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Variants in *TRIM22* that Affect NOD2 Signaling Are Associated With Very Early Onset Inflammatory Bowel Disease

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Abstract

Background & Aims—Severe forms of inflammatory bowel disease (IBD) that develop in very young children can be caused by variants in a single gene. We performed whole-exome sequence (WES) analysis to identify genetic factors that might cause granulomatous colitis and severe perianal disease, with recurrent bacterial and viral infections, in an infant of consanguineous parents.

Methods—We performed targeted WES analysis of DNA collected from the patient and her parents. We validated our findings by a similar analysis of DNA from 150 patients with very early onset IBD not associated with known genetic factors analyzed in Toronto, Oxford, and Munich. We compared gene expression signatures in inflamed vs. non-inflamed intestinal and rectal tissues collected from patients with treatment-resistant Crohn's disease who participated in a trial of ustekinumab. We performed functional studies of identified variants in primary cells from patients and cell culture.

Results—We identified a homozygous variant in the tripartite motif containing 22 gene (*TRIM22*) of the patient, as well as in 2 patients with a disease similar phenotype. Functional studies showed that the variant disrupted the ability of TRIM22 to regulate nucleotide binding

oligomerization domain containing 2 (NOD2)-dependent activation of interferon-beta signaling and NF- κ B. Computational studies demonstrated a correlation between the TRIM22–NOD2 network and signaling pathways and genetic factors associated very early onset and adult-onset IBD. The network also associated with antiviral and mycobacterial effectors and markers of inflammation such as fecal calprotectin, c-reactive protein, and Crohn's disease activity index scores.

Conclusion—In WES and targeted exome sequence analyses of an infant with severe IBD, characterized by granulomatous colitis and severe perianal disease, we identified a homozygous variant of *TRIM22* that affect the ability of its product to regulate NOD2. Combined computational and functional studies showed that the TRIM22–NOD2 network regulates antiviral and anti-bacterial signaling pathways that contribute to inflammation. Further study of this network could lead to new disease markers and therapeutic targets for patients with very early and adult-onset IBD.

Keywords

VEOIBD; CDAI; genetics; risk; susceptibility

Background

Very early onset inflammatory bowel diseases (VEOIBD) often presents with severe multi-systemic disease that is difficult to treat with conventional therapies. These young patients frequently have novel causal¹⁻³ and risk variants⁴⁻⁸ and common networks are now being established (reviewed in ⁹). For example, mutations in *IL10RA/B* genes cause a Mendelian form of VEOIBD with severe colitis and perianal disease¹⁰ and mutations in *TTC7A* cause a severe form of apoptotic enterocolitis¹.

Tripartite motif-containing 22 (TRIM22; also known as STAF50) is a RING finger E3 ubiquitin ligase¹¹ that is expressed in the intestine¹² and in macrophages¹³, and has a role in lineage-specific differentiation of lymphocytes¹⁴. TRIM22 was originally identified as an interferon inducible protein that possesses antiviral activity¹⁵⁻¹⁷ and activates NF- κ B signaling¹³. Here we identify *TRIM22* functional variants associated with a distinct VEOIBD phenotype characterized by granulomatous colitis and severe perianal disease and show the TRIM22-NOD2 network as a key antiviral and mycobacterial regulator.

Methods

Abbreviated Methods (see Supplemental Material for Full Description).

Subjects

All experiments were carried out with the approval of the Research Ethics Board (REB) at the Hospital for Sick Children. Informed consent to participate in research was obtained. A copy of the consent is available on the interNational Early Onset Pediatric IBD Cohort Study (NEOPICS) website at http://www.neopics.org/NEOPICS_Documents.html.

Whole exome sequencing

For Patient 1 and her parents (trio), whole exome sequencing (WES) was performed using the Agilent SureSelect Human All Exon 50Mb kit with high-throughput sequencing conducted using the SOLiD 4 System at The Center for Applied Genomics (TCAG) through the Hospital for Sick Children (Toronto, ON). Sanger sequencing was used to verify variant genotypes in the Family 1 and infantile patients from the collaborating institutions were screened for *TRIM22* variants.

Validation

In order to validate these findings, we examined WES results from 150 infantile international VEOIBD patients without a genetic diagnosis who were previously sequenced in Toronto (NEOPICS), Oxford, and Munich (Care-For-Rare) and targeted exome sequencing of the *TRIM22* gene in ten *IL10RA/B*, and *IL10* negative patients without previous WES.

Computational Analysis

Datasets

Biopsy data were collected at baseline from anti-TNF resistant Crohn's patients enrolled in the ustekinumab trial previously described¹⁸. The ustekinumab trial expression data were used for construction of the adult IBD network. RNA-seq from the RISK cohort as previously described¹⁹ was used for generation of the pediatric IBD Bayesian network.

Differential expression

To ascertain tissue-specific signatures, we examined inflamed versus non-inflamed tissue from various anatomical regions of the small intestine, colon, and rectum. To identify differential expression signatures, we used an unbiased univariate filter to select top- varying genes and then applied significance analysis of microarrays (SAM)²⁰ to whole-genome expression data collected from biopsy tissue samples and whole blood of individuals in the ustekinumab trial. To control for multiple hypothesis testing, we used the Benjamini & Hochberg adjustment on the raw p-values to control the family-wise error rate and set a false discovery rate threshold of 1% or 5%. All analyses were performed using the R statistical package, version 2.15.24.1

Bayesian Network

We employed Monte Carlo Markov Chain (MCMC)²¹ simulation to identify potentially thousands of different plausible networks that are then combined to obtain a consensus network. eSNP data were used as priors as follows: genes with cis-eSNP²² are allowed to be parent nodes of genes without cis-eSNPs, but genes without cis-eSNPs are not allowed to be parents of genes with cis-eSNPs.

Key Driver Analysis

Key driver analysis (KDA) takes as input a set of genes (G) and a directed gene network N (e.g. Bayesian network)^{19,23-26}. The objective is to identify the key regulators for the gene

sets with respect to the given network. KDA first generates a sub-network N_G , defined as the set of nodes in N that are no more than h -layers away from the nodes in G , and then searches the h -layer neighborhood ($h=1, \dots, H$) for each gene in N_G ($HLN_{g,h}$) for the optimal h^* , such that $ES_{h^*} = \max(ES_{h,g}) \forall g \in N_g, h \in \{1..H\}$ where $ES_{h,g}$ is the computed enrichment statistic for $HLN_{g,h}$. A node becomes a candidate driver if its HLN is significantly enriched for the nodes in G . Candidate drivers without any parent node (i.e., root nodes in directed networks) are designated as global drivers while the remaining are designated as local drivers.

Pathway Enrichment

eQTL analysis was performed on IBD, UC, and CD GWAS hits from the NHGRI catalog, plus all WTCCC IBD GWAS SNPs with p value < 0.001 . 10% FDR eGenes were thus extracted, and tested for enrichment of pathways from the Metacore database.

Clinical variable correlation

We used Spearman correlation to examine the relationship between the mRNA expression of TRIM22 in intestine and blood and several key IBD traits. The correlation test was performed with the R function “corr.test”. We applied a simple Bonferroni correction to a 0.01 significance level across all gene-trait correlation tests performed.

Transfection and Luciferase Reporter Assays

NF- κ B luciferase reporter plasmids (Promega) and ISRE promoter and IFNP β promoter luciferase reporter plasmids were obtained from Dr. Hong-bing Shu (Wuhan University). A total of HEK 293 cells (1×10^5) were seeded on 24-well dishes and transfected the following day using Lipofectamine 2000 method. Empty control plasmid was added to ensure that each transfection received the same amount of total DNA. To normalize for transfection efficiency, 0.01 μ g of pRL-TK (Renilla luciferase) reporter plasmid was added to each transfection. Approximately 24 hr. after transfection, luciferase assays were performed using a dual-specific luciferase assay kit (Promega). Firefly luciferase activities were normalized on the basis of Renilla luciferase activities. Cells were treated with recombinant TNF α , RSV, or L18-MDP (InvivoGen). Statistical analysis of luciferase assay data consisted of two-way ANOVA followed by Bonferroni post-hoc testing to compare across conditions. Statistical significance was assigned to tests with $p < 0.05$ after Bonferroni correction.

Coimmunoprecipitation and Immunoblot Analysis

For transient transfection and coimmunoprecipitation experiments, HEK 293 cells (1×10^6) were seeded for 24 hr. then transiently transfected for 18–24 hr. using Lipofectamine 2000 according to the manufacturer's protocols. Transfected cells were lysed in 1 mL of lysis buffer (150mM NaCl, 50mM HEPES, 1% Triton X-100, 10% glycerol, 1.5mM MgCl₂, 1.0mM EGTA) supplemented with protease and phosphatase inhibitors (aprotinin (1:1000), leupeptin (1:1000), pepsin (1:1000) and, PMSF (1:100). For each immunoprecipitation, a 0.9mL aliquot of the lysate was incubated with 0.5 μ g of the indicated antibody and 40 μ L of a 1:1 slurry of Protein G Sepharose (Bioshop) for 1 hr. Sepharose beads were washed 3 \times

with 1 mL of high salt lysis buffer containing 0.5 M NaCl. The precipitates were analyzed by standard immunoblot procedures using the following antibodies: mouse monoclonal anti-FLAG (Sigma), anti-HA (Origene), anti-NOD2 (Novus), anti-GFP (Invitrogen), rabbit polyclonal anti-TRIM22 (Abnova) and goat polyclonal anti-NOD2 (Ingenix). For endogenous coimmunoprecipitation experiments, HT29 cells (5×10^7) were stimulated with MDP (10 μ g/mL) for the indicated times or left untreated. The coimmunoprecipitation and immunoblot experiments were performed as described above.

Results

Case History

Patient 1 was born in Australia to consanguineous parents. She initially presented on day 8 of life with fever, oral ulcers, diarrhea and failure to thrive and later developed severe fistulising perianal disease (Figure 1A) and granulomatous colitis (Figure 1B). She deteriorated despite treatment with corticosteroid and biologic therapy. Symptomatic control was only achieved by faecal steam diversion with combined ileostomy and subtotal colectomy. Patients 2 and 3 had a very similar clinical course with granulomatous colitis and severe perianal disease (Figure 1C-D) requiring ileostomy followed by total proctocolectomy. Both Patients 2 and 3 presented with severe anemia and severe hypoalbuminemia secondary to colitis. Patients 1 and 2 had numerous bacterial and viral infections. The three patients did not have mutations in the *IL10RA/B*, *IL10*, *XIAP*, or *NOD2* genes and were all very difficult to treat medically and surgically with their disease non-responsive to anti-TNF α biologic therapy (Table S1A; Supplemental Material for full clinical description).

Whole Exome Sequencing

Whole exome sequencing of Patient 1 (and parents) resulted in greater than 100 times mean exome coverage and identified 152,020 variants including 16,097 rare variants (minor allelic frequency MAF < 0.01) predicted to be damaging (Supplemental Figure 1). From this list we identified 12 non-synonymous homozygous variants (Supplemental Table 2) that were inherited in an autosomal recessive manner. Based on known protein function, expression profiles, animal models, mutation conservation, and network analysis (discussed below), this list was narrowed down to a single candidate gene — *TRIM22*. Patient 1 had a homozygous non-synonymous variant in exon 7 of the *TRIM22* gene inherited from both parents (Figure 1E). This missense variant resulted in an arginine to cysteine substitution (c.1324c>t; p.Arg442Cys) predicted to be highly deleterious (Figure 1F and Table S1B). It is highly conserved in a functionally significant region of the exposed beta sheet around the variable loop 3 (V3) in the B30.2 (SPRY) domain thought to be responsible for subcellular localization^{27, 28} and the 3-dimensional structure suggest it results in an elongated or additional beta strand, which in the wild type, forms a relaxed loop segment.

To validate, we examined WES from 150 VEOIBD patients but did not identify damaging variants in *TRIM22*. Therefore we performed targeted exome sequencing in ten patients with a similar severe phenotype and identified two additional patients with *TRIM22* variants (Patients 2 and 3). Patient 2 had compound heterozygous *TRIM22* variants (Figure 1E)

including a novel arginine to threonine substitution (c.449G>C, p.Arg150Thr) inherited paternally; and a serine to leucine substitution (c.731C>T; p.Ser244Leu) inherited maternally. Both variants were predicted to be damaging and disrupt the highly conserved coiled-coil region of TRIM22 that facilitates homodimer/heterodimer multimers (Figures 1F, S2, and Table S1B). R150T is in an exposed region and S244L is buried and resides in the spacer-2 region that contains a bipartite nuclear localization sequence and was previously predicted to be a serine phosphorylation site²⁷.

Patient 3 had a homozygous *TRIM22* variant inherited from both parents (Figure 1E). This non-synonymous variant resulted in an arginine to lysine substitution (p.Arg321Lys; c.962G>A). The R321K variant was predicted to be deleterious and located in the highly conserved SPRY domain on an exposed residue of a beta sheet that has a functional role in the antiviral activity patch of the SPRY domain²⁷ (Figure 1F; Table S1B).

Integrative Analysis Supports TRIM22 as a Key Regulator in IBD Associated Networks Constructing a VEOIBD-informed network—To gain insight into the role of TRIM22 in the pathogenesis of IBD, we constructed a probabilistic causal network model by integrating 7,568 RNA-sequence derived gene expression traits from ileal biopsies from 322 treatment naïve pediatric Crohn's patients in the RISK cohort¹⁹ and expression quantitative trait loci (eQTL). To identify the VEOIBD component of this molecular network, we projected all genes known to be causally associated with VEOIBD⁹ (Table S3) onto this network, and then identified the most connected subnetwork containing these VEOIBD genes within the full RISK network, resulting in the VEOIBD network comprised of 665 genes, which included TRIM22 (Figure 2A).

To understand the regulation of this VEOIBD network, we employed a previously established procedure²⁶ to identify key regulator genes that are predicted to modulate the state of the network (Table S4). Interestingly, the most significant key regulators identified in the VEOIBD network included SNX10, STX11, CYBB and DRAM1, which directly connect TRIM22 to the IBD gene NOD2 via two equidistant paths. The first pathway, TRIM22→SNX10→STX11→DRAM1→NOD2, is linked to the known VEOIBD gene STAT1 via STX11 and GBP5, while the second pathway, TRIM22→SNX10→CYBB→DRAM1→NOD2, contains the known VEOIBD gene CYBB (Figure 2B). Both STAT1 and CYBB, in addition to IRF8, which is directly connected to TRIM22, are associated with Mendelian susceptibility to mycobacteria deficiency²⁹. As expected, we did not identify any common DNA variants from the IIBDGC GWAS³⁰ within the TRIM22 locus (Tables S5-6), however, the VEOIBD subnetwork is well represented in genes involved in TRIM22-relevant pathways, including p53, ubiquitin response, and antiviral signaling, which were among the top pathways enriched for genes associated with intestine and blood expression quantitative trait loci (eQTL) that were also associated with adult IBD loci identified in the IIBDGC cohort (Figure S3, Table S7). Further, we found that the VEOIBD network was 2.1 fold enriched ($p = 2.3 \times 10^{-5}$) for Crohn's genes annotated from adult IBD genetic studies that also give rise to blood eQTLs³¹ (Table S8).

Adult-onset IBD informed by a TRIM22-centered network—Given preliminary support for adult IBD in the VEOIBD network, we explored whether the TRIM22

component of the VEOIBD network was associated with adult IBD. We constructed an adult IBD causal gene network from gene expression and genotype data generated on 203 intestinal biopsies that included inflamed and non-inflamed tissues from ileum, ascending colon, descending colon, transverse colon, sigmoid and rectum. All of these tissues, in addition to blood, were collected at baseline from 54 anti-TNF α resistant Crohn's patients enrolled in the ustekinumab (IL12/IL23 p40 monoclonal antibody) clinical trial¹⁸ (Figure S4). We found that TRIM22 was a significantly up-regulated (1.33, $p=1.37e-5$), key driver ($p = 4.0e-6$) gene in inflamed sigmoid and inflamed rectum (1.24, $p=1.83e-6$), ($p = 1.0e-4$) compared to the non-inflamed samples of these same tissues.

We identified the homologous TRIM22 component of the above adult IBD network by first identifying all genes within a path length of 5 to TRIM22 in the VEOIBD network, which included NOD2, leaving a network comprised of 179 genes (referred to here as the TRIM22 network; Table S9). This TRIM22 network contained many macrophage/monocyte expressed genes associated with mycobacterial and anti-viral responses (including genes involved in autophagy, NF- κ B pathway and apoptosis; Figure S5; Table S10). Strikingly, of the 480 key driver genes we identified in the adult IBD network, 62 of these genes, including STX11, CYBB, and DRAM1 were represented in the pediatric TRIM22 network, 7.2-fold enrichment over what is expected by chance ($p = 6.5 e-38$) (Table S11). Further, the TRIM22 containing co-expression module from the blood of ustekinumab patients, which was 12.7-fold enriched for response to virus ($p = 6.3e-14$), was 9-fold enriched in the pediatric TRIM22 network ($p < 1e-16$). Further supporting the pediatric TRIM22 network's association to viral response was a 3.2-fold enrichment ($p = 2.5e-5$) of eQTLs associated with dendritic cell response to stimulation with flu or interferon beta³² and an 8.3-fold enrichment ($p = 1.1e-16$) of genes in a tuberculosis infection signature in dendritic cells³⁰.

The homologous TRIM22 network in the adult IBD network was then identified by projecting nodes from the TRIM22 network onto the adult IBD network, identifying those nodes that were either in the TRIM22 network or directly connected to such nodes (Figure 2C). In this TRIM22 pediatric oriented adult IBD network, TRIM22 is in close proximity to several drug targets that are either approved or in clinical development for IBD, including TNF α , the target of adalimumab (humira), certolizumab (cimzia), infliximab (remicade), and colimumab (simponi); IL12R β , a chain shared between the receptors that bind IL12/23, the molecular target of ustekinumab; JAK3, the target of tofacitinib; and IL17RA, the target of brodalumab.

Since S100A8 (calprotectin), a stool biomarker for inflammation, is also co-localized with TRIM22 in this network³³⁻³⁵ (Figure 2C), we decided to further investigate the role of TRIM22 in treatment refractory patients, exploring associations between TRIM22 and IBD-associated clinical traits in the ustekinumab clinical trial (Tables S12). We found that expression levels of TRIM22 in the colon were positively correlated with fecal calprotectin regardless of biopsy inflammation status. Overall, both inflamed and non-inflamed baseline intestine levels of TRIM22 revealed a correlation to blood c-reactive protein (CRP) of which consistently high levels is associated with subsequent development of perianal fistulae in Crohn's patients³⁶ and response to anti-TNF α ³⁷. TRIM22 expression levels in blood were positively correlated with Crohn's disease activity index (CDAI) (Tables S13).

Taken together, the intestinal network models provide causal support for the TRIM22 regulation of NOD2 and the correlation of the TRIM22-NOD2 pathway with predicted disease regulators, functionally relevant VEO and adult onset IBD genetic loci, effectors of anti-viral and mycobacterial function, correlation with inflammatory clinical variables and validated IBD drug targets.

Identification of TRIM22 as a NOD2 Interacting Protein

As NOD2 signaling plays a critical role in Crohn's disease susceptibility^{38, 39}; we further investigated this *in silico* TRIM22-NOD2 interaction. Immunofluorescence showed that NOD2 and TRIM22 colocalized in colonic biopsies and in the cytoplasm of transiently transfected HEK293 cells (Figure S6A-C). This NOD2 immunostaining was blocked by a NOD2-specific peptide and TRIM22 did not colocalize with NOD1 (Figure S6D-F) another member of the NOD family confirming the specificity of this TRIM22-NOD2 interaction. Immunostaining of colonic biopsy samples from Patients 1-3 with TRIM22 variants showed altered expression and localization of NOD2 and TRIM22 (Figure S6A-B).

TRIM22 Influences NOD2 Signaling Pathways

NOD2 senses muramyl dipeptide (MDP), a conserved structure in peptidoglycan present in most Gram-negative and Gram-positive bacteria. We found that TRIM22 was weakly associated with endogenous NOD2 in co-immunoprecipitation experiments in unstimulated HT29 cells, a human intestinal epithelial cell line, and this association increased after stimulation with MDP (Figure 3A). Furthermore, transiently transfected TRIM22 and NOD2 were found to reciprocally co-immunoprecipitate and these interactions were reduced with the TRIM22 variants (Figure 3B-C and S7A).

Since TRIM22 co-localized and co-immunoprecipitated with NOD2, we next determined if TRIM22 influenced MDP-induced NF- κ B signaling via NOD2. In reporter assays, co-expression of TRIM22 and NOD2 enhanced MDP-induced activation of the NF- κ B (Figure 3D) in HEK293 cells. As TRIM22 regulates viral activity¹⁵⁻¹⁷ and NOD2 functions as cytoplasmic viral pathogen recognition receptor by triggering interferon- β (IFN β) activation in response to Respiratory Syncytial Virus (RSV)^{40, 41}, we next determined whether TRIM22 also regulated NOD2-dependent RSV-induced IFN β signaling. As shown in Figure 3D, overexpression of TRIM22 and NOD2 both enhanced RSV-induced activation of the IFN β promoter and the Interferon Stimulated Response Element (ISRE). Neither wildtype (WT)-TRIM22 nor the TRIM22-R442C variant enhanced TNF α gene expression in response to TNF α (Figure S7B). As TNF α activates NF- κ B target genes independent of NOD2 signaling⁴², this suggested that TRIM22 regulation of MDP-induced NF- κ B activation is NOD2-dependent.

TRIM22 Variants Influence NOD2 Signaling

We next examined the role of TRIM22 to regulate NOD2-dependent NF- κ B and IFN β signaling. Luciferase assays using HEK293 cells and real-Time PCR experiments using HCT116 showed that expression of wild type TRIM22 potentiated NOD2 signaling in response to both MDP-induced NF- κ B and RSV-induced IFN β activation (Figures 3D and S7C-F). However, the TRIM22 variants (Figures 3D and S7C-E) and TRIM22 knockdown

using short hairpin RNA (shRNA; Figure S8A) showed impaired NOD2-dependent NF- κ B and IFN β signaling (Figure S8B). MDP-mediated NOD2-dependent signaling was also significantly reduced in only Patient 2 with the heterozygous *TRIM22* variants in the coiled-coil domain (Figure S9 and Supp. Material). Together this data suggests that TRIM22 is a positive regulator of antiviral and antibacterial NOD2-dependent signaling pathways.

TRIM22 mediates K63-linked Polyubiquitination of NOD2

As TRIM22 is a RING finger E3 ubiquitin ligase¹¹ and NOD2 has high confidence predicted ubiquitination sites at K436 and K445 (CKSAAP_UbSITE⁴³), we next determined if TRIM22 mediated ubiquitination of NOD2. Immunoprecipitation experiments using a HEK293 cell line co-expressing HA-NOD2 and Flag-TRIM22 showed that overexpression of TRIM22 enhanced NOD2 polyubiquitination (Figure 4A). The TRIM22 variants (Figure 4A and S10A) were unable to mediate this NOD2 polyubiquitination. TRIM22 did not increase polyubiquitination of other components (MAVS or RIPK2) of the NOD2 signaling pathways known to be ubiquitinated^{44, 45} (Figure S10B). Using the TRIM22 shRNA HT29 stable cell line, we found that only wildtype TRIM22 rescued NOD2 polyubiquitination and not the TRIM22-R442C variant (Figure 4B). Taken together, this suggested that TRIM22 specifically mediates NOD2 polyubiquitination.

K63-linked polyubiquitination regulates the localization and signaling activity whereas K48-linked polyubiquitination often mediates degradation⁴⁶. NOD2 has been previously shown to be K48-linked ubiquitinated by TRIM27 and results in NOD2 degradation⁴⁷. Using ubiquitin mutants, we showed that overexpression of TRIM22 mediated K63-linked but not K48-linked polyubiquitination of NOD2 while the TRIM22 variants were unable to mediate K63- or K48-linked NOD2 polyubiquitination (Figure 4C and S11A). We next determined that TRIM22 is required for MDP- and RSV-induced K63-linked NOD2 polyubiquitination and knockdown of TRIM22 reduced this MDP- and RSV-induced K63-linked (Figure 4D-E), but not K48-linked NOD2 polyubiquitination (Figures S11B-C).

Discussion

TRIM proteins are involved in a number of biological processes, including cell proliferation, apoptosis, innate immunity, autoimmunity, and inflammatory response^{48, 49}. Variants in *TRIM* genes result in monogenic diseases including familial Mediterranean fever (FMF) and Opitz syndrome type 1, and are implicated in a number of cancers and autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus^{48, 49}. TRIM22 belongs to a subfamily of TRIM proteins that contain a SPRY domain and are thought to have evolved to limit innate immune responses to viruses²⁰. Mutations in the SPRY domain of TRIM20 (pyrin) result in autoimmune diseases including FMF⁵⁰. Overexpression of TRIM22 is known to activate NF- κ B in a dose-dependent manner and both the N-terminal RING domain and C-terminal SPRY domain are crucial for TRIM22-mediated NF- κ B activation¹³ and antiviral activity¹⁵⁻¹⁷, and are implicated in the pathogenesis of autoimmune diseases⁵¹. Patients 1 and 3 both had homozygous variants in the SPRY domain and Patient 2 had compound heterozygous variants both predicted to disrupt the coiled-coil domain and had loss of NOD2-dependent MDP signaling. The

characterized *TRIM22* variants resulted in impaired NOD2 binding and K63-linked NOD2 polyubiquitination. The predicted NOD2 polyubiquitination sites described earlier are in the NACHT domain, a conserved domain required for NOD2 conformational change in response to MDP sensing through the LRR domain or interaction with binding adaptor proteins for NOD2⁵². Therefore, as all identified *TRIM22* variants disrupt both NOD2-dependent NF- κ B and IFN β signaling, this suggests that K63-linked polyubiquitination of NOD2 mediated by TRIM22 is critical for NOD2 function (Figure 4F).

NOD2 has long been recognized as a critical player in Crohn's disease pathogenesis^{38, 39} where it is proposed to regulate innate immunity through NF- κ B-induced pro-inflammatory responses triggered by PGN (reviewed in⁵³). Numerous studies have shown that the loss of function variants in NOD2 associated with Crohn's disease result in the loss of NF- κ B-induced pro-inflammatory cytokine response to MDP (reviewed in^{54, 55}) and therefore mirror the defect we observe in Patient 2 with TRIM22 mutations in the coiled-coil domain. Similarly, mutations in XIAP (also an E3 ubiquitin ligase) are associated with IBD with granulomatous colitis and perianal disease^{56, 57} and are associated with loss of NOD2-dependent mediated NF- κ B signalling⁵⁸⁻⁶⁰ and XIAP is also regulated by TRIM proteins⁶¹. XIAP deficiency usually presents in early childhood but there are cases of patients presenting with IBD at over 40 years of age⁹ with and without hemophagocytic lymphohistiocytosis (HLH) (reviewed in⁶²). Furthermore, XIAP not only regulates NOD2-dependent innate immunity responses but also is proposed to regulate inflammasome and adaptive immunity⁶². Loss of function NOD2 variants associated with IBD have low penetrance and do not cause the severe early onset disease observed in this group with either TRIM22- or XIAP-deficiency. Therefore it is most likely that in Patients 1 and 3, with TRIM22 SPRY domain variants, defects in other pathways regulated by TRIM22 result in the early onset and severity of disease. Alternatively hypomorphic TRIM22 variants act in an oligogenic manner with mutations in other genes similar to what has been observed with hypomorphic XIAP mutations⁶³ and as observed in Patient 3 who is also a carrier of a XIAP mutation. Together, this suggests that loss of NOD2-dependent mediated signaling in response to MDP contributes to a severe form of IBD and that other yet defined pathways must also be involved in disease pathogenesis. With regards to TRIM22 specifically, we anticipate that other pathways not identified in these studies play a role in disease pathogenesis.

Our computational studies further support our functional studies demonstrating that TRIM22 influences both NOD2-dependent IFN β and NF- κ B signaling pathways. While the loss of function TRIM22 variants in our VEOIBD patients may manifest as an immunodeficiency, the inflammation and tissue damage that ensues from a dysregulated response to microbial burden, may resemble the profile of adult-onset IBD. The functional outcome of pro-inflammatory NF- κ B activation may differ depending on the cell population in which TRIM22 is expressed, with circulating plasmacytoid dendritic cells (DCs) and monocytes predominating in the blood and resident intestinal DCs, macrophages and enterocytes in the intestine. We see from the intestine network that the regulation of causal TRIM22 results in modulation of a mycobacterial and viral response subnetwork that may also result in feedback to TRIM22 expression. Thus, either due to immunodeficiency arising from the TRIM22 variants or excessive inflammation in non-TRIM22 variant carried by anti-TNF α

refractory patients, TRIM22 driven NOD2 activation of NF- κ B in response to MDP or viral stimuli, contributes to dysregulation of NF- κ B and IFN β driven pathways as reflected in the networks. Together, this demonstrates that the TRIM22-NOD2 network plays a critical role in the development of IBD.

Investigation of VEO and adult onset IBD in treatment refractory disease will elucidate new treatment opportunities by revealing complexity of disease network dysregulation. Causal and correlative based network analysis allows for consideration of the synergistic effects between genes in disease, which can be a greater determinant than evaluation of single target genes, to inform disease drivers and opportunities for combinatorial therapy. Thus network analysis can be focused by novel variants, as seen with TRIM22 identified through WES in VEOIBD patients to generate hypothesis and potentially identify biomarkers and guide drug discovery and repositioning in both VEO and adult-onset IBD patient subsets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Avitzur Y, Guo C, Mastropaolo LA, et al. Mutations in tetratricopeptide repeat domain 7A result in a severe form of very early onset inflammatory bowel disease. *Gastroenterology*. 2014; 146:1028–39. [PubMed: 24417819]
2. Elkadri A, Thoeni C, Deharvengt SJ, et al. Mutations in Plasmalemma Vesicle Associated Protein Result in Sieving Protein-Losing Enteropathy Characterized by Hypoproteinemia, Hypoalbuminemia, and Hypertriglyceridemia. *Cell Mol Gastroenterol Hepatol*. 2015; 1:381–394 e7. [PubMed: 26207260]
3. Janecke AR, Heinz-Erian P, Yin J, et al. Reduced sodium/proton exchanger NHE3 activity causes congenital sodium diarrhea. *Hum Mol Genet*. 2015; 24:6614–23. [PubMed: 26358773]
4. Dhillon SS, Fattouh R, Elkadri A, et al. Variants in nicotinamide adenine dinucleotide phosphate oxidase complex components determine susceptibility to very early onset inflammatory bowel disease. *Gastroenterology*. 2014; 147:680–689 e2. [PubMed: 24931457]
5. Dhillon SS, Mastropaolo LA, Murchie R, et al. Higher activity of the inducible nitric oxide synthase contributes to very early onset inflammatory bowel disease. *Clin Transl Gastroenterol*. 2014; 5:e46. [PubMed: 24430113]
6. Moran CJ, Walters TD, Guo CH, et al. IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis*. 2013; 19:115–23. [PubMed: 22550014]
7. Muise AM, Walters T, Xu W, et al. Single nucleotide polymorphisms that increase expression of the guanosine triphosphatase RAC1 are associated with ulcerative colitis. *Gastroenterology*. 2011; 141:633–41. [PubMed: 21684284]

8. Muise AM, Xu W, Guo CH, et al. NADPH oxidase complex and IBD candidate gene studies: identification of a rare variant in NCF2 that results in reduced binding to RAC2. *Gut*. 2012; 61:1028–35. [PubMed: 21900546]
9. Uhlig HH, Schwerd T, Koletzko S, et al. The Diagnostic Approach to Monogenic Very Early Onset Inflammatory Bowel Disease. *Gastroenterology*. 2014
10. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med*. 2009; 361:2033–45. [PubMed: 19890111]
11. Duan Z, Gao B, Xu W, et al. Identification of TRIM22 as a RING finger E3 ubiquitin ligase. *Biochem Biophys Res Commun*. 2008; 374:502–6. [PubMed: 18656448]
12. Sawyer SL, Emerman M, Malik HS. Discordant evolution of the adjacent antiretroviral genes TRIM22 and TRIM5 in mammals. *PLoS Pathog*. 2007; 3:e197. [PubMed: 18159944]
13. Yu S, Gao B, Duan Z, et al. Identification of tripartite motif-containing 22 (TRIM22) as a novel NF-kappaB activator. *Biochem Biophys Res Commun*. 2011; 410:247–51. [PubMed: 21651891]
14. Obad S, Olofsson T, Mechti N, et al. Expression of the IFN-inducible p53-target gene TRIM22 is down-regulated during erythroid differentiation of human bone marrow. *Leuk Res*. 2007; 31:995–1001. [PubMed: 17257675]
15. Barr SD, Smiley JR, Bushman FD. The interferon response inhibits HIV particle production by induction of TRIM22. *PLoS Pathog*. 2008; 4:e1000007. [PubMed: 18389079]
16. Eldin P, Papon L, Oteiza A, et al. TRIM22 E3 ubiquitin ligase activity is required to mediate antiviral activity against encephalomyocarditis virus. *J Gen Virol*. 2009; 90:536–45. [PubMed: 19218198]
17. Di Pietro A, Kajaste-Rudnitski A, Oteiza A, et al. TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. *J Virol*. 2013; 87:4523–33. [PubMed: 23408607]
18. Sandborn WJ, Gasink C, Gao LL, et al. Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med*. 2012; 367:1519–28. [PubMed: 23075178]
19. Haberman Y, Tickle TL, Dexheimer PJ, et al. Pediatric Crohn disease patients exhibit specific ileal transcriptome and microbiome signature. *J Clin Invest*. 2014; 124:3617–33. [PubMed: 25003194]
20. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001; 98:5116–21. [PubMed: 11309499]
21. Madigan, Da; Y, J. Bayesian graphical models for discrete data. *International Statistical Review*. 1995; 63:215–232.
22. Schadt EE, Molony C, Chudin E, et al. Mapping the genetic architecture of gene expression in human liver. *PLoS Biol*. 2008; 6:e107. [PubMed: 18462017]
23. Tran LM, Zhang B, Zhang Z, et al. Inferring causal genomic alterations in breast cancer using gene expression data. *BMC Syst Biol*. 2011; 5:121. [PubMed: 21806811]
24. Wang IM, Zhang B, Yang X, et al. Systems analysis of eleven rodent disease models reveals an inflammatome signature and key drivers. *Mol Syst Biol*. 2012; 8:594. [PubMed: 22806142]
25. Yang X, Zhang B, Molony C, et al. Systematic genetic and genomic analysis of cytochrome P450 enzyme activities in human liver. *Genome Res*. 2010; 20:1020–36. [PubMed: 20538623]
26. Zhang B, Gaiteri C, Bodea LG, et al. Integrated systems approach identifies genetic nodes and networks in late-onset Alzheimer's disease. *Cell*. 2013; 153:707–20. [PubMed: 23622250]
27. Kelly JN, Barr SD. In Silico Analysis of Functional Single Nucleotide Polymorphisms in the Human TRIM22 Gene. *PLoS One*. 2014; 9:e101436. [PubMed: 24983760]
28. Herr AM, Dressel R, Walter L. Different subcellular localisations of TRIM22 suggest species-specific function. *Immunogenetics*. 2009; 61:271–80. [PubMed: 19212762]
29. Bustamante J, Boisson-Dupuis S, Abel L, et al. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-gamma immunity. *Semin Immunol*. 2014; 26:454–70. [PubMed: 25453225]
30. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012; 491:119–24. [PubMed: 23128233]
31. Westra HJ, Peters MJ, Esko T, et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. *Nat Genet*. 2013; 45:1238–43. [PubMed: 24013639]

32. Lee MN, Ye C, Villani AC, et al. Common genetic variants modulate pathogen-sensing responses in human dendritic cells. *Science*. 2014; 343:1246980. [PubMed: 24604203]
33. Sandborn WJ, Ghosh S, Panes J, et al. Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *N Engl J Med*. 2012; 367:616–24. [PubMed: 22894574]
34. Ho GT, Lee HM, Brydon G, et al. Fecal calprotectin predicts the clinical course of acute severe ulcerative colitis. *Am J Gastroenterol*. 2009; 104:673–8. [PubMed: 19262524]
35. Leach ST, Yang Z, Messina I, et al. Serum and mucosal S100 proteins, calprotectin (S100A8/S100A9) and S100A12, are elevated at diagnosis in children with inflammatory bowel disease. *Scand J Gastroenterol*. 2007; 42:1321–31. [PubMed: 17852869]
36. P226. Consistently high C reactive protein is associated with subsequent development of perianal fistulae in patients with Crohn's disease. *J Crohns Colitis*. 2015; 9(Suppl 1):S188.
37. Murdoch T, O'Donnell S, Silverberg MS, et al. Biomarkers as potential treatment targets in inflammatory bowel disease: A systematic review. *Can J Gastroenterol Hepatol*. 2015; 29:203–8. [PubMed: 25965441]
38. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001; 411:599–603. [PubMed: 11385576]
39. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001; 411:603–6. [PubMed: 11385577]
40. Kim YG, Park JH, Reimer T, et al. Viral infection augments Nod1/2 signaling to potentiate lethality associated with secondary bacterial infections. *Cell Host Microbe*. 2011; 9:496–507. [PubMed: 21669398]
41. Sabbah A, Chang TH, Harnack R, et al. Activation of innate immune antiviral responses by NOD2. *Nat Immunol*. 2009; 10:1073–80. [PubMed: 19701189]
42. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol*. 2002; 2:725–34. [PubMed: 12360211]
43. Chen Z, Chen YZ, Wang XF, et al. Prediction of ubiquitination sites by using the composition of k-spaced amino acid pairs. *PLoS One*. 2011; 6:e22930. [PubMed: 21829559]
44. Castanier C, Zemirli N, Portier A, et al. MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors. *BMC Biol*. 2012; 10:44. [PubMed: 22626058]
45. Yang S, Wang B, Humphries F, et al. Pellino3 ubiquitinates RIP2 and mediates NOD2-induced signaling and protective effects in colitis. *Nat Immunol*. 2013; 14:927–36. [PubMed: 23892723]
46. Walczak H, Iwai K, Dikic I. Generation and physiological roles of linear ubiquitin chains. *BMC Biol*. 2012; 10:23. [PubMed: 22420778]
47. Zurek B, Schoultz I, Neerinx A, et al. TRIM27 negatively regulates NOD2 by ubiquitination and proteasomal degradation. *PLoS One*. 2012; 7:e41255. [PubMed: 22829933]
48. Jefferies C, Wynne C, Higgs R. Antiviral TRIMs: friend or foe in autoimmune and autoinflammatory disease? *Nat Rev Immunol*. 2011; 11:617–25. [PubMed: 21866173]
49. Kawai T, Akira S. Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. *EMBO Mol Med*. 2011; 3:513–27. [PubMed: 21826793]
50. Chae JJ, Wood G, Masters SL, et al. The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts directly with caspase-1 to modulate IL-1beta production. *Proc Natl Acad Sci U S A*. 2006; 103:9982–7. [PubMed: 16785446]
51. Hattlmann CJ, Kelly JN, Barr SD. TRIM22: A Diverse and Dynamic Antiviral Protein. *Mol Biol Int*. 2012; 2012:153415. [PubMed: 22649727]
52. Zurek B, Proell M, Wagner RN, et al. Mutational analysis of human NOD1 and NOD2 NACHT domains reveals different modes of activation. *Innate Immun*. 2012; 18:100–11. [PubMed: 21310790]
53. Shaw MH, Kamada N, Warner N, et al. The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation. *Trends Immunol*. 2011; 32:73–9. [PubMed: 21251876]
54. Strober W, Watanabe T. NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease. *Mucosal Immunol*. 2011; 4:484–95. [PubMed: 21750585]

55. Philpott DJ, Sorbara MT, Robertson SJ, et al. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol.* 2014; 14:9–23. [PubMed: 24336102]
56. Marsh RA, Rao K, Satwani P, et al. Allogeneic hematopoietic cell transplantation for XIAP deficiency: an international survey reveals poor outcomes. *Blood.* 2013; 121:877–83. [PubMed: 23131490]
57. Zeissig Y, Petersen BS, Milutinovic S, et al. XIAP variants in male Crohn's disease. *Gut.* 2014
58. Abbott DW, Yang Y, Hutti JE, et al. Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. *Mol Cell Biol.* 2007; 27:6012–25. [PubMed: 17562858]
59. Damgaard RB, Fiil BK, Speckmann C, et al. Disease-causing mutations in the XIAP BIR2 domain impair NOD2-dependent immune signalling. *EMBO Mol Med.* 2013; 5:1278–95. [PubMed: 23818254]
60. Krieg A, Correa RG, Garrison JB, et al. XIAP mediates NOD signaling via interaction with RIP2. *Proc Natl Acad Sci U S A.* 2009; 106:14524–9. [PubMed: 19667203]
61. Ryu YS, Lee Y, Lee KW, et al. TRIM32 protein sensitizes cells to tumor necrosis factor (TNF α)-induced apoptosis via its RING domain-dependent E3 ligase activity against X-linked inhibitor of apoptosis (XIAP). *J Biol Chem.* 2011; 286:25729–38. [PubMed: 21628460]
62. Latour S, Aguilar C. XIAP deficiency syndrome in humans. *Semin Cell Dev Biol.* 2015; 39:115–123. [PubMed: 25666262]
63. Rigaud S, Lopez-Granados E, Siberil S, et al. Human X-linked variable immunodeficiency caused by a hypomorphic mutation in XIAP in association with a rare polymorphism in CD40LG. *Blood.* 2011; 118:252–61. [PubMed: 21543760]

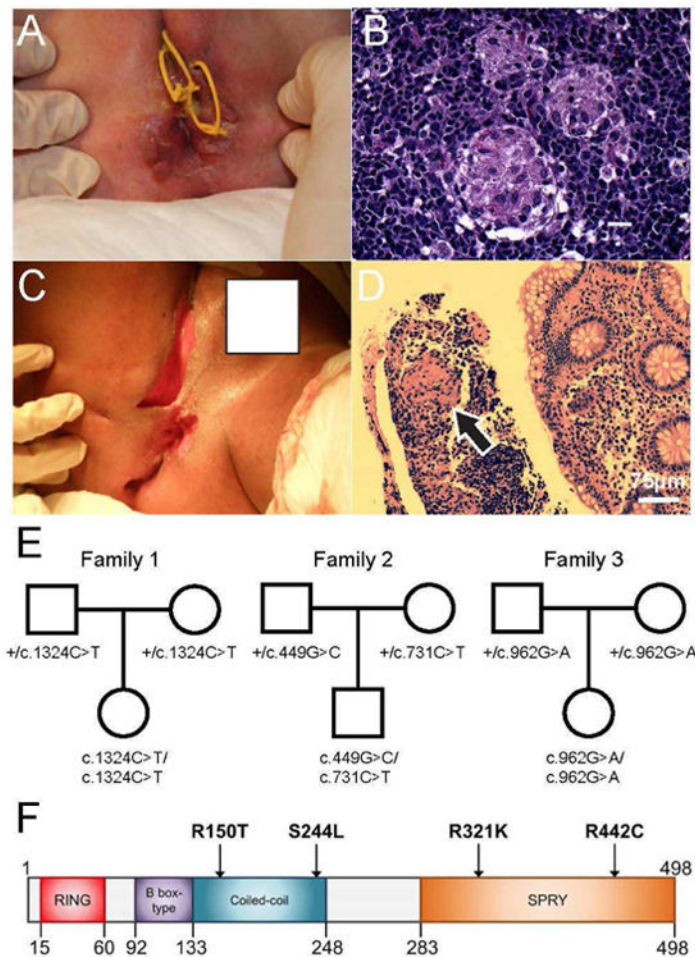


Figure 1. Clinical Features and Pedigrees of Patients with *TRIM22* Variants

A, B) *Clinical presentation and histopathologic findings in colonic biopsy from Patient 1.*

Panel A shows severe perianal fistulization with seton placement. Recto-vaginal fistulae were also present. Panel B shows non-caseating granulomas on a background of chronic inflammatory cell infiltrates (H&E stain).

C, D) *Clinical presentation and histopathologic findings in colonic biopsy from Patient 2.*

Panel C shows multiple, severe cavitating perianal fistulae. Panel D shows a colonic biopsy showing chronic inflammation with non-caseating granuloma (black arrow) (H&E stain, scale bar represents 75 μm).

E) *Pedigree analysis of the genetic inheritance pattern for each family trio.*

F) *Schematic view of the protein domain architecture of *TRIM22* with the relative position of each identified variant depicted.*

Number indicates amino acid position.

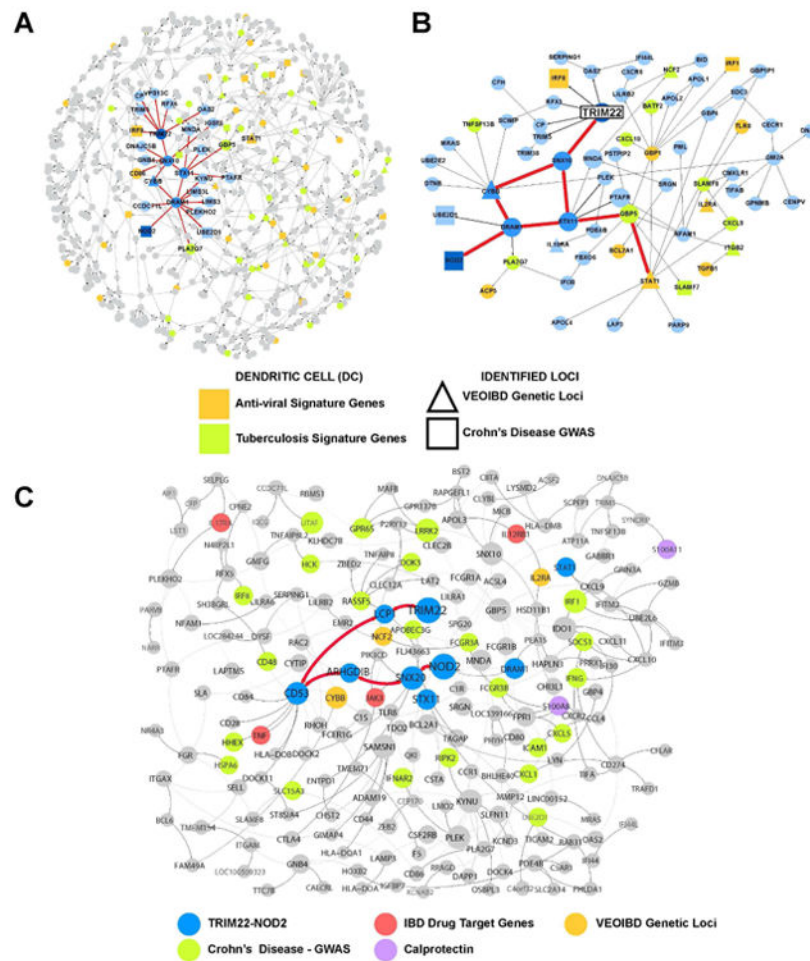


Figure 2. TRIM22 - NOD2 Network

A) VEOIBD loci focused subnetwork within RISK Pediatric Crohn's ileum network.

To define the context of VEOIBD in the ileum network, genes known to be causally associated with VEOIBD (not including TRIM22) were projected onto the RISK network and extended out a path length of two. The resulting VEOIBD focused sub network is comprised of 665 genes and contains TRIM22 and NOD2. The TRIM22-NOD2 paths are highlighted in bold and are connected to the known VEOIBD loci CYBB, and STAT1, as well as the GWAS locus IRF8.

B) Local network structure surrounding the NOD2/TRIM22 paths in the 665-node VEOIBD focused subnetwork.

Representative nodes from the 179-gene TRIM22 network within the 665 node VEOIBD loci focused RISK subnetwork.

C) TRIM22-NOD2 interaction is conserved in the adult IBD intestine.

This network provides the context of the TRIM22/NOD2 interaction in the adult IBD intestine. The 179-gene TRIM22 network was projected and extended out an additional path length on the adult IBD pan intestine Bayesian network. IBD GWAS (green), VEOIBD loci (orange), IBD drug targets approved or in clinical development (red), and calprotectin (S100A8) (purple) are all represented in this conserved subnetwork.

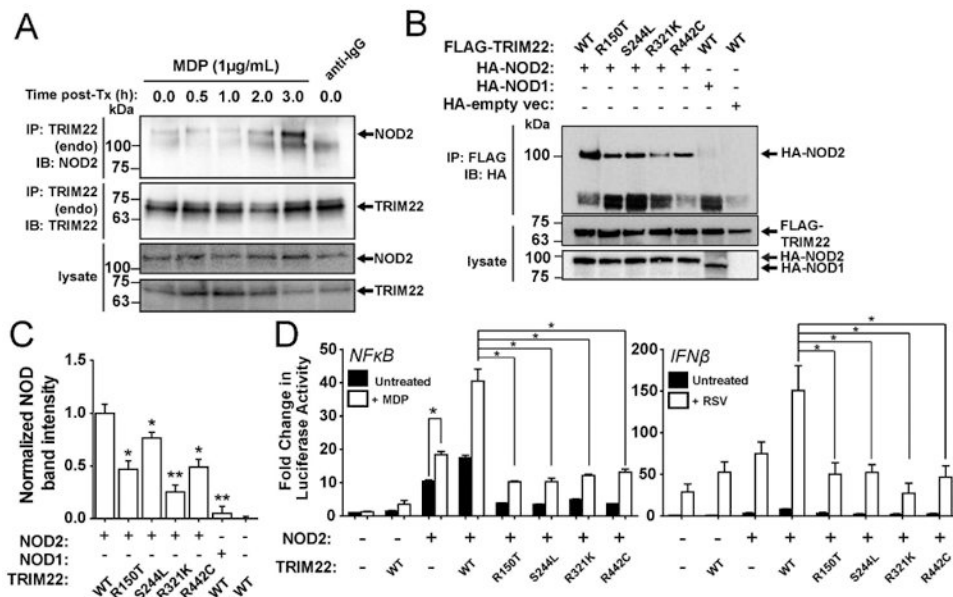


Figure 3. TRIM22 Interacts with and Activates NOD2 Signaling

A) *Endogenous NOD2 and TRIM22 co-immunoprecipitate following MDP stimulation.*

HT29 cells were stimulated with MDP (1 $\mu\text{g}/\text{mL}$) for various time periods. Following treatment, cells were lysed and endogenous TRIM22 was immunoprecipitated using anti-TRIM22 antibody conjugated to Protein G-Sepharose. Bound levels of endogenous NOD2 were evaluated using Western blotting. As stimulation time with MDP increased, the binding of endogenous NOD2 to TRIM22 increased. Immunoprecipitation with IgG bound to Protein G-Sepharose was used as a negative IP control.

B) *TRIM22 interacts with NOD2.*

HEK293T cells were transiently co-transfected with HA-NOD2 and either wildtype (WT) or variant (R150T, S244L, R321K, and R442C) FLAG-epitope tagged TRIM22. Twenty-four hours post-transfection, cells were lysed followed by immunoprecipitation of the FLAG-TRIM22 by anti-FLAG-agarose. Reduced binding of NOD2 to TRIM22 was observed in varying degrees for each variant compared to the wildtype protein. HA-NOD1 and an empty vector construct were used as negative controls.

C) *Quantification of NOD2 co-immunoprecipitation with wild-type or mutant TRIM22.*

Densitometry analysis was performed on Western blots performed in 3B. Each TRIM22 point mutation significantly reduced NOD2 co-immunoprecipitation (Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, $n = 3$).

D) *TRIM22 mutants fail to activate NF- κ B and IFN β downstream of NOD2.* HCT116 cells were transiently co-transfected with (or without) NOD2, TRIM22-WT, or TRIM22 point mutants (R150T, S244L, R321K, R442C). Twenty-four hours post transfection, cells were stimulated with either MDP (10 $\mu\text{g}/\text{mL}$) or respiratory syncytial virus (RSV) (0.1 μg) for 18 hr before NF- κ B or IFN β promoter activation was evaluated by luciferase assay. Cells transfected with TRIM22 mutants showed significantly reduced NF- κ B and IFN β promoter activation following stimulation compared to wild type TRIM22 transfected controls (* $p < 0.001$ after Bonferroni post-hoc testing, $n = 3$).

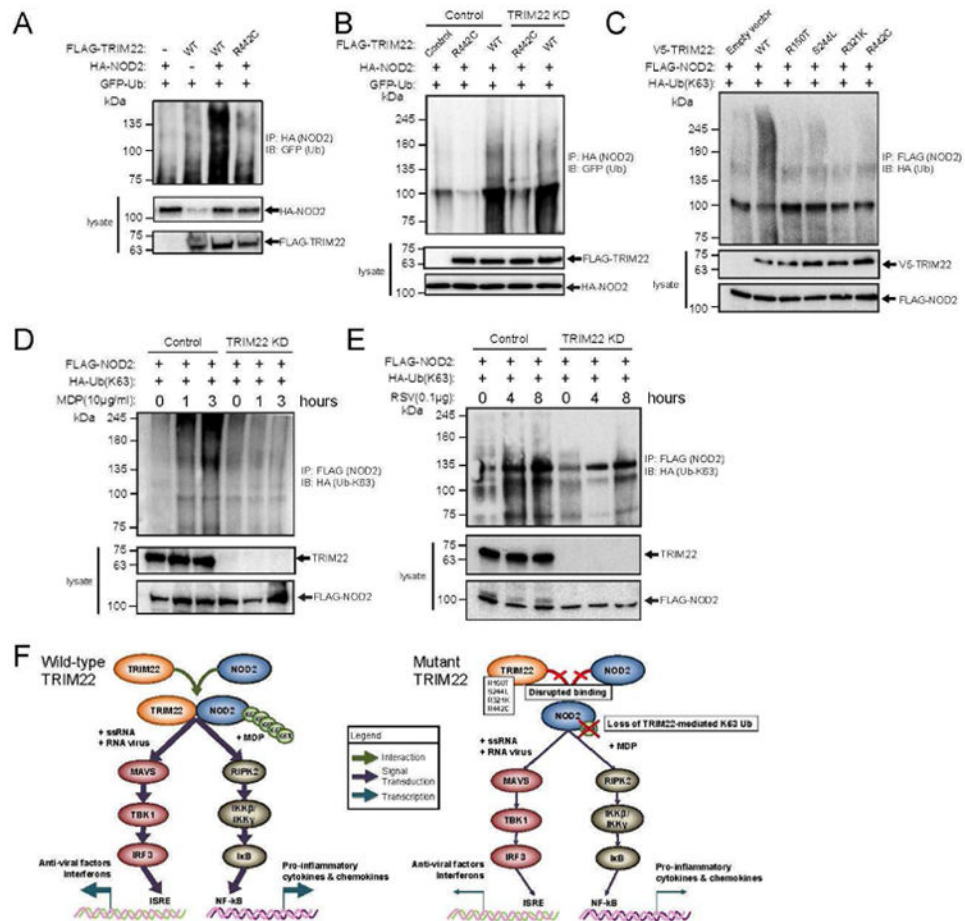


Figure 4. TRIM22 Mediates K63-linked NOD2 Polyubiquitination

A) TRIM22 enhances NOD2 polyubiquitination in HEK293 cells.

HEK293T cells were transiently co-transfected with HA-NOD2, GFP-ubiquitin, and either wildtype (WT) or variant (R442C) FLAG-TRIM22. Twenty-four hours post-transfection, cells were lysed followed by immunoprecipitation of HA-NOD2. Ubiquitination of NOD2 was evaluated by immunoblotting for GFP (ubiquitin). Co-transfection with wildtype TRIM22 enhanced ubiquitination of NOD2, which was abrogated with the R442C variant TRIM22. All experiments were carried out in triplicate, independent experiments.

B) Transgenic TRIM22 rescues NOD2 polyubiquitination in TRIM22-shRNA-HT29 cells.

HT29 cells stably transduced with control (scramble) or TRIM22 shRNA were transiently co-transfected with HA-NOD2 and either wildtype (WT) or variant (R442C) FLAG-TRIM22. Twenty-four hours post-transfection, cells were lysed followed by immunoprecipitation of the HA-NOD2. Evaluation of NOD2 poly-ubiquitination by GFP immunoblotting demonstrated rescued ubiquitination following transfection of WT-TRIM22 in the TRIM22 shRNA cell line that was not observed following transfection of the variant R442C. All experiments were carried out in triplicate, independent experiments.

C) Variants in TRIM22 abrogates K63-linked NOD2 polyubiquitination.

HEK293T cells were transiently co-transfected with FLAG-NOD2, HA-(K63-specific) ubiquitin, and either wildtype (WT) or variant (R150T, S244L, R321K, or R442C) V5-

TRIM22. Twenty-four hours post-transfection, cells were lysed followed by immunoprecipitation of the FLAG-NOD2. Evaluation of NOD2 K63 specific-ubiquitination by HA immunoblotting demonstrated significantly reduced ubiquitination in cells co-transfected with TRIM22 variants compared to wildtype. All experiments were carried out in triplicate, independent experiments.

D and E) *TRIM22-shRNA reduces MDP- and RSV-induced K63-linked NOD2 polyubiquitination.*

HEK293T cells stably transduced with control (scramble) or TRIM22 shRNA were transfected with HA-tagged K63-specific ubiquitin and FLAG-NOD2. Forty-eight hours after transfection, cell lysates were stimulated by MDP (D) or RSV (E) for indicated the time points followed by lysis and immunoprecipitation using anti-FLAG-agarose. K63-polyubiquitination of NOD2 was evaluated by immunoblotting using anti-HA antibody. Following stimulation by either MDP or RSV, NOD2 K63-specific ubiquitination increased over treatment time. However, in TRIM22 knockdown cells, this ubiquitination was significantly reduced or absent. All experiments were carried out in triplicate independent experiments and blots are representative.

F) *Schematic overview of the proposed impact of TRIM22 and its identified variants on the NOD2 signaling axis.*

Wildtype TRIM22 binds and mediates the K63-specific polyubiquitination of NOD2, potentiating downstream signaling through the MAVS and RIPK2 signaling axes depending on antigen. TRIM22 variants disrupt binding to NOD2, abrogating this polyubiquitination thus attenuating downstream pro-inflammatory signaling