflamed samples (UCa and CDa) and Ctrl, but only minor separation between UCa and CDa (Fig. 3d). We used EBSSeq³⁰ to

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**Fig. 2** Differential expression of TSSs and genes in IBD. a Principal component analysis (PCA) based on CAGE TSS regions. X- and Y-axes show principal components (PCs) 1 and 2, percent of variance explained is indicated. Dots correspond to subjects, colored by group. Boxplots at the bottom and right show the distribution of PC. Circles show three major groups: CDa, UCa, and non-inflamed samples (UCi, CDi, and Ctrl). b Number of differentially expressed TSSs. Bar plot shows the number of differentially expressed TSSs in the four defined groups. c Gene ontology (GO) term overrepresentation analysis of differentially expressed genes. X-axis shows GO term overrepresentation FDR values on −log₁₀ scale for differentially expressed genes in the IBDup, IBDonw, and CDpec sets. Y-axis shows the top 10 GO terms, ordered by FDR. d Identification of IBD-upregulated genes with extreme variance across IBD patients. Y-axis shows the variance of CAGE expression across CDa, UCa, and Ctrl subjects. X-axis shows the F-statistic from edgeR. Dots correspond to genes, where size indicates the average CAGE expression in respective group. Five outliers are highlighted, corresponding to antibacterial peptides. e FANTOM5 cell type enrichment of differentially expressed TSSs. Differentially expressed TSS sets were analyzed for overlap with TSSs specifically expressed in cell types in FANTOM5. X-axis shows under/over-representation of a given cell type expressed as log₂(odds). Whiskers indicate 95% confidence intervals. Whiskers with black lines indicate statistical significance (Fisher’s exact test, FDR < 0.05). Cell types are ordered by their log-odds ratio in IBDup. Numbers in parentheses indicate the number of IBD-expressed TSSs specifically expressed in respective cell type in FANTOM5. Red shading shows cell types only enriched in CDpec TSSs, blue indicates cell types only enriched in UCpec TSSs. f Correspondence of TSSs upregulated in IBD with TSSs upregulated after TNF stimulation in epithelial organoids and blood monocytes. Y-axis shows the number of TSSs (out of 36) upregulated in IBD (UCa or CDa vs. Ctrl), upregulated after TNF stimulation in gut epithelia organoids or blood monocytes, measured by qPCR. Upregulation after 4 and 24 h of TNF stimulation are pooled; see Supplementary Fig. 3c for time-specific measurements.
define four differentially expressed enhancer groups, analogous to the TSS groups defined above: shared CDa and UCa up/downregulated vs. Ctrl (IBDup and IBDdown), upregulated in CDa vs. UCa (CDspec) and vice versa (UCspec) (Supplementary Data 12 and Supplementary Fig. 4d). In agreement with the PCA, the IBDup and IBDdown enhancer sets were much larger than CDspec and UCspec sets (Fig. 3e).

Next, we used HOMER to identify shared predicted TF binding sites around IBDup and IBDdown enhancers (±300 bp around enhancer midpoints; CDspec and UCspec sets were too small for meaningful analysis). The same analysis was used for promoter regions of corresponding TSS groups (defined as −500/+100 bp around TSSs) (Fig. 3f). IBDup promoters and IBDup enhancers were enriched for sites linked to TFs previously associated with inflammation, including ETS1, IRF1, STAT4, and RELA. IBDdown promoters and enhancers site enrichments were also highly similar: in particular, HNF4A and CDX2 sites were over-represented, consistent with their important roles in...
differentiation of gut epithelial cells. Decreased CDX2 and HNF4A expression were previously observed in IBD and indeed, site enrichments generally correlated with the expression of the associated TF (Fig. 3). We observed similar enrichment patterns when analyzing enhancer and promoter regions for overlapping ENCODE TF ChIP-seq peaks (Supplementary Fig. 5). Thus, IBD-induced enhancers and promoters share similar TF binding pattern enrichments, which are distinct from those of IBD-repressed enhancers and promoters.

To understand enhancer regulation, enhancers must be linked to target gene TSSs. As shown previously, enhancer-TSS pairs can be predicted based on CAGE co-expression across samples. Following the same approach, we identified 21,502 enhancer-TSS pairs within 500 kb with positive and significant Pearson correlation (correlation test, FDR < 0.05) (Supplementary Data 13). Of these pairs, 8507 (39.6%) were between a differentially expressed TSS and an enhancer, regardless of its differential expression status. Around half of IBD up and IBD down TSSs (54.7% and 47.9%, respectively) were linked with at least one enhancer, compared to smaller fractions for CD4 spec, UC spec (40.9% and 18.3%) and remaining TSSs (Fig. 3g). Enhancer-linked TSSs in IBD up and IBD down groups were commonly linked with more than one enhancer: in particular, IBD up TSSs linked to >3 enhancers were as frequently observed as TSSs linked to a single enhancer (Fig. 3g).

The NOD2 locus, a cluster of chemokine genes (CXCL1-3, CXCL5-6, and CXCL8), and two receptors for these chemokines (CXCR1 and CXCR2), represent examples of enhancers linked with neighboring TSSs of genes associated with IBD pathogenesis (Fig. 4a–c). The examples show putative distal regulators for key IBD genes, and highlighted a potential regulatory SNP that may affect the chemokine response pathway. Due to the common occurrence of enhancers linked to cytokine-related genes, we annotated the cytokine–cytokine receptor interaction pathway with linked enhancers and alternative TSSs (Supplementary Fig. 6).

Characterization of IBD upregulated enhancer clusters. Previously, large regions having enhancer-like chromatin features—so-called “super”—or “stretch”—enhancers—were identified as drivers of central biological processes. By eye, clusters of CAGE-defined enhancers were evident, for instance in the region surrounding CEBPB, a key inflammatory response TF (Fig. 5a). We defined CAGE-based enhancer clusters by requiring >2 consecutive enhancer midpoints spaced ≤15 kb from each other, resulting in a set of 693 enhancer clusters (Supplementary Data 14), where 67% overlapped enhancer clusters defined by ChIP-seq in immune and gut cells. CAGE-defined enhancer regions within clusters often co-occurred with ENCODE TF ChIP-seq peaks (Fig. 5a).

The number of CAGE-defined enhancers within a cluster correlated with the average fraction of IBD up but not IBD down enhancers in the same cluster (Fig. 5b). The same trend was observed for predicted enhancer targets: TSSs linked to at least one enhancer within a cluster had a higher IBD vs. Ctrl fold change if the enhancer cluster contained >6 enhancers compared to TSSs linked to clusters with fewer enhancers (P < 2.2e−16, two-sided t-test) (Fig. 5c and Supplementary Fig. 7a). Consistent with these observations, if an IBD up TSS was linked to an enhancer in a cluster with >6 enhancers, it was typically linked with all or most enhancers within the cluster (Supplementary Fig. 7b). Altogether, this suggests that enhancer clusters with many members and singleton enhancers have distinct functions in IBD.

To further explore the relation between the number of enhancers within a cluster and their regulatory function in IBD, we compared (i) IBD up TSS-linked enhancers from enhancer clusters with >6 members with (ii) IBD up TSS-linked singleton enhancers (not belonging to any cluster). Singleton enhancers had a greater overlap with ENCODE ChIP-seq peaks corresponding to TFs associated with inflammation (including RELA, SP1, STAT factors, and IRF1) (Fig. 5d), while enhancer clusters were overrepresented for ChIP-seq peaks for chromatin remodelers and insulators, including SMARCA4 and CTCF. As SMARCA4 has a function in activation of repressed genomic regions and CTCF is an insulator, these larger enhancer clusters might reflect large-scale changes in chromatin structure induced by the inflammatory response, while smaller enhancer clusters may be driven by the binding of one or a few TFs.

SNP overrepresentation in enhancers and promoters. As discussed above, a majority of IBD-associated SNPs are located in intergenic regions, and may impact gene regulation. The UC-associated SNP overlapping an IBD up enhancer located between cytokine receptors CXCR1 and CXCR2 (Fig. 4c) exemplifies an SNP with potential regulatory function. As we did not have access to the genotypes of our subjects, we investigated the overlap between publically available IBD-associated SNPs and enhancer and promoter regions using two complementary approaches; GWAS SNP enrichment and partitioned heritability of IBD.

First, we obtained all SNPs significantly associated with a disease/trait from the GWAS catalog. For each GWAS SNP, we merged the region covered by other SNPs in linkage disequilibrium (LD, R2 > 0.75 and within 500 kb) into a single block, using...
the 1000 genomes CEU reference population. If LD blocks overlapped, they were merged into an LD clump.

For every GWAS disease, we assessed the overlap between LD clumps with our identified promoter (−500/+100 bp around TSS peaks) and enhancer regions (±300 bp around enhancer midpoints) by using empirical Bayes to shrink fraction of overlapping LD clumps toward the average of all GWAS diseases. Only IBD, CD and UC LD clumps had a high degree of overlap with both the promoter and enhancer sets (Fig. 6a and Supplementary Fig. 8a). Next, we analyzed whether differentially expressed TSSs and enhancers were enriched for LD clump overlaps. IBDup promoters and enhancers were enriched for LD clumps associated with several immune-related diseases, but only IBD, CD, and UC LD clumps were enriched in both promoters and enhancers.
(Fig. 6b). Enhancers, but not promoters, were highly enriched for other inflammatory intestinal disease LD clumps. Conversely, IBD\textsubscript{down} TSSs and enhancers were not enriched for LD clumps associated with IBD, UC, or CD, but showed modest enrichment for LD clumps associated with heart disease (Fig. 6c).

The above analysis only assessed enrichments of genetic variants reaching genome-wide significance. However, many genetic variants might collectively make important contributions to IBD pathogenesis without individually reaching genome-wide significance. To this end, we performed partitioning of heritability analysis using the stratified LD-score regression method\textsuperscript{40} to investigate if TSSs and enhancers were enriched for SNPs explaining the heritability of IBD. Briefly, this method measures SNP heritability enrichment in a set of genomic regions (e.g., enhancers) as the proportion of SNP heritability $\left(Pr \left(\hat{h}^2\right)\right)$ divided by the proportion of SNPs (Pr(SNP)) in the same regions, based on GWAS summary statistics. Previously, this method was employed on 24 classes of annotated functional regions and GWAS summary statistics from 17 complex diseases, to establish a "baseline model" describing the contribution of each annotation class\textsuperscript{40}. To extend this analysis with our enhancer and TSS sets in an IBD setting, we obtained GWAS summary statistics for ~12 million SNPs from the International IBD Genetics Consortium\textsuperscript{5}. We then analyzed the partitioned heritability of IBD in the 24 annotated functional regions from\textsuperscript{40} but added our enhancer (+300 bp around enhancer midpoints) and promoter (−500/+100 bp around TSS peaks) regions, and the subsets of these belonging to the IBD\textsubscript{up} or IBD\textsubscript{down} groups, to the original model. We found that our enhancers and promoter sets were significantly enriched for IBD SNP heritability. This was even more evident in the IBD\textsubscript{up} enhancers and promoters, while IBD\textsubscript{down} was not significantly enriched (Fig. 6d, Supplementary Fig. 8b, c). The combined IBD\textsubscript{up} enhancer and promoter set had the highest heritability enrichment of all regions included in the baseline model, including FANTOM5 enhancers. Overall, IBD heritability enrichment was considerably higher for annotation classes corresponding to regulatory regions (enhancers, promoters etc.) than coding exons.

Overall, these results suggest that there is an association between IBD-associated SNPs and the regulatory regions we define. While the genotypes of the samples investigated here would be necessary to establish the causal effect of individual SNPs, our analysis indicate that the enhancer and promoter resources we provide are useful starting points for future investigations of non-coding IBD-associated SNPs, since they allow for a more detailed interpretation of otherwise characterized intergenic regions.

Classification of UC, CD, and controls from TSS expression. The statistical analyses above could identify TSSs and enhancers that were up- or downregulated between subject groups, but cannot assess whether expression data for a single biopsy contain enough information to accurately classify the subject as active UC, active CD, or control. This is a relevant question as the diagnosis between active UC and CD is often clinically difficult, and expression data might be useful to increase overall diagnosis accuracy. We reasoned that CAGE-based analysis is not realistic in a clinical setting; instead, we sought to define a small set of CAGE-based biomarkers for active UC, active CD and controls that were amendable to robust targeted methods such as qPCR.

To achieve this, we used successive steps of biomarker selection and testing (Fig. 7a). First, we analyzed the CAGE data from the 25 UCa, 20 CDa, and 29 Ctrl subjects in cohort 1 with an ensemble of statistical and manual curation methods to extract 274 TSSs/enhancers distinguishing UCa, CDa, and Ctrl groups (Supplementary Data 15). A Random Forrest (RF) classification framework trained on CAGE data for these 274 biomarkers could predict UCa, CDa, and Ctrl diagnosis with an overall accuracy of 95%, assessed by five-fold cross-validation (Fig. 7b, left and middle panels). This high accuracy could be retained when reducing the number of biomarkers (Fig. 7b, right panel). We were able to design and validate qPCR primers corresponding to 161 of these biomarkers (Supplementary Data 15, 16), and analyzed their expression using microfluidic qPCR on the same biopsies as above (cohort 1: 161 primer pairs analyzed in biopsies from 74 subjects) (Supplementary Data 17). An RF trained on the microfluidic qPCR expression data gave an overall accuracy of 84% assessed by five-fold cross-validation (Fig. 7c, left and middle panels). Thus, accurate classification of IBD based on these biomarkers was achievable across experimental methods. Similar accuracy levels were achieved using fewer biomarkers: we retained comparable accuracy using 30–40 biomarkers (Fig. 7c, right panel). Encouraged by this, we reduced the number of biomarkers to a final set of 35. In this final selection no enhancers were retained, consistent with their lower RNA abundance compared to gene TSS.

To test whether the classification power of our biomarkers generalized beyond cohort 1, we enrolled a second independent validation cohort (cohort 2: 18 CDa, 46 Ctrl, 37 UCa; Supplementary Table 1), which included biopsies acquired at two different hospitals by different physicians, and measured the expression of same 35 biomarkers using microfluidic qPCR (Supplementary Data 18). We then trained a RF on microfluidic qPCR data from the 35 biomarkers from cohort 1 and predicted the diagnosis of the subjects in cohort 2. We achieved an overall accuracy of 85% in cohort 2 (Fig. 7d, left and middle panels), strongly suggesting that our selected biomarkers generalize to the larger population. Although the CD sensitivity was lower when using qPCR than with CAGE (see Discussion), our results were in all cases substantially better than expected by chance, as estimated by training on randomly shuffled cohort 1 labels and then...
Fig. 5 Characterization of enhancer clusters in IBD. a Example of an enhancer cluster in the CEBPB locus. Genome browser screenshot of the CEBPB locus, organized as in a but also showing chromatin-defined enhancer clusters from dbSUPER36, and a CAGE derived enhancer cluster located ~150 kb downstream of CEBPB. Because CEBPB has two nearby alternative TSSs with similar activity, most enhancers are linked to both. Lower panel shows a zoom-in of the enhancer cluster where ENCODE transcription factor ChIP-seq peaks are displayed: each black line corresponds to one ChIP-seq peak.

b Relation between enhancer IBD-up/downregulation and number of enhancers within an enhancer cluster. Bar plot shows the fraction of enhancers that are significantly downregulated (IBDown, gray) or upregulated (IBDup, white) in UCa and CDa vs. Ctrl, grouped by the number of enhancers within an enhancer cluster. Enhancers not part of clusters are included for comparisons (singleton enhancers). The expected overlap by chance for each bar is indicated as dotted lines, with 95% confidence intervals.

c Relation between the number of enhancers within an enhancer cluster and IBD upregulation of linked TSSs. Boxplots show the distribution of IBD vs. Ctrl log fold changes of TSSs linked to singleton enhancers or enhancer clusters as in b. TSSs are grouped by how many enhancers the linked enhancer cluster contains.

d Overrepresentation of ENCODE TF ChIP-seq peaks in singleton enhancers vs. enhancer clusters with >6 members linked to IBD-upregulated TSSs. X-axis shows the log2 fold change in ENCODE ChIP-seq peak over-representation in single enhancers vs. enhancers within enhancer clusters having >6 enhancers, where 0 indicates no difference between sets. Only enhancers linked to IBDup TSSs are analyzed. Y-axis shows the associated over-representation P-value. Each dot corresponds to one type of ChIP-seq peak, colored by whether they are annotated as inflammation-associated factors (purple), SMARC- or CTCF factors (orange), or other factors (gray). Factors of interest are highlighted.
predicting the labels of cohort 2 (Fig. 7d, right panel and Supplementary Figure 9a, b). To explore whether CD sensitivity could be improved further, we employed the gradient boosting decision tree method implemented in XGBoost\(^{41}\), training on the same 35 biomarkers cohort 1 and predicted the diagnosis of the patients in cohort 2. This resulted in an increase in CDA classification sensitivity by 13% points with no overall classification performance decrease (Supplementary Figure 9c). Thus, our initial results are not specific to machine learning method employed and may be improved by the use of more complex techniques. In summary, we have shown that a small set of qPCR primers, selected from CAGE data, could distinguish control, active UC, and active CD biopsies.

**Discussion**

Here we have profiled the enhancer and TSS landscape of biopsies taken in the descending colon of admitted IBD patients and controls. Such biopsies are identical to biopsies used in current...
diagnosis methods. This has the advantage that samples are highly clinically relevant, and that transcriptional changes in the disease states measured will be close to the in vivo reality. Thus, from a diagnostic perspective, such tissue samples are more relevant than selected primary cells isolated from tissue. As with all tissue samples, expression changes may be partially due to changes in cell composition. We showed that aside from a shared increase of immune cell-linked TSSs, active CD and UC seem to have different composition of cells, where UC-specific gene expression was more strongly linked to epithelial cells.

CAGE data interpreted on gene level identified similar pathway and GO term enrichments as previous RNA-seq studies. A recent study identified two distinct CD populations based on RNA expression data: we could not replicate this finding but the CAGE data did show the active CD population to be much more transcriptionally diverse than active UC or controls, as discussed below.

This is a large-scale analysis of enhancer activities across a total of 94 CD, UC, and control subjects. We investigated two smaller CD populations (N = 10 and 9) using FAIRE-seq, a method identifying accessible DNA, while H3K27ac ChIP-seq has been performed in gut tissue for even fewer individuals, and not in an IBD context. With our data, we could establish that promoters and enhancers that are upregulated in IBD share the same DNA-binding patterns enrichments, and that enhancer clusters upregulated in IBD are distinct from corresponding singleton enhancers.

The enhancer component of IBD reported here gives new possibilities for understanding the disease and its genetics. We show a clear overrepresentation of IBD-associated SNPs in both IBD-upregulated enhancer and promoter regions, and these regions had the largest IBD heritability enrichment compared to a large set of other genomic regions. Thus, the IBD TSS and enhancer sets reported here are unique resources for interpretation of the functional impact of noncoding genetic variants.

Aside from adding to the functional and genetic understanding of IBD, CAGE data can be used to distinguish between active UC, CD, and control subjects. This accuracy could be retained using a secondary feature selection process used to reduce the set of biomarkers to 35. We analyzed the expression of these biomarkers in an independent validation cohort (cohort 2) using microfluidic qPCR. Classification analysis was performed at each step (panels b–d). b Prediction of UCa/CDa/Ctrl diagnosis labels based on CAGE expression. CAGE expression data from cohort 1 from 274 selected biomarkers were used to train and evaluate a Random Forest model based on fold cross-validation. Left panel: average accuracy, sensitivity, and specificity are shown for each subject group as bar plots along with overall accuracy. Error bars show 95% confidence intervals across cross-validations. Dotted lines indicate 0.8 and 0.9. Middle panel: confusion matrix showing average fractions of predictions that fall into each of the actual subject groups (columns add to 100%). Right panel: average prediction accuracy (Y-axis) as a function of number of biomarkers used for training (X-axis). Shaded areas indicate 95% confidence intervals across cross-validations. c Prediction of UCa/CDa/Ctrl diagnosis labels based on microfluidic qPCR expression. Plots are organized as in panel b, but on microfluidic qPCR expression data from cohort 1 using 161 primers corresponding to selected biomarkers. d Validation using an independent cohort feature reduction based on the data in panel c resulted in the selection of 35 features. We trained a Random Forest model on microfluidic qPCR data from these biomarkers from cohort 1 and evaluated it on corresponding data from an independent cohort (cohort 2). Left and middle panels show classification results, as in panel b. Right panel shows a comparison between the confusion matrix (as in panel b) of our predictions and the confusion matrix obtained by repeating the analysis with randomly shuffled training labels. Numbers indicate the average fold changes of fractions (actual vs. shuffled).
in clinical settings in its current form, it may provide an inroad for new qPCR-based diagnostic methods complementing current diagnostic methods. Several studies have used microarray- or qPCR-based approaches to classify UC, CD and controls previously (e.g.16,18,19), with varying degrees of success ranging from high separation in small cohorts to little or no separation: a meta-study analyzing multiple microarray experiments indicated that UC-CD differences are not substantial23, and the accuracy of one of the reportedly best-performing methods could not be verified in an independent laboratory45. These variations in accuracy may be due to small patient cohorts, and that different standards may have been applied in the initial patient diagnosis and inclusion criteria. For comparison, we tested the classifying power of gene expression markers previously selected by two previous studies16,44 in our set, using CAGE data in cohort 1. These markers performed consistently worse than ours (Supplementary Fig. 9d).

There are two important limitations with our analysis. First, biopsies were collected from inflamed segments of the descending colon only: expression patterns may be different in other parts of the intestinal system, and while UCa patients typically are inflamed all along the colon, this is less common for CD patients. Similarly, it is possible that non-macroscopically inflamed biopsies from IBa patients have better classification potential, but on the other hand macroscopically inflamed biopsies are the primary type of samples used for histology assessment in the clinical diagnosis of IBa and it is clear that IBa-upregulated regions we identified in such biopsies were highly overrepresented in SNPs associated to the disease. The latter argues that the regions identified are disease-relevant and involved in pathogenesis. Second, the sensitivity of CDa prediction was lower than that of UCa and Ctrl. This may be related to the above, but as we noticed a much higher variance among CDa patients than UCa (Supplementary Fig. 10), it might reflect subtypes of CDa.28,46 Although a higher CDa sensitivity would be desirable, a high sensitivity in UCa prediction is more clinically important than in CDa, because the surgical option of ileal pouch-rectal anastomosis exists for UC only. Thus, CD in general is considered a contraindication for ileal pouch-rectal anastomosis, due to high risk of pouch dysfunction, fistula formation, and peripouch sepsis, ultimately leading to a higher rate of pouch excision and the potential for short bowel syndrome47.

As a summary, the data sets presented here comprise comprehensive maps of TSSs and enhancers in clinical IBa samples, and show the feasibility of predicting active UC/CD and control status using such data.

Methods

Human samples and cohorts. Two independent cohorts, including IBa patients and controls, were analyzed. Cohort 1 (N = 94) was used for CAGE assay and subsequent microfluidic real-time quantitative reverse transcription polymerase chain reaction (qPCR) validation. This cohort included 25 patients with active UC (UCa), 17 patients with UC in remission (UCr), 20 patients with active CD (CDa), 3 patients in remission (CDr) and 29 healthy controls (Ctrl). Cohort 2 (N = 101) was used for independent qPCR validation of selected target genes. The cohort included 37 UCa, 18 CDa, and 46 Ctrl subjects. All included subjects underwent a routine sigmoidoscopy or colonoscopy as part of their clinical evaluation at the Department of Gastroenterology, Medical Section, Herlev Hospital, Denmark due to their clinical symptoms. They were included into the study as UC or CD patients undergoing lower endoscopy to assess possible disease activity, extension of disease or surveillance for dysplasia, or as controls (endoscopy was performed due to gastrointestinal symptoms but all investigations returned normal). IBa patients were diagnosed established using well-defined criteria43, UC patients were graded by Mayo score: a score ≤2 with endoscopic sub-score of 0 (i.e., no macroscopic inflammation) as quiescent (in remission) UC and >2 as active UC, and CD patients were graded in accordance with the Harvey-Bradshaw score: a score <5 as quiescent (in remission) CD and ≥5 as active CD. Exclusion criteria were as follows: age >80 or <18, clinical evidence of infection, use of antibiotics or probiotics within 14 days, severe mental illness and pregnancy. Additional data for each cohort is available in Supplementary Table 1 and Supplementary Data 1.

Ethics. The IBa sample collection and study was approved by the Scientific Ethics Committee of the Capital Region of Denmark, filed under the ethical per- mission numbers 5558/H-3-2014-057, 22848/H-2009-0057, 43691/H-6-2014- 051, and 53619/H-6-2014-051, covering both biopsy collection and the application of data to next-generation sequencing approaches. All individuals provided written informed consent to participate in this study. In this context, participation meant that five extra biopsies were obtained in addition to the number of biopsies required on medical grounds, extending the examination time by 2–3 min.

Tissue isolation and RNA extraction. Mucosal pinch biopsies (weight on average 15 mg) were obtained from the descending colon using endoscopic forceps. We choose the descending colon in order to avoid intersegmental variation. The endoscopic diagnosis of active or inactive disease was confirmed by histopathology conducted on parallel biopsies taken within an inch of the 1st biopsy. The biopsies were immediately placed in RNAlater® Stabilization Solution (Ambion®, Life Technologies) and kept at 4°C for 24 h before long-term storage at −80 °C. The RNA was extracted using PureLink® RNA Mini Kit (Ambion®, Life Technologies) (supplemented with lysa buffer) and polyadenylated, fibroblast marker genes were homogenized directly in the lysis buffer using an ULTRA-TURRAX® (IKA Works, Inc). The purification was performed following the manufacturer’s instructions including an on-column DNase treatment with the PureLink® DNase Set (Ambion® Life Technologies) and equilibrated with 2× qPCR samples RNase-free water. The RNA was quantitatively described in ref.48. Quantity and purity of the RNA was determined for all the samples on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The purity was measured as the A260/280 ratio and was between 1.9 and 2.1. RNA quality (integrity) was determined using the 2100 Bioanalyzer Instrument (Agilent Technologies) with the Agilent RNA 6000 Pico Kit (Agilent Technologies) and manufactured by Agilent Technologies. RNA quality was also confirmed by manufacturers (GE Healthcare, Uppsala, Sweden). Harvested PBMCs were used for monocytosis. CD14+ monocytes were isolated from PBMCs by negative immunomagnetic bead separation using Monocytes Isolation Kit II (Miltenyi Biotec, CA, USA). After monocytosis, 1 × 10⁶ monocytes were plated in each well of 24-well plates (TPP, Trasadingen, Switzerland) in 1 ml RPMI medium containing 10% (v/v) FCS, 100 μg/ml penicillin, 50 μg/ml streptomycin, and 0.5 mg/ml gentamycin) at 37 °C in a cell culture incubator. Cells were cultured overnight and stimulated with 10 ng/ml TNFa the next day and harvested at indicated time. Control cells were not treated with TNFa but otherwise treated in the same way. Cells were pelleted, snap frozen on dry ice and stored at −80 °C until RNA was collected. RNA was purified using the MicroNeasy Micro Kit as recommended by manufacturer with lysa buffer containing 1% 2-mercaptoethanol (Sigma) and on column DNase digestion. The average yield was 700 ng, with RIN values between 8 and 9.2. TSS expression analysis of 36 TSS targets (as defined below) was done using the microfluidics Fluidigm platform in parallel with biopsy qPCR, as described below.

Epithelial organoid growth and stimulation. Biopsies of morphologically normal epithelium from patients undergoing surgery for colorectal cancer were asexually collected. After washing thoroughly with PBS, biopsies were cut into ca. 4 mm pieces and incubated in freshly prepared chelator buffer (96 mM NaCl, 55 mM D-sorbitol, 44 mM sucrose, 10 mM EDTA, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 1.6 mM KCl, 0.5 mM DTT) for 45 min at agitation on ice. After replacing chelator buffer with fresh buffer, the explants were transferred to low calcium medium, filtered, and kept on dry ice and stored at −80 °C until use. RNA was purified using the MicroNeasy Micro Kit (Qiagen). Filtered crypts were suspended in ice cold Matrigel, plated in 48-well plates and maintained in organoid culture medium: Advanced DMEM/F12 (Life Technologies), 1× penicillin/streptomycin (Life Technologies), 10 mM HEPES (Life Technologies), 2 mM GlutaMAX (Life Technologies), 10 mM Nicotamide (Sigma), 1× N2 (Life Technologies), 1× B27 (Life Technologies), 1 mM N-acetylcysteine (Sigma), 10 μM Y-27632, 0.5 μM A-83-01 (Tocris), 10 μM SB202190 (Sigma), 100 ng/ml mNoggin (R&D systems), 50 ng/ml hEGF (Peprotech), 2.5 μM PGE-2 (Sigma). For TNFa stimulation experiments, primary organoid lines from different patients (passage number < 3) were run in technical triplicates and maintained in organoid culture medium supplemented with 10 nM hTNFa (R&D systems) for 0, 4, or 24 h in duplicate wells. Duplicate wells were pooled together and RNA was...
isolated using TriZol (Invitrogen) in combination with PureLink minicolumns (Life Technologies). The PurElunker minikit was used as recommended by manufacturer including the on column DNase step. The average yield was 5540 ng with a RIN between 6.8 and 9.8. TSS expression analysis of 36 TSS targets (as defined below) was done using the microfluidics Fluidigm platform in parallel with biopsy qPCR, as described below.

**CAGE library preparation, sequencing, mapping, and processing.** CAGE libraries were prepared according to ref. 44 with an input of 1500 ng of total RNA as starting material. Libraries were prepared using the microCAGE library preparation, sequencing, mapping, and processing command from BEDTools package51. Libraries were summed into a joint CTSS profile and subbed. If mentioned, only TCs having at least two tags in at least eight samples (Supplementary Fig.4a). Ten TSSs were annotated using GENCODE v19 annotation17. Canonical TSSs were annotated using GENCODE v19 annotation17. Canonical TSSs were defined as the most upstream annotated TSS in the GENCODE gene model. CAGE-defined TSSs within ±100 bp from the most upstream annotated TSS for a CAGE-annotated gene were thus labeled as canonical TSSs. CAGE-defined TSSs > 100 bp distant from gene annotation were annotated as novel alternative TSSs. **Enhancer identification.** Enhancer regions were identified from CAGE-derived bidirectionally transcribed loci, as in ref. 15 with the following modifications: only enhancer regions candidates whose bidirecionality score [4] < 0.6 (D ranges from –1 to 1 where 0 corresponds to a perfectly balanced transcription between strands around the enhancer midpoint, as defined in ref. 15) were used. The permissive set was defined by requiring at least two CAGE tags in at least one sample within the enhancer region (as in ref. 15). Evolutionary conservation analysis of enhancer regions. Enhancer regions from the strict set were aligned by their midpoint. Enhancers whose ±2000 bp flanks from their midpoint overlapped the empirical genome regions were discarded (see main text Fig. 4a). Technical replicate libraries were included at this stage, but discarded in final expression measurement table. Overlap with FANTOM5 enhancers (http://fantom.gsc.riken.jp/5/datasets/latest/extra/Enhancers/) was calculated using the intersect command from BEDTools package51.
Differential expression analysis of enhancer regions. Because edgeR analysis has limited power for globally lowly expressed entities like enhancer RNAs, we used an alternative approach to assess differential expression between conditions for these data. The enhancer RNA data were normalized using the median-based method implemented in the DESeq2 package (default)35. Instead of normalizing directly on the counts of enhancer RNA, we used the counts from TSSs from the same samples. These signals are typically orders of magnitude higher and the measurement error is therefore associated with a smaller variability of expression levels within conditions compared to the variability of expression levels between conditions. The R-package EBSeq (version 1.10.0) was used for the identification of differentially expressed enhancers based on the normalized data30. EBSeq assumes that any given data row (enhancer count data for CDa, UCa, and Ctrl samples, respectively) can follow one of a number of possible expression patterns, corresponding to different plausible hypotheses about that specific enhancer. For the three conditions considered here, there are a total of five possible patterns

Pattern 1: Enhancers expressed at the same level in CDa, UCa, and Ctrl (non-differential expression).

Pattern 2: Enhancers with the same expression level in CDa and UCa, and a different expression level in Ctrl (general IBd enhancers).

Pattern 3: Enhancers having similar expression levels in CDa and Ctrl, while being expressed differently in UCa (enhancers specific for UCa).

Pattern 4: Enhancers having similar expression levels in UCa and Ctrl, while being expressed differently in CDa (enhancers specific for CDa).

Pattern 5: Enhancers expressed differently in all three conditions.

For each enhancer dataset, EBSeq computes the posterior probabilities that it follows each of these five patterns. EBSeq also produces posterior estimates of the fold change in expression levels between each pair of the three conditions for any given enhancer. Another feature of the EBSeq method is that it uses hierarchical (or non-parametric) modeling of the expression level (read count) of individual enhancers is assumed to follow negative binomial distributions whose \( r \) - and \( q \) -parameters are inferred from the data. For any given enhancer, these parameters may be identical across conditions (non-differential expression) or different across conditions (differential expression). Different enhancers each have their own \( r \) - and \( q \) -parameters, but all \( r \) -parameters are assumed to be related to each other, as they are drawn from a higher-level beta distribution whose (hyper) parameters are also estimated from data. This multi-level modeling approach results in “borrowing of strength” (inference from data on one enhancer will help inform inference for other enhancers), and “shrinkage” (parameter estimates for enhancers with little data will be “shrunk toward the mean”). Since the majority of all enhancers are not differentially expressed the approach is conservative (it requires a substantial amount of data to pull log-fold estimates away from zero) and thereby helps avoiding issues with multiple testing.

Specifically, the analysis was performed using the EBMultiTest function in the EBSeq package. The analysis was set to 100 iterations. Convergence was checked by inspecting plots of the hyper-parameters Alpha and Beta and the mixture parameter \( P \) and ensuring their values had settled at stable values at the end of the iterations. The function QQP was used to generate QQ-plots confirming that the beta prior used in the analysis was appropriate (data not shown). We used a posterior probability cutoff of 0.9 for deciding when to label an enhancer as differentially expressed, corresponding to a false discovery rate of 0.1. Labeled enhancers were then reconciled with the classification of differential expression made for TSSs as follows (\( P = \) posterior probability):

\[
\text{IBd:} \quad P(\text{Pattern 2}) > 0.9 \quad \text{and} \quad (\text{CDa} \cup \text{UCa} \cup \text{Ctrl})
\]

\[
\text{IBdn:} \quad P(\text{Pattern 2}) > 0.9 \quad \text{and} \quad (\text{CDa} \cup \text{UCa} \cup \text{Ctrl})
\]

\[
\text{IBup:} \quad P(\text{Pattern 2}) > 0.9 \quad \text{and} \quad (\text{CDa} \cup \text{UCa} \cup \text{Ctrl})
\]

\[
\text{UCspec:} \quad (\text{Pattern 4}) > 0.9
\]

\[
\text{CDspec:} \quad (\text{Pattern 3}) > 0.9
\]

There is no simple way of accounting for batch effects in the software used here, and we have therefore performed the analysis independently for the two batches. Results reported in this paper are for the larger batch (58 subjects), but we found very similar results for the smaller batch (Supplementary Fig. 4d). All analyses were performed both on the larger (permissive) enhancer set, and the (smaller) restricted enhancer set. On the whole, enhancers identified as being differentially expressed in the strict data set were a subset of those identified based on analysis of the permissive data set (data not shown).

Genotype and protein interaction analysis. For differential expression analysis performed at the gene level, we performed enrichment tests for GO-terms using the gProfiler online tool31. We considered all the possible interactions of the STRING database of protein–protein interaction networks (using default settings, but removing proteins with no connections to any other proteins).

FANTOM5 cell specificity enrichment analysis. For TSSs that were differentially expressed, we performed enrichment tests for FANTOM5 cell specificity of TSSs. We downloaded the table of cell type specificity of CAGE tag clusters from http://fantom.gsc.riken.jp/p5/datasets/phase1/1extra/Sample_ontology_enrichment_of_ CAGE peaks/.

Selection of biomarkers. In order to create an initial candidate list for machine learning-based selection and classification, we reasoned that any single prioritization method has its own disadvantages. Therefore, we performed an ensemble approach where we integrated the results of multiple analysis methods each aimed to extract TSSs with high power to distinguish the subject groups. As our main goal was classification by machine learning, we will refer to these TSS regions as “features” or “biomarkers as in the main text” (with the associated gProfiler R-package [https://CRAN.R-project.org/package=gProfilerR] using the gProfiler function. Default settings were used, except the background set (i.e., “universe”) was set to all expressed genes. The results of the enrichment analysis on each of the four sets can be found in Supplementary Data 7. Additionally, we also queried each of our gene-level differential expression sets against the STRING database of protein–protein interaction networks (using default settings, but removing proteins with no connections to any other proteins).

Transcription factor binding site enrichment analysis. Promoter regions were defined as follows: for each TSS defined as differentially expressed in the respective sets (IBdp, IBdn, as defined above), we extracted the genomic positions corresponding to \( \pm 100 \) bp around each such TSS peak. Background promoters were defined as corresponding regions from TSSs, which were not part of any of the four differential expression sets (IBdp, IBdn, UCspec, CDspec). For enhancer regions, we selected enhancer regions that were differentially expressed in the three datasets (IBdp, IBdn). We matched the genomic positions corresponding to \( \pm 300 \) bp around the midpoint of each such enhancer region. Background enhancers were defined as corresponding regions from enhancers not part of any differentially expressed set. Next, we used the HOMER tool31 to score enrichment of sites corresponding to known motifs in the regions and background described in the previous paragraph (using default settings, except genome = hg19 and size = given, and mset = auto which defaults to “vertebrate”). Sequence logos for these motifs were acquired from http://homer.salk.edu/homer/motif/HomerMotifDB/. HOMER motifs were manually paired with CAGE-defined transcription factor expression (ComBat-corrected gene-level expression).

ENCODE transcription factor ChIP peaks enrichment. ChIP-Seq peaks were downloaded from the ENCODE consortium (161 TFS from 91 cell types; see above section for heatmap visualization for details). We analyzed the enrichment of each set of TF peaks in a given set of enhancer regions (e.g., IBdp enhancers) by constructing a contingency table as defined in Supplementary Table 2.

Enrichment of a given TF in the region of interest was defined as log2 (counts in Supplementary Table 2) and significance of enrichment was tested by Fisher’s exact test for each TF based on the above contingency table. For the analysis in Supplementary Fig. 5 the regions of interest were IBdn or IBup enhancers and the background regions were non-differentially expressed enhancers. In Fig. 5d, we only focused on enhancers that were associated with IBdn or IBup TSSs by expression correlation as defined previously. Among these enhancers, we defined the regions of interest as the enhancers from large enhancer clusters (>6 enhancers in a cluster) and the background regions as singleton enhancers (not part of any cluster).
responsive inflammatory response), while controlling for batch effects. Extracted TSSs had a Benjamini–Hochberg FDR < 0.05 and a log2 fold change >1, for a total of 1000 sets.

ii. Partial Least Squares Discriminant Analysis (PLSDA): The plsDA function from the R-package Discriminer (https://cran.r-project.org/package=Discriminer) was used to perform PLSDA with two components on the variance stabilized expression values from above, using all amplified intergenic and gene body TSSs, in three groups of patients, in three genotypes: CDa, Ctrl, UCa. The 500 TSSs with the highest and lowest loadings for the component separating Ctrl from the remaining samples were extracted for a total of 1000 TSSs.

iii. ComBat54 followed by PCA: ComBat corrected values were used for PCA analysis. We identified the component that contributed the most to the difference between the two states and sampled 1000 TSSs from each side with the highest rotation values. We focused on the PCAs that gave the most explanations for each of the comparisons (PCA1 for shared IBD-specific signal and PCA2 for CDspec and UCepec), producing two lists for IB (from each side one list) and one list for CDspec and UCepec, (from each side one list).

iv. ComBat followed by limma52: Combat normalized data (as described above) was used for differential expression analysis using the limma package and the limFit function. TSSs that passed the threshold of log fold change >1 and FDR < 0.05 were taken into account making two lists for IB (over-represented in UCa vs. Ctrl and overrepresented in CDa vs. Ctrl) and one list for CDa vs. UCa.

v. Random Forest analysis. We utilized the inherent ability for Random Forests (RFs) to rank features by their importance for classification accuracy. To do this, we used the Gini function from the randomForest R package (http://CRAN.R-project.org/package=randomForest) on Combat-normalized TC expression data (as described above in the “Exploratory data analysis” section). To circumvent the “Large p, small N” problem with our data (74 samples, 48,593 TCS), which makes difficult to find the most predictive features, we used an iterative feature selection approach where we in each step remove a subset of the least important features. More specifically: in each iteration we first evenly divided all the data into subsets each containing 500 features. For each of these data subsets a RF was trained to classify the patient labels based only on the data in that particular subset. Next, for each of the data subsets, we removed the 5% of features that had the lowest classification power (lowest Mean Decrease Accuracy (values) resulting in a list of features that is analyzed in the next iteration of the procedure. To make sure an important feature was not lost due to a challenging subset of data, in each iteration we randomly divided all our data into subsets 10 times. In other words, features had several chances of showing its importance. This iterative procedure was terminated when less than 200 features were left. Each RF was made with default parameters except specifying that 501 trees per forest should be generated and specifying the number of variables randomly sampled as candidates at each split by setting the “mtry” parameter to sqrt(8x−1)2, where x was the number of features supplied to the individual RF. Using this approach we generated four candidate lists: two that enabled classification of IBD patients (CDa and UCa) from healthy patients (Ctrl) and two lists that aimed at differentiating between CDa and UCa. For the two lists in each category, one was based on the full data set (N = 48,593 TCS) and the other was built on a high confidence dataset (N = 9699 TCS). The high-confidence data set was obtained by requiring that (i) the tag cluster was annotated as “5_prime_UTR”, “intergenic”, “known_alternative_cds”, “known_n_alternative_tss”, “proximal” or “tss”. (ii) the width of the TC was in the range of 2–100 nucleotides (both included). (iii) the TC was expressed more than 10 TPM in at least 3 subjects. As a summary, the ensemble produced 10 candidate sets for separating CDa and UCa from Ctrl (3 edger, 4 ComBat, 2 RandomForest, 1 PLSDA) and 5 candidate sets for separating CDa and UCa (edgeR, limma, 2 RandomForests, and PCA).

Next, we overlapped the lists from each comparison to find a consensus set of classification candidates. For the IB vs. Ctrl comparison, we selected features that appeared in at least 9 out of 10 lists, resulting in 63 features. From the UC/CD set we selected the 169 features that appeared in at least 2 out of 5 lists. The reasoning behind this unbalanced selection was to have a higher number of features for the more difficult task of distinguishing CDa from UCa, compared to the easier task of distinguishing Ctrl from UCa or CDa.

The two lists were merged and a short list of expert-curated known and novel biomarkers (21 TSSs and 10 enhancers) was added. The combined list was then further pruned by manual curation, taking into account overall expression strength and genomic loci complexity. In total, the initial list comprised 263 features, which was used for initial classification using CAGE data (see below).

**Microfluidic qPCR analysis.** For qPCR analysis, the 263 features identified above were assessed for qPCR primer targeting suitability. 181 candidates were taken into the primer design stage, where genomic complexity and primer design feasibility was evaluated manually.

Primers were designed using Primer3 (v.0.4.0)39 aiming for an amplicon length of 70–110 bp and a primer melting temperature of 60 °C calculated by Breslauer thermodynamics. Primers were designed to be intron-spanning when possible, and each designed primer-set was analyzed with the UCSC browser in-silico PCR tool in order to ensure a unique region would be amplified. Primers were synthesized by Integrated DNA Technologies (IDT). Primer sequences are presented in Supplementary Data 16. Primer efficiency and dynamic ranges were acquired from standard curves constructed from several separate dilution series of pooled cDNA: with the dilutions 1.5, 1.25, 1, 1:100, 1:500, and 1:2500. Melting curves of amplicons were measured to ensure primer specificity. 161 primer pairs (features) successfully passed our quality control and were used for analysis of cohort 1 on the Fluidigm platform.

The CDNA synthesis and preamplification for cohort 1 was performed as described previously39. Total RNA (500 ng RNA, from biopsies) was converted into cDNA using Quantitect Reverse Transcription kit (Qiagen), using a mix of random and oligo-dT primers, as per the manufacturer’s instructions. We used the specific separate cDNA reactions for each DNA sample. Preamplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems). A 500 μl primer mix (200 nM) combining all primers to be used on the Fluidigm plate was prepared by pooling 5 μl of all primer pairs (20 μM) and filling up the remaining volume with low EDTA TE-buffer (VWR International). TaqMan PreAmp Master Mix (5 μl) was mixed with 2.5 μl of the 200 nM primer mix, 2.5 μl diluted cDNA, and incubated at 95 °C for 10 min, followed by 15–21 cycles of 95 °C for 15 s and 60 °C for 4 min (the number of cycles depended on the expression measured by CAGE). 16 U of Exonuclease I (New England Biolabs) was added to the preamplified cDNA, and incubated 30 min at 37 °C, 80 °C for 15 min. The preamplified cDNA was diluted with low EDTA TE-buffer (VWR International) either 1:5 if the cDNA was preamplified for 21 cycles or 1:10 if the cDNA was preamplified for 19 or 15 cycles.

RNA expression was analyzed by real-time qRT-PCR in the microfluidics system BioMark™, 96.96 Dynamic Array (Fluidigm) following the protocol as described previously39. The following cycle parameter were used: Thermal Mix with 2 min at 50 °C, 30 min at 70 °C, 10 min at 25 °C, UNG and Hot start with 2 min at 50 °C, 10 min at 95 °C, followed by 35 cycles with denaturing for 15 s at 95 °C and annealing/elongation for 1 min at 60 °C. Melting curves were generated after each run to confirm a single PCR product (from 60 °C to 95 °C, increasing 1 °C per 3 s). We performed reactions in duplicates. Non-template controls were used to indicate problems with sample contaminations or non-specific amplification. To assess potential DNA contamination, non-reverse transcriptase controls were used. We used the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm) to acquire Ct values. These were exported to GenEx (MultiD) for data pre-processing including normalization to reference genes, interplate calibration, individual PCR efficiency correction for each primer assay, and averaging of cDNA technical replicates. Using GeNorm60 and NormFinder61, we identified the most stable reference genes of the 10 reference genes included on the plates. This was done separately for all plates run with the same number of preamplification cycles. The used reference genes for each preamplification batch are specified in Supplementary Data 16. By normalizing to these reference genes, ΔCt values were calculated and used in the down-stream analysis.

**Feature selection for cohort 2.** The 161 features were reduced to 36 features by ranking the features for classification power. Briefly, a RF was trained to predict subject groups, both using all groups and one-vs.-rest specification (i.e. UCa vs. non-UCa) using the randomForest R package.65 The optimal value for the mtry parameter was found using 100 five-fold cross-validations using the caret package (https://cran.r-project.org/web/packages/caret/index.html) train-function and mtry = 1000. Feature importance for both classification tasks were extracted using the importance function with type = 2 (mean decrease in node impurity or gini index). Primer pairs with a high importance in both complete and one-vs.-rest classification were selected as the most predictive. In addition to these RF predictive features, six gene-based features were selected to validate pathology-based findings in the first cohort.

cDNA synthesis and preamplification for cohort 2 was performed as described above for cohort 1 with an adjustment of the number of pre-amplification cycles. In this cohort cDNA was either preamplified 15 or 20 cycles, depending on expression level of the primer targets. The preamplified cDNA was diluted with low EDTA TE-buffer (VWR International) either 15 if the cDNA was preamplified for 20 cycles, or 1:10 if the cDNA was preamplified for 15 cycles. RNA expression was analyzed by real-time qRT-PCR in the microfluidics system BioMark™. Dynamic Array (Fluidigm) following the same protocol as used for the cohort 1 runs, with volumes adjusted to the 192.24 format, as described by manufacturer. Expression data (ΔCt values) were acquired and treated similar to cohort 1 above.

qPCR analysis of monocyte and organoid samples. cDNA synthesis and preamplification was performed as described for cohort 1 and 2, but with an input of 300 ng RNA (250 μl of RNA in 250 μl water) due to limited amounts of RNA. RNA expression was analyzed by real-time qRT-PCR in the microfluidics system BioMark™. Dynamic Array (Fluidigm) as described...
for cohort 2. Monocyte samples and organoid samples were placed on two independent plates.

**Batch correction and expression normalization of qPCR data.** For each primer all ΔΔCq values from cohort 1 and cohort 2 were batch corrected using the removeBatchEffect function from limma, giving the subject/group labels (CDa, UCa, Ctrl) as covariates and cohort and hospital of origin as batch effects and specifying the ‘robust’ method should be used for the linear models. To make organoid and monocyte data comparable with the batch corrected data a batch correction factor, obtained as the mean of the batch correction factors for the two hospitals, was applied to the organoid and monocyte data. Afterwards the batch corrected ΔΔCq values were calculated in a two-step process: (1) \( x = 2^{-\Delta \Delta Cq} \), (2) \( \Delta \Delta Cq = \log_2 (x/\text{min}(x)) \).

**Preprocessing of microfluidic qPCR data.** For classification analysis of cohorts 1 and cohort 2 (see below), only monocyte, UCa, and CDa subjects were considered. We removed one subject and one primer pair from the analysis which both had >10% “NA” values from the ΔΔCq Fluidigm data (Supplementary Data 1B), making the final feature count 35. The remaining missing values (N = 16, 0.26% of total) were imputed via bagged regression trees using the preprocess function from the “caret” R package (https://CRAN.R-project.org/package=caret) specifying methods to the lmStepAIC function. Experimental practice and reporting were performed according to MIQE guidelines.

**Comparison of organoid, biopsy, and monocyte qPCR data.** Only measurable ΔΔCq Fluidigm data were analyzed (values labeled “missing” or “infinite” were discarded). For each qPCR primer we performed the following quantifications:

1. Inflammatory response in UCa or CDa as \( \log_2(\Delta \Delta Cq_{\text{CDa-UCa}}) \) (average \( \Delta \Delta Cq\) of all individuals across UCa and CDa).
2. 4 or 24 h TNFα response in gut epithelia organoid as \( \log_2(\Delta \Delta Cq_{\text{organoid-TNF4h/24h(all)}}) \) (average \( \Delta \Delta Cq\) of all organoid-TNF4h/24h).
3. Medians of responses across replicates or subject group (CDa, UCa, monocyte TNF4h, monocyte TNF24h, organoid TNF4h and organoid TNF24h) was then used to indicate the IBD or TNFa response of the group. Specifically, if the median IBD or TNFa response of a qPCR primer in a sample group was >0, it was labeled upregulated in the sample group, and vice versa. qPCR primers with missing median in any of the sample groups were discarded, resulting in analysis of 30 qPCR primers in Fig. 2f and Supplementary Fig. 3b, c.

**Real-time quantitative PCR.** For specific enhancer RNA expression validations (Fig. 4), we used the Applied Biosystems® QuantStudio™ 6 Fast Real-Time PCR System, with the 384-well plate. This platform allows for higher reaction volumes compared to the Fluidigm system. The cDNA preparations were done as described for cohort 1. All samples were run in triplicates in 5 μl reaction volumes using SYBR® Green Master Mix (Life Technologies) as described by manufacturer, with a primer concentration of 0.5 μM, and under the following conditions: 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 95 °C for 15 s, 58 °C for 1 min. All results were normalized to the reference gene PIA54.

**Prediction of subject diagnosis using random forests.** For all three classification analyses in Fig. 7, we used a two-step RF classification approach with the randomForest package from the randomForest package (https://CRAN.R-project.org/package=randomForest). In this approach, the first step was a RF trained to distinguish IBD (CDa and UCa) from Ctrl and the second step was a RF trained to distinguish CDa from UCa. The two-step approach was chosen over an all-in-one approach because, based on five-fold cross-validation in the CAGE data, resulted in better CDa sensitivities (data not shown).

To optimize the parameters most important for a RF model, \( m \) indicating the number of variables randomly sampled as candidates at each split and \( n \) indicating the minimum size of terminal nodes (corresponding to the mtry and nodesize options in the R ForestRandom package), we used a grid search approach testing each pair of \( (m, n) \) where \( m \in \{2, 3, 4, 5\} \) and \( n = (\sqrt{x} - 1) \cdot x \), where \( x \) is the number of features supplied to the RF and \( y = \lfloor 1/2 (3.4, 1, 6.4, 2) \rfloor \). For each of the three classifications analyses performed, the final \( m \) and \( n \) parameters were chosen as the combination given the highest overall accuracy in the two-step RF approach based on the average result of five-fold cross-validation in cohort 1. Apart from this all RFs were created with default parameters except specifying that each RF should have 101 iterations.

To assess the accuracy of the classification model we performed 101 RF iterations of 5-fold cross-validation. The only exception to this was the classification of subject labels on cohort 2, where 101 RFs were created based on data from cohort 1 and used for prediction in cohort 2. For each of the 101 iterations, the overall accuracy and CDa, UCa, and Ctrl-group specific accuracy, sensitivity, and specificity were calculated. For each of these, we reported the average and 95% confidence interval across the 101 iterations. The CDa, UCa and Ctrl-group specific performance measures were calculated by for each label recasting the problem in a binary setting (e.g. for the “Ctrl” label converting both CDa and UCa to “non-Ctrl”) and then assessing accuracy, sensitivity, and specificity. The overall accuracy was assessed on all three categories.

To assess the observed vs. expected performance in classification, we trained 1001 RF models on randomly shuffled labels (again only based on cohort 1 data). These models were then used to predict the patient labels of cohort 2 and these predictions were compared to the true labels. Performance measures were calculated as described above.

To analyze the relation between number of features in the model and classification accuracy, for a selected number of features referred to as \( i \), we randomly selected \( i \) features from the total set of features and performed five-fold cross-validation with the same parameters as identified by the grid search above. This procedure was repeated 11 times for each selected \( i \). The average accuracy and 95% confidence interval of these repeated analyses were reported for each \( i \).

**Prediction of subject diagnosis using XGBoost.** We used the XGBoost framework41 to perform the same classification as done with Random Forests in Fig. 7c (training on cohort 1 qPCR data and evaluating on the independent cohort 2 qPCR data). For additional power, each sample was scored using a database of curated pathways (wikiPathways.org). Briefly, for each patient, we computed pathway expression scores, based on the sum of overlapping genes in each pathway and the qPCR data. From the 569 original pathways in the database, 16 returned a non-null score. These scores were then used to provide 16 supplemental features, on top of the 35 original ones, for the training of the classifier. After parameter optimization on the training data (max_depth, min_child_weight and subsample), the final classifier was used on the cohort 2.

**Analysis of GWAS data.** A recent version of the GWAS catalog was downloaded (2016-12-15) and lifted to hg19 using the gwasucr R-package (http://bioconductor.org/packages/gwasucr/). To calculate linkage disequilibrium (LD), we used genotype data from whole-genome sequencing of Europeans. This was obtained from the VCF files from the 1000 Genomes CEU population release 20130502. We use only diallelic SNPs with <5% missing genotypes that were polymorphic in the CEU population45. SNPs from the GWAS catalog were merged with 1000G SNPs by genomic position, and LDpeak46 was used to calculate LD between all SNPs within 500 kb (using the function plink -r2 -ld-window-kb 500 -ld-window 99999 -ld-window-r2 0.5). Pairs of SNPs were considered to be in LD if \( R^2 > 0.75 \). LD clumps were found by expanding all SNPs with their associated LD-SNPs, and then reducing the set by merging overlapping regions.

To assess LD clump enrichment, Empirical Bayes shrinkage was performed with the ebb R-package (https://github.com/drgrtwo/ebb) package using the ebb_lit.prior and augment functions (with default arguments). Prior means were calculated as \( \alpha = \alpha_1 + \alpha_2 \cdot \alpha_1 \) and added to the plot of shrunken proportions. LD-clump (one-sided) enrichment was calculated using the kegg function from the limma R-package47, but using user-supplied sets: universe was set to all TSSs/ enhancers, de the respectively differentially expressed sets and gene/pathway set to all promoters. LD clumps for a GWAS disease. No trend based on an additional covariate was used.

Partitioned heritability analysis was performed using the ldsc tool from (https://github.com/bulik/lscr). The two analyses below were done based on recommended practice by the creators of the tool: https://github.com/bulik/lscr/wiki/LD-Score-Enumeration-Tutorial. https://github.com/bulik/lscr/wiki/Partitioned-Heritability. LD-scores were calculated for TSSs, enhancers, IBG and IBG, sets (including both promoter (~500–100 bp around TSS peaks) and enhancers (~300 bp around enhancer midpoints)). IBG SNP summary statistics were obtained from the International IBG Genomics Consortium, https://www. ibgdgenetics.org/downloads.html. Downloaded 2016-12-22. SNP summary statistics were processed using munge_sumstats.py (with settings -N-cas 12882 -N-con 21770 for sample sizes (as described in ref. 4)). Base model enrichment statistics for this set of GWAS summary statistics can be seen in Supplementary Fig. 8b. All four extra categories were added to the baseline model for Fig. 6d. Additionally, each new annotation was separately added to the baseline model in sequence, similar to the cell-type group analysis from the original paper40. Proportions, enrichments and coefficients were obtained from each model and compared (Supplementary Fig. 8c).

**Data availability.** CAGE data from this study has been deposited in GEO database under accession number GSE95437. Microfluidics qPCR data are available in Supplementary Data 9, 17, 18.
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Author contributions

M.B. and J.B. made CAGE libraries and qPCR validations. M.T., M.V., K.V.S., M.B., Y.C., A.G.P., R.A., T.B.T., A.T., N.R., and A.S. analyzed the data. M.B., J.B., M.C., P.K., B.L., A.M.P., B.Z.S.L., P.T., I.G., J.B.S., O.H.N., and J.T.T. collected and characterized patient materials. K.S. assisted in microfluidic qPCR analysis. K.D. and J.T.T. characterized targeted promoters. P.W.S. and K.B.J. made organoid experiments. Y.L. made monocyte experiments. M.B., M.T., M.V., J.B., K.V.S., Y.C., and A.S. wrote the paper with input from all authors.

Additional information

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Competing interests: The authors A.S., M.B., J.B., M.V., M.T., K.V.S., O.H.N. and J.B. have filed a patent for the method for classifying CD/UC vs. control based on qPCR, based on this study. The remaining authors declare no competing interests.

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