Supplemental Material

Supplemental Methods

BN-PAGE. BN-PAGE (1) followed by Western blotting was performed using the NativePAGE™ Bis-Tris Gel System (Thermo Fisher) including 3-12% Bis-Tris gels, sample preparation buffer, running buffer, and transfer buffer according to the manufacturer's instructions. The resolved proteins were transferred to a polyvinylidene difluoride membrane and IL-23Rα protein was detected using anti-IL-23Rα (R&D Systems).

RNA isolation and RT-PCR. Total RNA was extracted using a guanidine thiocyanate procedure or prepared using RNeasy Mini Kit (Qiagen), and cDNA was prepared using oligo(dT) primer and SuperScript III RT (Invitrogen). Semiquantitative PCR was performed using *Taq* DNA polymerase as previously described (2). Cycle conditions were 94°C for 40 s, 60°C for 20 s, and 72°C for 40 s. Hypoxanthine phosphoribosyl transferase (*HPRT*) was used as housekeeping gene. Real-time quantitative PCR was performed using SYBR Premix Ex Taq II and a Thermal cycler Dice real-time system according to the manufacturer's instructions (TAKARA). Glyceraldehyde-3-phosphate (*GAPDH*) was used as housekeeping gene to normalize mRNA. Relative expression of real-time PCR products was determined by using the ΔΔCt method to compare target gene and housekeeping gene mRNA expression. The specific primer pairs for each gene in semiquantitative RT-PCR analysis used were as follows: *EBI3*, 5′-AACTCCACCAGATCCACGTC-3′ and 5′-GCGGAGTCGGTACTTGAGAG-3′; *p35*, 5′-ACCTCAGTTTGGCCAGGGTC-3′ and 5′-CAAGGCACAGGGTCATCATC-3′; *p28*, 5′-TGAGGTTCAGGGCTATGTCC-3′ and 5′-AGGGGCAGCTTCTTTTCTTC-3′; *HPRT*, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and 5′-GAGGGTAGGCTGGCCTATAGGCT-3′. The specific primer pairs for each gene in quantitative RT-PCR analysis used were as follows: *EBI3*, 5′-AGAGCCACAGAGCATGTCCAA-3′ and 5′-TGCACTCTGGGCTGGCTTAG-3′;

IL-23Rα, 5′-GAGATGCTCAGTGCTACAATCTTCA-3′ and 5′-AAAGCCACTTTGGGATCATCAG-3′; *IL-12Rβ1*, 5′-GATGGCTGCTGCGTTGAGAA-3′ and 5′-CATTGTCCTCAGGGCCATCATAC-3′; GAPDH, 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG -3′.

Reporter gene assay. HEK293T cells were transiently transfected by using FuGENE 6 with p3×GAS-Luc reporter construct (3) (firefly luciferase, kindly provided by Dr. R. Pine and Dr. J. J. O'Shea), p3×FLAG-CMV14-IL-23Rα, pME18S-IL-12Rβ1-HA, pCXN2-EBI3, and pRL-TK [sea pansy (*Renilla reniformis*) luciferase expression plasmid under control of thymidine kinase promoter] (Promega) as an internal control. The total amount of DNA was adjusted to be kept equal with empty vector. After approximately 40 h, half of the cells were harvested and used for Western blotting with antibodies against FLAG (M2), HA (6E2, Cell Signaling), EBI3 (Santa Cruz), calnexin, and actin, and the remaining half was stimulated with 20 ng/ml IL-23 for 6 h and then harvested for measurement of activities of firefly and sea pansy luciferases using a Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's instructions. Firefly luciferase activity in each sample was normalized by the corresponding sea pansy luciferase activity to correct transfection efficiency. Data are shown as normalized firefly luciferase activity.

Immunofluorescence. HEK293T cells were transiently transfected by using FuGENE 6 with p3×FLAG-CMV14-IL-23Rα, pCXN2-EBI3, and p3×Myc-CMV14-calnexin. The total amount of DNA was adjusted to be kept equal with empty vector. The transfected HEK293T cells grown in the Lab-Tek II 4-well chamber slide (Thermo Scientific) were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton at room temperature for 10 min each. For co-localization staining of IL-23Rα and EBI3 with the ER marker calnexin, 293T cells were incubated overnight with anti-IL-23Rα goat antibody (R&D Systems), anti-EBI3 rat antibody (Santa Cruz), and anti-calnexin

rabbit antibody (Santa Cruz) at 4°C. Then, the cells were incubated with a mixture of donkey antibody against goat IgG conjugated with Alexa Fluor 488 (Invitrogen), donkey antibody against rat IgG conjugated with Alexa Fluor 594 (Abcam), and donkey antibody against rabbit IgG conjugated with Alexa Fluor 647 (Abcam) for 2 h at 4°C. For nonpermeabilized staining of surface proteins, live transfected HEK293T cells were first labeled with antibodies for 1 h at 4°C, then incubated with secondary antibodies for 30 min at 4°C as described above, and fixed with Fixation Buffer (eBioscience) for 30 min. Finally, cytospins were prepared by seeding the cells on a cytospin funnel and spun at 500 rpm for 5 min onto a microscope slide. The cells were examined using a ×60 oil-immersion objective with confocal imaging (Olympus FV10I).

Small interfering RNA (siRNA) transfection. Human calnexin siRNA (AAGACGAUACCGAUGAUGAAA, AAUGUGGUGGUGCCUAUGUGA) (4) was synthesized by Sigma-Aldrich and negative control siRNA was purchased from Sigma-Aldrich. HEK293T cells were plated in 12-well plates and transfected with these siRNAs using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocols. After 24 h, these cells were also transfected with p3×FLAG-CMV14-IL-23Rα, pME18S-IL-12Rβ1-HA, and pCXN2-EBI3. The total amount of DNA was adjusted to be kept equal with empty vector. After another 48 h, total cell lysate was prepared and subjected to Western blotting.

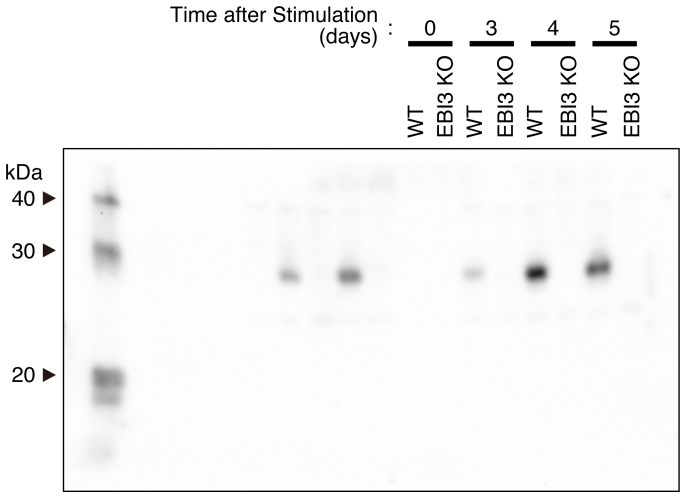
Preparation of calnexin knockout (KO) HEK293T cells by CRISPR/Cas9. Calnexin-specific single-guide RNA was designed as described previously (5). Its sequence was as follows: 5′-GCTTGGAACTGCTATTGTTG-3′, and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (5) (Addgene). Then, the plasmid was transfected into HEK293T cells and selected by puromycin (Thermo Fisher). Two cell lines, containing a single clone, were obtained, and the deletion of two nucleotides in

the exon II of calnexin genome was confirmed by sequencing. One of these clones was used in the present study.

References

1. Schagger H, and von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal Biochem.* 1991;199(2):223-231.
2. Yoshimoto T, et al. A pathogenic role of IL-12 in blood-stage murine malaria lethal strain Plasmodium berghei NK65 infection. *J Immunol.* 1998;160(11):5500-5505.
3. Pine R, Canova A, and Schindler C. Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. *EMBO J.* 1994;13(1):158-167.
4. Swanton E, High S, and Woodman P. Role of calnexin in the glycan-independent quality control of proteolipid protein. *EMBO J.* 2003;22(12):2948-2958.
5. Ran FA, et al. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc.* 2013;8(11):2281-2308.

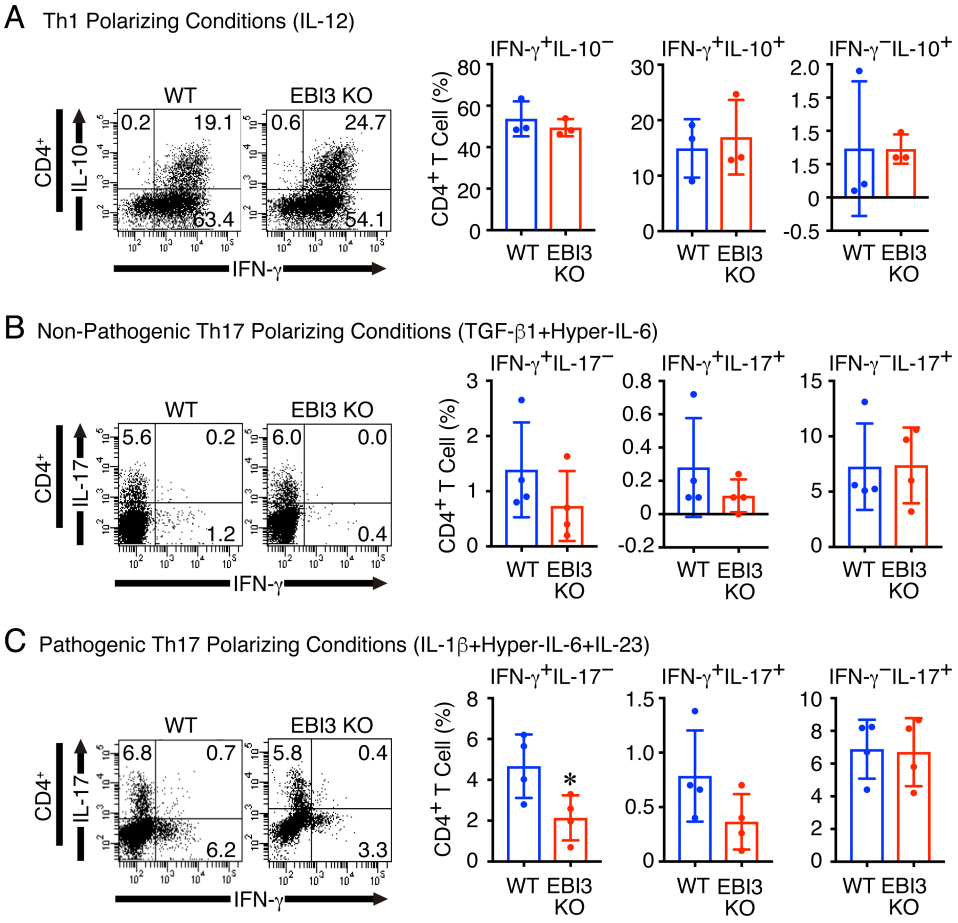
Supplemental Figures



Supplemental Figure 1

High specificity of antibody against EBI3 from Santa Cruz.

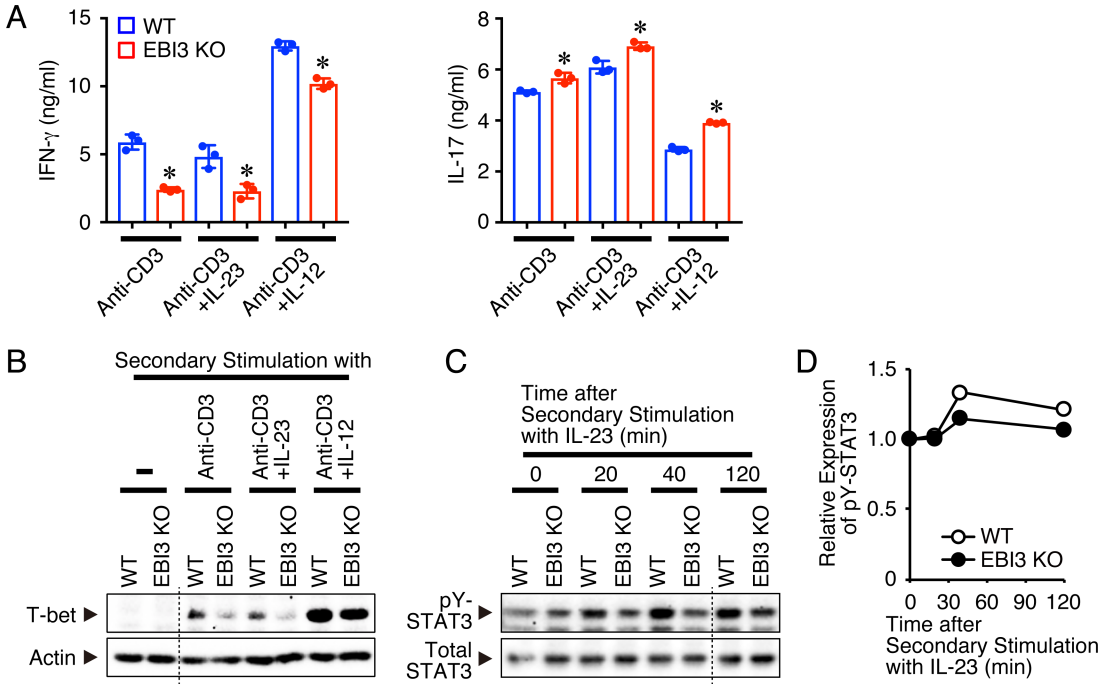
Naive $CD4^+$ T cells from WT mice or EBI3-deficient mice were stimulated with plate-coated anti-CD3 and anti-CD28 under pathogenic Th17 polarization conditions for the indicated time points. Resultant cell lysates were analyzed by Western blotting using anti-EBI3 purchased from Santa Cruz. A whole picture of the blot with molecular markers shows almost no non-specific binding with this antibody.



Supplemental Figure 2

Decreased IFN-γ production in EBI3-deficient CD4⁺ T cells differentiated under pathogenic Th17 polarizing conditions with IL-23 in vitro.

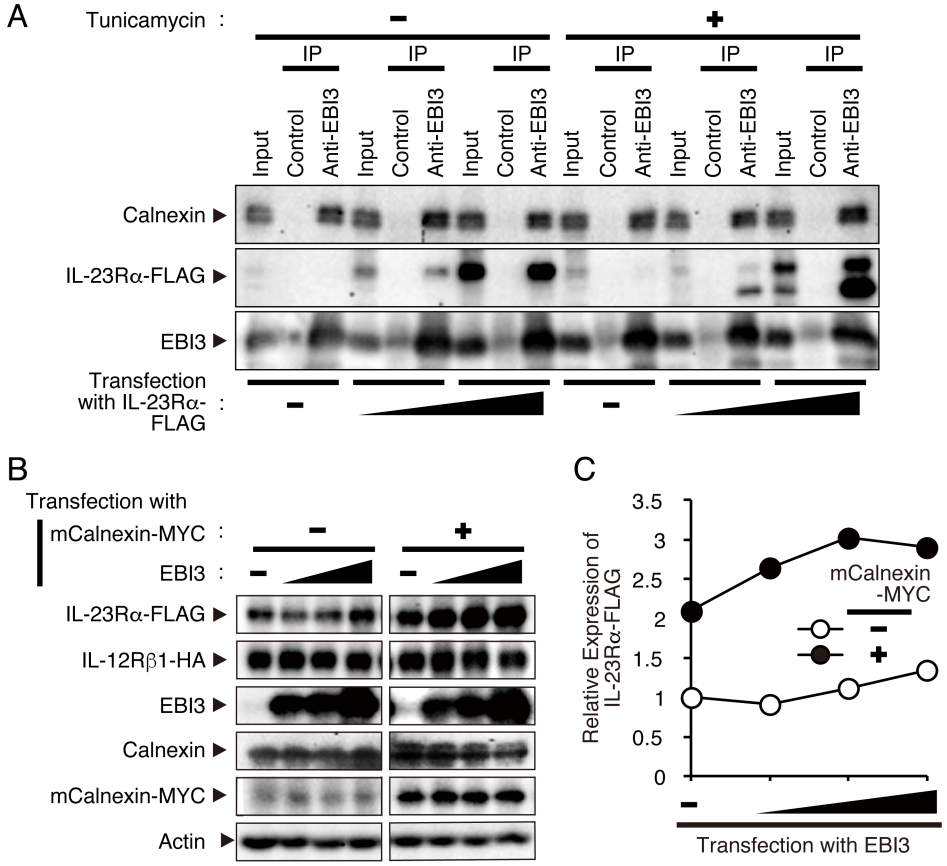
(A–C) Naive CD4⁺ T cells from WT mice or EBI3-deficient mice were stimulated with soluble anti-CD3 in the presence of irradiated spleen cells depleted of T and NK cells under Th1 (A), nonpathogenic Th17 (B), and pathogenic Th17 (C) polarization conditions for 4 days. These cells were restimulated with PMA and ionomycin, and the intracellular staining was performed. Representative dot plots of IFN-γ and IL-10 or IL-17 in CD4⁺ T cells are shown, and the frequencies of respective CD4⁺ T cells were calculated. Data are shown as mean ± SD (n = 3-4) and are representative of three independent experiments. *P* values were determined using unpaired, two-tailed Student's *t*-test. $*P < 0.05$.



Supplemental Figure 3

Decreased IFN-γ production in EBI3-deficient CD4⁺ T cells is due to reduced IL-23R signaling.

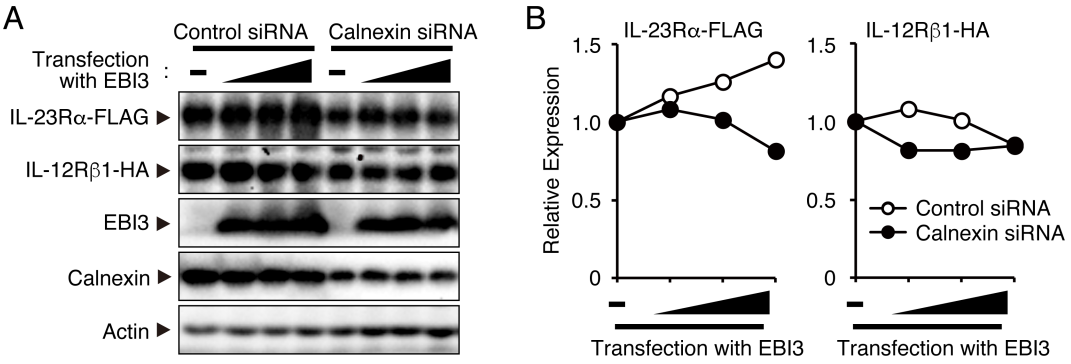
(A–D) Naive CD4⁺ T cells from WT mice or EBI3-deficient mice were stimulated with soluble anti-CD3 in the presence of irradiated spleen cells depleted of T and NK cells under pathogenic Th17 polarization conditions for 3 days and expanded with IL-2 for another 2 days. These cells were restimulated with plate-bound anti-CD3 in the presence or absence of IL-23 or IL-12 for 48 h. Cytokine production in the culture supernatant was measured by ELISA (A), and total cell lysate was prepared for Western blotting with anti-T-bet and actin (B). These cells were also stimulated with IL-23, and total cell lysate was prepared at the indicated times followed by Western blotting with anti-pY-STAT3 and anti-total STAT3 (C). The intensities of each band of pY-STAT3 and total STAT3 were densitometrically quantified, and the pY-STAT3 expression was normalized to total STAT3 and is shown as the relative expression respective to time 0 (D). Data are shown as mean ± SD (n = 3, A) and are representative of two independent experiments. *P* values were determined using unpaired, two-tailed Student's *t*-test (A). **P* < 0.05.



Supplemental Figure 4

EBI3 binds to calnexin and IL-23Rα in a peptide-dependent but not glycan-dependent manner and EBI3 and calnexin additively augment IL-23Rα protein expression.

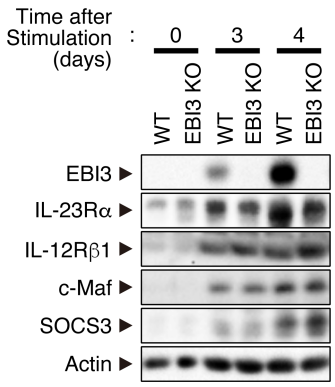
(A**)** HEK293T cells were transfected with expression vectors of IL-23Rα-FLAG and EBI3, and 48 h later cells were treated with tunicamycin or DMSO for further 6 h. Total cell lysate was then prepared and immunoprecipitated with anti-EBI3, followed by Western blotting with anti-calnexin, anti-FLAG, and anti-EBI3 as indicated. (B and C) HEK293T cells were transfected with expression vectors of IL-23Rα-FLAG, IL-12Rβ1-HA, EBI3, and mouse calnexin-MYC. After 72 h total cell lysate was prepared and subjected to Western blotting (B). The intensity of each band was quantified, and the expression of IL-23Rα was normalized to actin and is shown as the relative expression to untransfection without mouse calnexin (C). Data are representative of two independent experiments.



Supplemental Figure 5

Decreased IFN-γ production in EBI3-deficient CD4$^+$ T cells is due to reduced IL-23Rα signaling.

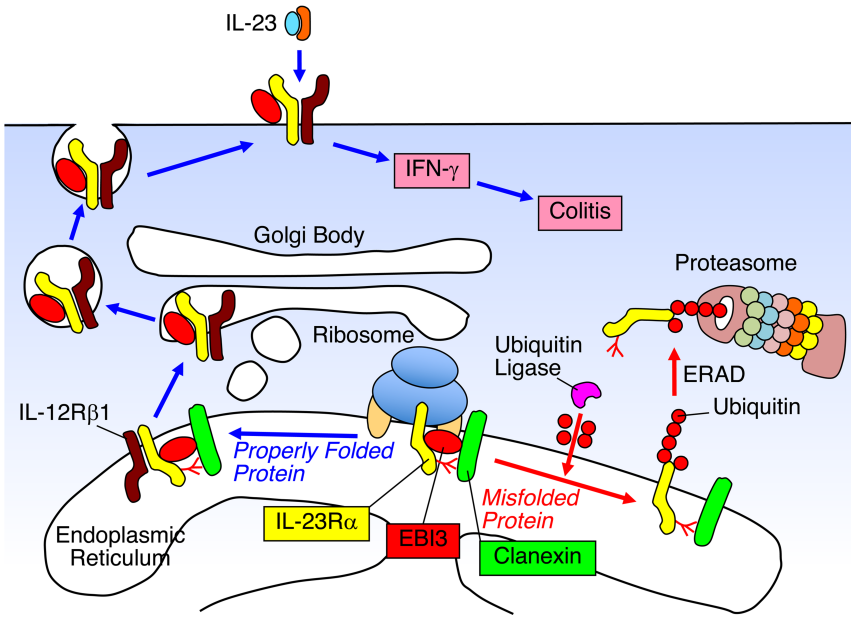
(A and B) HEK293T cells were transfected with siRNA specific to calnexin or control siRNA for 24 h, and further transfected with expression vectors of IL-23R-FLAG, IL-12Rβ1-HA, and EBI3. After 72 h, total cell lysate was prepared for Western blotting (A), and the intensity of each band was quantified, and the expression of IL-23Rα and IL-12Rβ1 was normalized to actin and is shown as the relative expression to respective untransfected cells (B). Data are representative of two independent experiments.



Supplemental Figure 6

No difference in the expression level of c-Maf and SOCS3 between WT and EBI3-deficient naive CD4$^+$ T cells stimulated under pathogenic Th17 conditions

Naive CD4$^+$ T cells from WT mice or EBI3-deficient mice were stimulated with plate-coated anti-CD3 and anti-CD28 under pathogenic Th17 polarization conditions. Cell lysates were then prepared after the indicated time points and subjected to Western blotting analysis for expression of c-Maf and SOCS3 in addition to EBI3, IL-23R□, IL-12R□1, and actin. Data are representative of two independent experiments.



Supplemental Figure 7

EBI3 promotes the proper protein folding of IL-23Rα by binding to calnexin and IL-23Rα, leading to development of colitis.

Calnexin expression is constitutive and ubiquitous, while EBI3 expression is inducible in response to ligation of T-cell receptor and toll-like receptor ligands. Under pathogenic Th17 polarizing conditions with IL-23, IL-23Rα protein is greatly synthesized and properly folded in association with IL-12β1 in the presence of EBI3, which directly binds to IL-23Rα and calnexin through peptide interaction, consequently followed by transportation to the cell surface, leading to the development of colitis. In marked contrast, in the absence of EBI3, the newly synthesized IL-23Rα protein is largely misfolded and degraded at the proteasome of ERAD after ubiquitination. Thus, EBI3 plays a critical role in the augmentation of IL-23Rα expression at the protein level, possibly through promoting the chaperone activity of calnexin.