

## Autophagy receptor CALCOCO2/NDP52 takes center stage in Crohn disease

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**Abbreviations:** CD, Crohn disease; NOD2, nucleotide-binding and oligomerization domain containing 2; PAMP, pathogen-associated molecular pattern; TLR, toll-like receptor; XBP1, X-box binding protein 1

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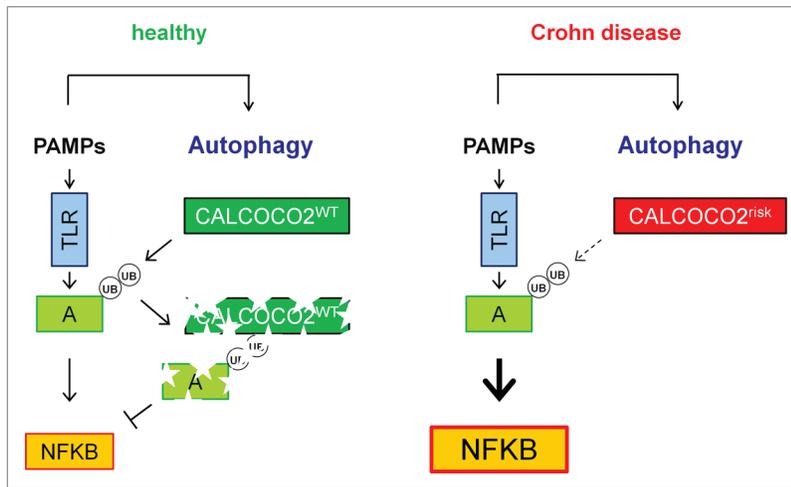
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To advance understanding of the complex genetics of Crohn disease (CD) we sequenced 42 whole exomes of patients with CD and five healthy control individuals, resulting in identification of a missense mutation in the autophagy receptor *calcium binding and coiled-coil domain 2* (*CALCOCO2/NDP52*) gene. Protein domain modeling and functional studies highlight the potential role of this mutation in controlling NFkB signaling downstream of toll-like receptor (TLR) pathways. We summarize our recent findings and discuss the role of autophagy as a major modulator of pro-inflammatory signaling in the context of chronic inflammation.

Crohn disease is a chronic inflammatory bowel disease presenting with recurring episodes of local inflammation that can affect all parts of the gastrointestinal tract. CD manifests as a consequence of a disturbed immune response to environmental triggers in susceptible individuals. The strong genetic component of the disease is documented by a high twin concordance rate and familial clustering. Based on its high prevalence in Western civilizations, lifelong manifestation and ensuing economic impact, CD is among the best-studied multifactorial inflammatory diseases. Collaborative efforts involving multicenter studies on several thousand individuals have revealed a total of 140 loci bearing genetic risk variants that predispose for CD. Pathway analyses of the proposed risk genes from susceptibility regions emphasize the principal role of distinct cellular mechanisms in disease manifestation:

These include epithelial barrier function, interleukin (IL) 23 signaling, pathogen sensing, ER stress and autophagy. The link between CD-susceptibility and autophagy is based on the identification of several autophagy core and mediator genes as pivotal risk factors, including *ATG16L1*, *LRRK2*, *IRGM* and *ULK1*. Moreover, several CD risk genes link cellular stress pathways to autophagy. Prominent examples are the pathogen-associated molecular patterns (PAMP) receptors, NOD2 and TLR4, and the ER stress regulator XBP1. Importantly, the spectrum of CD-associated autophagy-related genes uncovers the multifaceted nature of this pathway since several aspects and subtypes of autophagy are implicated in CD etiology, including general nonselective ('classical') autophagy, antibacterial xenophagy, IL1B production and secretion, antigen presentation and release of antimicrobial peptides by intestinal Paneth cells.

In our recent study (Ellinghaus et al.) we identified coding allelic variants contributing to CD susceptibility by means of whole-exome enrichment and subsequent next-generation sequencing (Fig. 1). We sequenced entire exomes of 42 unrelated subjects with CD and five healthy individuals, and then filtered single-nucleotide variants by incorporating association results from a previous meta-analysis of CD genome-wide association studies and in silico mutation effect predictions. We genotyped 9,348 patients with CD, 2,868 with ulcerative colitis, 14,567 controls, and associated variants were analyzed in functional studies using materials from patients, controls and in vitro model



**Figure 1.** Hypothetical model: Putative role of allelic variants of autophagy receptor CALCOCO2/NDP52 in susceptibility to Crohn disease. Under physiological conditions (left), the wild-type allele of CALCOCO2 is capable of inhibiting proinflammatory NFKB signaling by facilitating selective degradation of adaptors of TLRs or other PAMP receptors ('adaptophagy'). In CD, the risk allele of CALCOCO2 (Ala248, caused by a low-frequency missense mutation) fails to recognize polyubiquitinated adaptors, resulting in adaptor stabilization and excessive NFKB activity (right). A, adaptor; UB, ubiquitin chains.

systems. This approach identified two novel genes as genetic risk factors for CD: Whereas two rare single nucleotide variants were located within the gene *PR domain containing 1, with ZNF domain* (*PRDM1*, involved in regulating T cell proliferation), the second identified locus harbors a low-frequency missense mutation in the gene encoding the autophagy receptor CALCOCO2. Structural modeling of the CALCOCO2 coiled-coil region harboring the mutation suggested a potential impact of the Val248Ala substitution in affecting the structure and binding capacity of a proposed ubiquitin binding motif within this region. Functional analyses were performed for the two CALCOCO2 alleles using overexpression of fluorescently-tagged protein variants in human cell lines under varying conditions. One major focus of these experiments was to elucidate the role of CALCOCO2 variants in modulating proinflammatory signaling, based on the emerging concept that nonclassical autophagy, and receptors like CALCOCO2, are capable of dampening excessive NFKB signaling. After stimulating HeLa cells with the TLR3 agonist, poly(I:C), we found that overexpression of CALCOCO2 wild type (Val248), but not the mutant Ala248 allele, impaired NFKB activation. This is

important in the context of CD pathology since excessive NFKB activation is implicated as a major driver of chronic inflammation. We hypothesize that only wild-type CALCOCO2, but not the mutant form, represents a molecular circuit breaker that tunes down NFKB activation after receptor engagement. The proposed mechanism suggests that signaling adaptors downstream of TLRs (or other receptor families) might represent selective targets for a special type of selective autophagy that we name "adaptophagy" for purposes of this article. Previous studies have demonstrated adaptophagy of TLR adaptor TICAM1/TRIF (facilitated by CALCOCO2 and controlled by the signal modulator TNFAIP3/A20), and likewise the role of BCL10 degradation downstream of the T cell receptor, suggesting that other signaling cascades may also be fine-tuned by adaptophagy. The identity of the target proteins of this pathway, and the exact molecular mechanisms involved, remain to be unraveled.

Another finding may be functionally related to the aforementioned phenomenon or represent a distinct aspect of CALCOCO2 biology: Our data reveal that the stability of CALCOCO2 protein itself is affected by the polymorphic amino acid Val248Ala. While the

wild-type allele Val248 is destabilized and degraded after activation of TLR3 signaling by poly(I:C), the mutant variant is significantly more stable. This effect may represent a direct consequence of adaptophagy in that CALCOCO2 is degraded along with components of the TLR signaling cascade, just like autophagy receptor SQSTM1/p62 is degraded along with its protein aggregate cargos. Alternatively, CALCOCO2 itself may be polyubiquitinated after TLR engagement and degraded via the ubiquitin proteasome system. Indeed, the region surrounding amino acid 248 contains several lysine residues that may represent ubiquitination targets. Consequently, the mechanism of CALCOCO2 turnover will be a major aspect of future studies.

Our data do not exclude the possibility that the Val248Ala mutation affects other aspects of CALCOCO2 biology. Although we did not find apparent evidence for allele-specific differential colocalization of CALCOCO2 with the autophagy marker LC3 or intracellular bacteria, more detailed analyses may reveal subtle effects evoked by the polymorphism that could also support a role of the mutation in the context of pathogen sensing and defense mechanisms.

Taken together, our study for the first time identifies a low-frequency missense mutation in an autophagy receptor to be associated with susceptibility to Crohn disease. This highlights a potentially druggable mechanism that links noncanonical autophagy mediated by CALCOCO2 or other autophagy receptors to fine-tuning of proinflammatory signaling cascades.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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