Ileal Crohn's Disease Is Linked to Wnt/Tcf-4 α-Defensin Deficiency of Paneth Cells

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The Paneth Cell α-Defensin Deficiency of Ileal Crohn’s Disease Is Linked to Wnt/Tcf-4

Jan Wehkamp,‡ Guoxing Wang,* Irmgard Kübler,* Sabine Nuding,* Alex Gregorieff,§ Anke Schnabel,* Robert J. Kays,* Klaus Fellermann,† Oliver Burk,* Matthias Schwab,*† Hans Clevers,§ Charles L. Bevins,‡ and Eduard F. Stange‡

Ileal Crohn’s disease (CD), a chronic mucosal inflammation, is characterized by two pertinent features: a specific decrease of Paneth cell-produced antimicrobial α-defensins and the presence of mucosal-adherent bacteria. A mutation in NOD2, the muramyl dipeptide recognition receptor, is found in some patients, which leads to an even more pronounced α-defensin decrease. However, the underlying mechanism remains unclear for the majority of patients. In this study, we report a reduced expression in ileal CD of the Wnt-signaling pathway transcription factor Tcf-4, a known regulator of Paneth cell differentiation and α-defensin expression. Within specimens, the levels of Tcf-4 mRNA showed a high degree of correlation with both HD5 and HD6 mRNA. The levels of Tcf-4 mRNA were decreased in patients with ileal disease irrespective of degree of inflammation, but were not decreased in colonic CD or ulcerative colitis. As a functional indicator of Tcf-4 protein, quantitative binding analysis with nuclear extracts from small intestine biopsies to a Tcf-4 high-affinity binding site in the HD-5 and HD-6 promoters showed significantly reduced activity in ileal CD. Furthermore, a causal link was shown in a murine Tcf-4 knockout model, where the comparably reduced expression of Tcf-4 in heterozygous (+/-) mice was sufficient to cause a significant decrease of both Paneth cell α-defensin levels and bacterial killing activity. Finally, the association between Paneth cell α-defensins and Tcf-4 was found to be independent of the NOD2 genotype. This new link established between a human inflammatory bowel disease and the Wnt pathway/Tcf-4 provides a novel mechanism for pathogenesis in patients with ileal CD. The Journal of Immunology, 2007, 179: 3109–3118.

Inflammatory bowel disease (IBD) is a chronic inflammation of the intestine. Details of disease pathogenesis are emerging and the current concept holds that in genetically susceptible individuals, an inappropriate and ongoing activation of the mucosal immune system driven by normal luminal bacterial flora results in chronic intestinal inflammation (1–3). On the basis of its clinical features and histopathology, IBD is commonly classified into two major entities: ulcerative colitis (UC) and Crohn’s disease (CD) (3). Whereas UC is typically restricted to the colon, CD can occur at many sites, predominantly in the small intestine (ileum) the colon, or in both locations. Recent studies have supported a link between CD and reduced expression of defensins, a group of endogenous antimicrobial peptides (for review, see Ref. 4). CD of the colon is associated with a low-gene copy number polymorphism of the human β-defensin locus resulting in an attenuated epithelial induction of β-defensin antimicrobial peptides in the colonic mucosa (5, 6). In contrast, CD of the ileum is associated with a deficiency in another group of defensins, Paneth cell α-defensins (7, 8).

Paneth cells reside in the base of small intestinal crypts of Lieberkühn where they secrete high levels of antimicrobial peptides and proteins in response to bacterial stimuli such as muramyl dipeptide, CpG DNA, and LPS (8–13). Among their broad arsenal of antimicrobials, the α-defensins human defensin (HD) 5 and HD6 are the most abundant products (14, 15). These Paneth cell antimicrobials protect the host from food and waterborne pathogens and likely help regulate the composition of bacterial colonization in the small intestinal lumen. CD of the small intestine is characterized by a specific reduction of Paneth cell α-defensins, which results in decreased antimicrobial mucosal barrier function and a shift in the composition of the luminal microbiota (7, 8).

Paneth cells also express NOD2, an intracellular receptor of MDP (for review, see Refs. 16 and 17). A pivotal advance for the field came with the discovery that mutations in the gene encoding NOD2 (CARD 15) confers genetic susceptibility to approximately one-third of patients with CD (18, 19). Clinical analysis has revealed that this mutation is not linked to all patients with CD, but rather CD with ileal involvement (20). In the subset of ileal CD patients harboring NOD2 mutation, the aforementioned decrease in Paneth cell α-defensins is particularly pronounced (7, 8). Consistent with, and extending this correlative finding, NOD2 knockout mice have a selective decrease in Paneth cell α-defensin expression (21). Even though a causal link between NOD2 and Paneth cell defensins is now generally accepted, the question remains what other factors are responsible for the decrease in Paneth cell...
α-defensins in the majority of patients with ileal CD who do not carry mutations in NOD2.

Paneth cells, like other cells of the small intestine are derived from a compartment of progenitor cells residing in the crypts adjacent to the basal location of the Paneth cells (22). Wnt signaling, which can be transduced through β-catenin/Tcf-4, helps maintain the undifferentiated state of the progenitor cells (23, 24) and, paradoxically, also induces positioning, differentiation, and maturation of Paneth cells in intestinal crypts (25, 26). The Paneth cell gene program is critically dependent on Tcf-4 (also known as transcription factor Tcf-4 resulting in the observed decrease of Paneth cell α-defensins in small intestine CD. In this study, we report that CD of the small intestine is associated with a decrease of the Wnt signaling transcription factor Tcf-4 resulting in the observed decrease of Paneth cell α-defensins. Furthermore, the effects of Tcf-4 levels on α-defensin expression were independent of the influence of the NOD2 genotype.

Materials and Methods

Patients and patient material

Ileal biopsies from healthy individuals (controls) and CD patients were obtained and processed at the Robert Bosch Hospital (Stuttgart, Germany).

Table II. Tcf-4 potential binding sites in HD5 and HD6 promoter region

<table>
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<td>Human</td>
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<td>Mouse</td>
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<td>NOD-2</td>
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In all cases, control plasmids were cloned to allow assessment of absolute mRNA copy numbers.

The Tcf-4 potential binding sequence is (A/T)(A/T)CAA(A/TG). The analysis was performed in the HD5 and HD6 promoter region minus 3 kb. For the competitor, the core binding sequence for Tcf-4 was mutated from (A/T)(A/T)CAA(A/TG)→GC CAA(A/TG). Numbering of binding sites was performed from proximal to distal with respect to transcriptional start site. This order is consistent with the order in Fig. 6. mut, Mutation.
As a second cohort, surgical specimens of ileal mucosa were obtained at the Cleveland Clinic Foundation (Cleveland, OH). This cohort was previously described (8). The protocols were approved by the respective institutional review boards at these locations. The diagnosis at both institutions was based on standard criteria using clinical, radiological, endoscopic, and histopathological findings. Exclusion criteria included the diagnoses of backwash ileitis, indeterminate colitis, concurrent CMV or Clostridium difficile infection. Samples were immediately snap frozen in liquid nitrogen.

Real-time PCR
For quantification of the mRNA, real-time PCR was performed by using a fluorescence detection monitor (LightCycler; Roche Diagnostics). Briefly single-stranded cDNA from human and mouse tissue corresponding to 10 ng of RNA (or gene-specific plasmids as controls) served as a template with specific oligonucleotide primer pairs (Table I) as described previously (7, 8).

NOD2 mutation analysis
Genotyping of genomic DNA for the functionally relevant NOD2 mutations (SNP8, SNP12, and SNP13) was performed in all patient samples using TaqMan technology (Applied Biosystems) as described previously (7, 8).

Histological analyses
Surgical specimens from CD and non-IBD controls from a previous study (8) with known histological inflammation score were used to assess the
possible association of small intestinal inflammation and Wnt pathway expression.

Promoter analysis

Previous studies (25, 27) have shown that there are multiple consensus Wnt signaling Tcf-4 binding sites in the promoter regions of mouse and human Paneth cell cryptdin/defensin (HD5, HD6) genes, respectively. Other potential binding sites in these promoter regions were identified using LaserGene 7.0 DNA analysis GeneQuest software (DNASTAR). In the 3-kb upstream flanking regions, we found 9 potential Tcf-4 binding sites for HD5 and 11 consensus sequences (A/T A/T CAA A/T G) in the HD6 gene promoter region (Table II).

Nuclear extracts

Nuclear protein extracts were obtained from both Caco-2 cells and small intestine biopsies of healthy controls and CD patients using the NE-PER kit from Pierce (catalog no. 78833).

EMSA

Gel mobility shift assays were performed as previously described (28). Human Tcf-4 protein was synthesized from expression vector pK-Myc-FLH-Tcf-4 (generously provided by V. Korinek, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic) using the TNT T7 Quick-Coupled Transcription/Translation System (Promega). Nuclear response elements were prepared by annealing 10 μl of two complementary oligonucleotide stocks (100 μM) in 180 μl of 25 mM NaCl, 25 mM Tris-Cl (pH 7.5), and 5 mM MgCl2. For radioactive labeling, 2 μl of the annealed oligonucleotides, 5 μl of 10× buffer (500 mM NaCl, 500 mM Tris-Cl (pH 7.5), 100 mM MgCl2, 25 μCi of [32P]dCTP, 5 μl of 2 mM dATP, dGTP, dTTP, 2 U of Klenow fragment, and H2O to a final volume of 50 μl were incubated at 37°C for 1 h and then purified through Sephadex columns (MinicropSpin G-25 columns; GE Health). Binding reaction contained 10 mM HEPES (pH 7.8), 60 mM KCl, 0.2% Nonidet P-40, 40% glycerol, 2 mM DTT, 0.25 μg of poly(dI:dC), 2 μl of 10 μM nonspecific oligonucleotides (5′-AGC TTG CGA AAA TTG TCA CTT CCT GTG TAC ACC CA-3′), 50,000 cpm of labeled probe, and 4 μl of recombinant Tcf-4 in a final volume of 20 μl. Samples were incubated on ice for 20 min after the recombinant protein (or nuclear extract) had been added. For supershift experiments, 4 μg of mouse anti-Tcf-4 mAb (Upstate Biotechnology) was preincubated with the recombinant protein or the nuclear extract 1 h at room temperature before the addition of the oligonucleotide. In competition experiments, unlabeled competitor oligonucleotides were added before the addition of the in vitro-translated protein. The protein-DNA complex was resolved on a pre-electrophoresed 5% polyacrylamide gel in 44.5 mM boric acid and 1 mM EDTA (pH 8.3) at 200 V at 4°C. Gels were dried, autoradiographed overnight at room temperature, and then analyzed with the BAS-1800 II phosphor-storage scanner (Fuji) and AIDA software (Raytest). For comparison, the mean of Tcf-4 transcription activity in healthy controls was defined as 100.

Analysis of Tcf-4 in heterozygous knockout mice

Heterozygous Tcf-4 knockout (strain Tcf7l2) and wild-type littermate mice were genotyped as described previously (23). The reduced levels of Tcf-4 in the heterozygous (vs no expression in homozygous knockout mice) resembles the in vivo decreases as observed in human disease. Tissue samples from the distal (ileum) and middle (jejunum) part of the small intestine were taken from Tcf7l2+/- mice and control Tcf7l2+/- mice. The samples were used for real-time PCR gene expression analysis and functional assessment of antimicrobial activity.

Antimicrobial activity in mouse small intestinal mucosa

Cationic proteins from mouse small intestine mucosal biopsies were isolated by weak cation exchange matrix chromatography as described elsewhere (8, 29). Mid-logarithmic growth phase suspensions of Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923) were incubated with the cationic protein fraction at 37°C in a final volume of 100

FIGURE 3. Expression of Wnt/Tcf-4-related genes (Dkk-1, EphB3, EphrinB1, EphrinB2) in the ileal mucosa of healthy controls and CD samples. The mRNA copy number per 10 ng of total RNA was determined with quantitative real-time RT-PCR using external standards as described. Mean values are presented along with their SE.
μl of 1/6 diluted Schaedler broth (BD Biosciences). Bacterial suspensions incubated with vehicle (0.01% acetic acid) served as negative controls. After 120 min, [bis-(1,3-dibutylbarbituric acid)-rimethine oxonol] (Molecular Probes), a dye sensitive to membrane potential, was added at a concentration of 1 μg/ml. Bacterial pellets were isolated by centrifugation, resuspended in 300 μl of FACSFlow (BD Biosciences), and analyzed by flow cytometry by using a FACSCalibur (BD Biosciences) as described previously (8, 29). A total of 30,000 events was analyzed in each sample. The flow cytometry enabled us to distinguish viable from nonviable bacteria at the single-cell level. The antimicrobial activity was determined as percentage of depolarized bacteria compared with untreated controls.

**Statistics**

Statistical analyses of quantitative real-time PCR were performed nonparametrically or parametrically (in case of normal distribution) by using the U test of Wilcoxon, Mann-Whitney, or Student t test, respectively. Tcf-4 relative binding ability in the HD5, HD6 promoter region was subjected to t test and ANOVA analysis by using GraphPad Prism version 4.0 software. Values of p < 0.05 were considered to be statistically significant. Regression using the Spearman rank analysis was performed to assess the relationship between the transcript of HD5, Tcf-4, and other gene products. For illustration, mean values are presented along with their SE.

**Results**

**Tcf-4 expression is specifically reduced in ileal CD**

We measured Wnt signaling protein Tcf-4, Paneth cell α-defensin, and control mRNA expression in the ileal mucosa of three groups: controls, CD patients with solely colonic disease, and CD patients with ileal disease. Consistent with previous findings (7, 8), we confirmed in ileal biopsy specimens from ileal CD patients that there was reduced expression of HD5 (Fig. 1a) and HD6 mRNA (data not shown) compared with controls. We previously reported that this decrease reflected a corresponding decrease in α-defensin peptide levels (8). In these specimens, we observed that Tcf-4 mRNA levels were also significantly reduced compared with controls (p = 0.026; Fig. 1b). Compared with patients with an inflammatory course, those with progressive disease according to the Vienna classification (fibrostenosing and penetrating disease) showed a trend of even lower Tcf-4 expression (40% reduction, p = 0.06; data not shown). Similar decreases of Tcf-4 mRNA were observed in an independent cohort of surgical specimens from the small intestines of CD patients (n = 35) vs controls (n = 12, p = 0.0198; Fig. 2, inset). Within specimens, the levels of Tcf-4 and HD5 and HD6 mRNA showed a high degree of correlation (Fig. 1c). In contrast, both Tcf-4 and Paneth cell α-defensin expressions were unchanged in ileal biopsies from patients with colonic CD compared with controls (Fig. 1, b and a, respectively). The small intestine mRNA expression of β-catenin, the key adaptor for activation of Tcf-4, did not show any significant differences in any of the groups (Fig. 1d). Four Tcf-4/Wnt-related genes known to be important in other tissues were unchanged and showed no correlation with Tcf-4 (Fig. 3).
To test whether the observed decrease in Tcf-4 might be a direct consequence of inflammation, we examined Tcf-4 mRNA expression levels in a group of specimens that were previously categorized by a gastrointestinal pathologist based on degree of histological inflammation and which showed a significant correlation with the expression of the proinflammatory cytokine IL-8 (8). In these specimens (data not shown), as well as in ileal biopsies corresponding to Fig. 1, Tcf-4 did not show any correlation with IL-8 in contrast to the transcription factor’s tight link with Paneth cell α-defensin levels (Fig. 4). Moreover, the levels of Tcf-4 mRNA were similarly decreased in all patients with ileal CD, irrespective of whether the mucosal inflammation in intestinal biopsies was absent, moderate, or severe (Fig. 2). Furthermore, we tested Tcf-4 levels in macroscopically uninfamed as well as inflamed UC pouch biopsies as an inflammatory non-CD control. Like HD5 and HD6 (8), the levels were similar in both groups (n = 8 normal UC pouch, n = 10 inflamed UC pouch; data not shown). Together, these data indicate that the inflammation itself does not affect expression levels of Tcf-4.

To examine whether the observed effect was due to therapy, we analyzed the possible effects of different common treatments on Tcf-4 expression in the group of ileal CD. Neither treatment with 5-aminosalicylate nor oral steroids nor azathioprine had a significant effect on Tcf-4 expression levels (Fig. 5). Also, we compared the treatment schemes between ileal and colonic CD. The treatment in ileal and colonic CD was almost identical (data not shown) even though Tcf-4 and Paneth cell α-defensins were significantly decreased in ileal disease. These observations argue against a systematic change caused by one of these drugs.

Reduced binding activity of Tcf-4 in ileal CD

Complementing previous studies that reported 5 potential binding sites of Tcf-4 in the HD5 promoter and a total of 11 in the HD6 promoter region (25, 27), we identified 4 additional putative sites in the 3-kb upstream region of the HD5 gene. Using recombinant Tcf-4 protein, a comparison of each potential binding site for the quantity of Tcf-4-binding activity by EMSA revealed that binding site 2 in the HD5 promoter region has the strongest binding capacity (Fig. 6a). In the HD6 promoter region, binding sites 1 and 10 bound the recombinant Tcf-4 protein most avidly (Fig. 6b); the same results were obtained in independent experiments using nuclear extracts of Caco-2 cells as a source of Tcf-4 (data not shown). In each case, the specificity of the Tcf-4-binding was confirmed by supershift experiments using a Tcf-4 Ab (Fig. 6, a and b). Because binding site 2 in HD5 and binding site 1 in HD6 contained nearly the same sequence, we selected this probe for further analysis in human tissue. We used this EMSA-binding activity as a functional indicator of Tcf-4 protein levels, since available Abs were not sufficiently sensitive to provide satisfactory quantitation of the protein by Western blot analysis. Quantitative binding analysis of this common Tcf-4 high-affinity binding site probe using EMSA

![EMSA analysis of the Tcf-4 binding in Paneth cell α-defensin gene promoters.](http://www.jimmunol.org/)

**FIGURE 6.** EMSA analysis of the Tcf-4 binding in Paneth cell α-defensin gene promoters. EMSA was performed using recombinant Tcf-4 protein and radiolabeled double-stranded oligonucleotides (probes) corresponding to the Tcf-4 motif in the HD5 and HD6 promoters (a and b). Location of putative Tcf-4 binding sites (○) are presented in the graph of HD5 and HD6 promoter regions. The binding reactions contained (+) or lacked (−) indicated recombinant Tcf-4 protein, Tcf-4 Ab, unlabelled wild-type (wt) oligonucleotide, or competitor oligonucleotide with scrambled sequence as indicated. Migration of bound complexes of protein and oligonucleotide are marked by an arrow. The relative binding activity of each probe to human Tcf-4 protein was quantified by densitometry and relative values were normalized to the highest value which was defined as 1.0. Mean values are presented along with their SE (three independent experiments for HD5 and HD6 promoters, respectively). Representative (left) and quantitative comparisons (right) of Tcf-4-binding activity in extracts of small intestine tissue of healthy control and ileal CD are shown in c. For these experiments, probe #2 of HD5, which is nearly identical to probe #1 of HD6, was used. The value of \( p < 0.05 \) was considered to be statistically significant. mut, Mutation.
showed significantly reduced activity ($p = 0.0028$) in nuclear extracts from small intestine biopsies in ileal CD as compared with healthy control tissue (Fig. 6c), which is consistent with the reduced expression of Tcf-4 mRNA (Fig. 1b). Two patients with heterozygote NOD2 SNP13 mutations were in the range of the ileal CD patients (data not shown).

**Reduced Tcf-4 levels cause decreased Paneth cell α-defensin expression**

To directly assess whether modestly reduced Tcf-4 levels could cause a decrease in Paneth cell α-defensins, we turned to a Tcf-4 knockout murine model. We reasoned that the reduced levels of this transcription factor in heterozygous (+/−) knockout mice compared with wild-type littermates would be comparable to the differences in Tcf-4 expression observed in ileal CD compared with controls (Fig. 1). Similar to human ileal CD, there was no decrease in Paneth cell numbers in the Tcf-4 heterozygote (+/−) mice (data not shown). The Tcf-4−/− heterozygous knockout mice had a significant reduction of both Paneth cell α-defensin-4 (called cryptdin-4), an abundant mouse Paneth cell α-defensin (Fig. 7a), as well as cryptdin-1 (Fig. 7a). The expression levels of cryptdin-related sequence (CRS-1C) (Fig. 7a) and mouse lysozyme (data not shown) were also diminished, but the decreases were not statistically significant. To test whether the decrease of Paneth cell antimicrobial products in this murine model is functionally relevant, we performed an antimicrobial killing assay using small
Reduced Tcf-4 is independent of NOD2 genotype

Because NOD2 significantly affects human and mouse Paneth cell α-defensin expression (7, 8, 21), we tested whether Tcf-4 expression was also directly dependent on NOD2. In patient biopsies, the expression level of Tcf-4 was independent of whether the patients had a NOD2 mutation or not (Fig. 8a). Conversely, in heterozygous (+/−) Tcf-4 knockout mice, NOD2 expression levels were unchanged compared with wild-type control mice (Fig. 8a, inset). To further test whether the decrease of Tcf-4 is independent of NOD2, we assessed whether the Tcf-4 and HD5 levels correlated in two groups: ileal CD patients with the common heterozygote NOD2 L1007fs mutation (SNP13) and individuals without this mutation. Included in this correlation were patients with SNP8 or SNP12 missense mutations, which we previously showed had HD5 levels indistinguishable from those of wild-type NOD2 (8). Consistent with the patient cohort from Stuttgart with NOD2 wild-type ileal CD patients (Fig. 1c), there was a strong correlation of Tcf-4 with HD5 ($r_s = 0.74, p < 0.0001$) and HD6 ($r_s = 0.6824, p < 0.0001$; data not shown) in the surgical group of ileal CD patients not harboring the SNP13 mutation (Fig. 8b). Strikingly, the correlation was absent in the ileal CD patients with NOD2 SNP13 mutation (Fig. 8b). Specimens we previously characterized with SNP13 mutations have a significantly more pronounced decrease of Paneth cell α-defensins (7) and this decrease was prospectively confirmed in patient cohorts from the United States (8), Australia (G. Radford-Smith, unpublished observations), and Japan (33). Interestingly, the latter population does not exhibit NOD2 mutations (34). Furthermore, this diminished expression was not observed in CD of the colon or in UC in any of these studies. The decrease in α-defensins was even more pronounced in patients with a frameshift (SNP13) mutation in NOD2, as compared with those with either missense mutations (SNP8 and SNP12) or the majority of ileal CD patients with a normal NOD2 genotype (8). In this study, we report an explanation for these findings where a decrease of the Wnt pathway transcription factor Tcf-4 is responsible for the reduced expression of both Paneth cell α-defensins HD5 and HD6. CD patients with ileal disease showed a consistent decrease in Tcf-4 levels and activity, whereas patients with strictly CD of the colon had normal levels of ileal Tcf-4 and downstream Paneth cell α-defensins. Furthermore, the decreased expression of Tcf-4 in ileal CD was not a secondary effect of inflammation itself. We propose that the Tcf-4 dependent decrease of Paneth cell α-defensins in ileal CD leads to the changes in the luminal and/or surface bacteria composition, which have been implicated by others (35–38) as triggers of inflammation in CD (Fig. 9).

The current data show, both in the mouse and humans, that the decrease in Paneth cell α-defensins/cryptdins is Tcf-4 dependent and weakens enteric antimicrobial defense. Previous studies showed that compared with wild-type mice, HD5-transgenic mice were resistant to the otherwise lethal concentrations of orally administered Salmonella, indicating that HD5 had a biologically significant effect on pathogenic microbes in the intestinal lumen (9). Furthermore, using this same transgenic model, we reported that modest changes in HD5 expression levels, similar in magnitude to those observed in ileal CD compared with controls, significantly altered the composition of the luminal microbiota (8). Other studies in murine models have elegantly demonstrated that decreased Paneth cell α-defensin expression weakens mucosal defense against pathogenic bacteria (21, 39). In patients, the decrease of Paneth cell α-defensins in ileal CD results in a significantly

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**FIGURE 8.** Analysis of interdependence of NOD2, Tcf-4, and Paneth cell α-defensin expression levels in human and mouse ileal tissue. The expression of Tcf-4 mRNA is shown with respect to NOD2 mutation status in a cohort of surgical specimens with ileal CD, where adequate numbers of patients harboring SNP13 mutations were available (a). The expression of NOD2 mRNA was determined in Tcf-4 heterozygous (+/−) knockout and wild-type mice (inset, a). The correlation of Tcf-4 and HD5 mRNA levels is shown in individuals harboring the common SNP13 NOD2 frameshift mutation (●) as well as in individuals without this mutation (NOD2 wild type and SNP 8 and 12; b). A linear regression using the Spearman analysis for nonparametric data was performed. The value of $p < 0.05$ was considered to be statistically significant.
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