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Supplemental Data

Germline Mutations in *NFKB2* Implicate the Noncanonical NF-κB Pathway in the Pathogenesis of Common Variable Immunodeficiency

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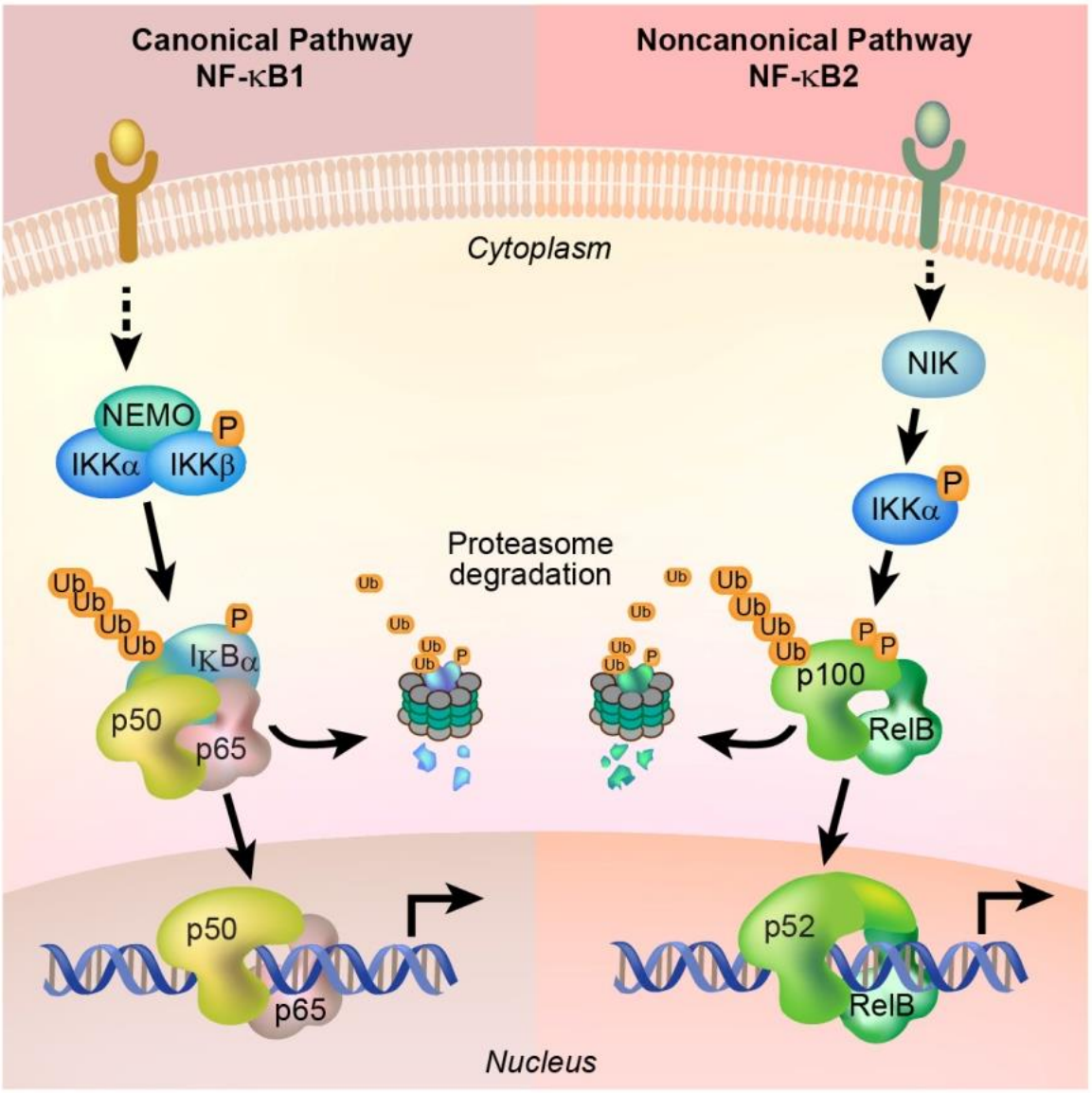

Figure S1. Canonical and noncanonical NF-κB pathways. Ligand mediated receptor activation of the NF-κB pathways on the cell surface leads to activation and nuclear translocation of NF-κB proteins into the nucleus where they bind their respective gene targets to initiate gene transcription and downstream cellular responses. The canonical pathway (left) is stimulated by a number of different ligand-receptor interactions. Receptor activation of the canonical pathway leads to stimulation of a kinase cascade that phosphorylates the IκB kinase (IKK) complex that consists of IKKα, IKKβ, and NF-κB essential modulator (NEMO, also known as IKKγ). IKKβ kinase phosphorylates the inhibitor IκBα, which normally sequesters NF-κB1 in the cytoplasm. Phosphorylation stimulates ubiquitination and subsequent proteasomal degradation of IκBα, resulting in release and nuclear localization of the p50/p65 dimer. The

p50/p65 dimer binds its genomic targets in the nucleus to initiate downstream inflammatory responses against infection. The noncanonical pathway (right) is stimulated by a limited number of ligand-receptor interactions. Receptors of this signaling pathway include BAFFR, RANK, lymphotoxin β receptor, and CD40. A key difference between the canonical and noncanonical NF-κB pathways is that while nuclear localization of p50/p65 is NEMO-dependent, the inactive form of NF-κB2, p100, acts as its own inhibitor, and activation of the noncanonical pathway is NEMO-*independent*. Receptor stimulation of the noncanonical pathway results in accumulation of the NF-κB inducing kinase (NIK), which, under non-activating conditions, is rapidly turned over in the cytoplasm. NIK is a member of the mitogen activated pathway 3 kinase family and phosphorylates the IKKα kinase upon its cytoplasmic accumulation. Activation of IKKα leads to phosphorylation of p100 at serines 866 and 870 and subsequent ubiquitination of lysine 855. Ubiquitination of p100 signals its processing by the proteosome to remove the C-terminus, forming p52. p52 in its heterodimeric configuration with RelB is then translocated into the nucleus where the active complex acts as a transcription factor to initiate downstream responses including peripheral lymphoid organogenesis, B-cell maturation, osteoclastogenesis, and thymic development.

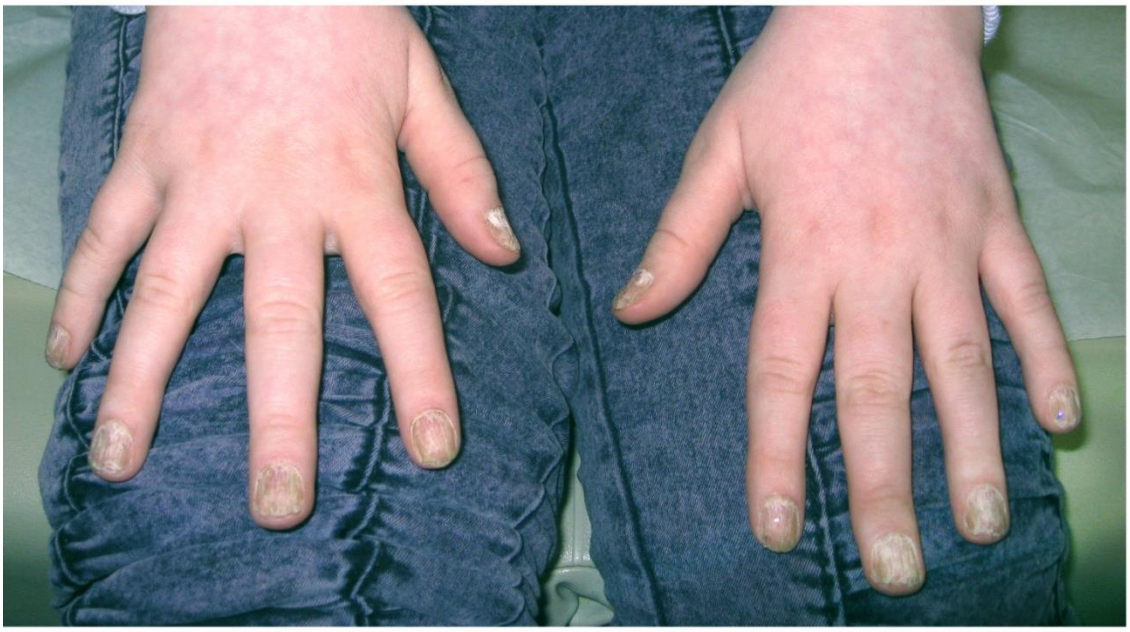

Figure S2. Fingernail dystrophy with onychomycosis seen in CVID-affected individual P2.

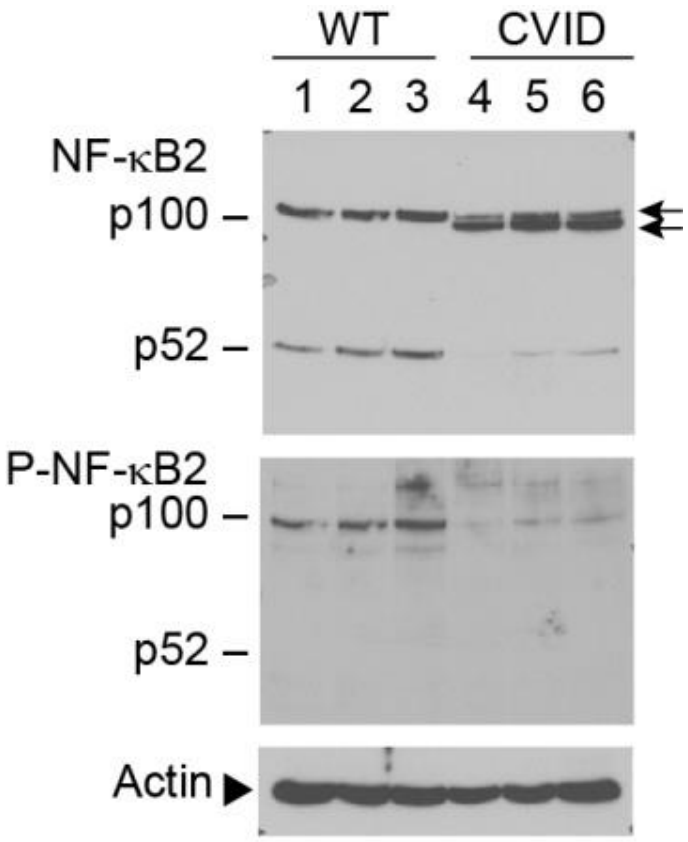

Figure S3. Mutant NF-κB2 proteins are defective for p100 phosphorylation. Western blot analysis from EBV-B cell whole cell lysates derived from unaffected (A.I.2 (lane 1), A.II.1 (lane 2), and pediatric control (lane 3)) and affected individuals (P2 (lane 4), P3 (lane 5), and P4 (lane 6)) with antibodies to NF-κB2, phosphorylated NF-κB2 (P-NF-κB2), and actin. Arrows indicate wildtype (100kDa) and mutant (94kDa) p100. Actin is shown as a loading control.

	Primer sequences 5'-3'
Sanger sequencing c.2564delA and c.2557C>T	Forward ACCTCATTCCTCTGTCTTCTC Reverse TGTCTTCCTTCACCTCTGCT
Long range PCR 9,648 bp amplicon	Forward 5AmMC6/CCTGGCCCGCTGGGAACCTGTCACTTC Reverse 5AmMC6/CCGAGAGCCCCTTTTTGTGATAA
Long range PCR 9,695 bp amplicon	Forward 5AmMC6/GGTGGATAAACACTTCATTTCCCTTCTCCTGAGCAG Reverse 5AmMC6/CAGATGGTGGGGTGGGCAGAAGGCAGAGAAGAG

Table S1. Primer sequences used in this study

Pedigree Number	Individual	SNP Concordance (%)
A.II.1	Father	99.2
P1	Mother	98.8
P2	Daughter	98.7
P3	Son	99.2

Table S2. SNP Concordance between Cytoscan HD array data and exome sequencing data.

	P2	P1	A.II.1	P3
Raw data (Gb)	13.2	13.8	15.9	16.1
Base quality >= 30 (%)	80%	82%	79%	77%
Data mapped to target region (Gb)*	4.0	3.4	4.4	3.9
Bases with 1x coverage*	98%	98%	98%	98%
Bases with 10x coverage*	95%	94%	95%	94%
Bases with 20x coverage*	91%	88%	91%	88%
Mean coverage*	92x	78x	100x	89x
SNVs detected (k)**	83.4	82.7	82.2	81.5
Indels detected (k)**	12.1	11.7	12.8	11.9

*after duplicate removal, base quality >=17 (infers 98% correct base calls), and mapping quality >=10
**Nimblegen capture target regions +/- 100 bp, variant quality score >= 4

Table S3. NGS data output for exome sequencing of Family A.

Filtering step	# of variants		
Total variants in each family member	~150,000		
Remove low quality and non-coding variants[a]	~22,000		
Remove common variants from daughter[b]	2,415		
Remove familial homozygous variants[c]	1,822		
Remove daughter's homozygous variants	1760		
Intersect and difference[d]	314		
Variants affecting protein function[e]	160		
Divide into MAF bins[f]	0-1%	1-2.5%	2.5-5%
	98	30	32
Prioritize by function	3	1	0
Variants with supporting mouse model	1	0	0

[a] Removed variants with base quality score $\leq$ 10, read depth < 8, and outside of exons ±10 bases.
[b] Removed variants with a 1000 Genomes minor allele frequency >5% in individual P2.
[c] The homozygous variants found in the father (A.II.1), mother (P1), and brother (P3) were removed from the daughter's (P2) variant set.
[d] The heterozygous variants from P1 and P3 were removed from the heterozygous variants in P2. The heterozygous variants from A.II.1 were removed from the heterozygous variants in P2.
[e] Only missense, nonsense, and frameshift variants were further analyzed.
[f] 1000 Genomes frequencies.

Table S4. Heuristic filtering of exomes from Family A.

Genomic Position	*NFKB2* gene Position	Genotype	Protein Change	dbSNP rs#	1000 Genomes Frequency	5400 Exomes Frequency	# of Alleles in Pool
104153698	5´UTR	c.-1928C>A					1
104154876	5´UTR	c.-750A>G					2
104155345	5´UTR	c.-281T>C		36226954	1.10%		1
104155520	Noncoding Exon 2	c.-106T>C					1
104155591	Intron 2	c.-73+38C>T					2
104155597	Intron 2	c.-73+44C>T					2
104155750	Intron 3	c.21+13G>A				0.28%	1
104156383	Intron 5	c.145-99T>G		11574845	25%		1
104156911	Intron 7	c.395+99A>G		12772374	16.80%		7
104157711	Intron 9	c.662-27T>G		7897947	28%	24%	17
104157947	Intron 10	c.767-22C>A		45487496	1.80%	1.80%	1
104158993	Intron 13	c.1118-52T>C					1
104158994	Intron 13	c.1118-51G>T					1
104159323	Intron 14	c.1328-11G>A					1
104160643	Intron 18	c.1969-61A>G					1
104160959	Exon 20	c.2094C>T	p.Asn698Asn	11574851	4.50%	3.70%	1
104161191	Intron 20	c.2224-15C>T			0.23%	0.03%	1
104161475	Intron 21	c.2294-27A>C		11574852	3.50%	2.30%	1
104161737	Intron 22	c.2466+63C>T					1
104161796	Intron 22	c.2467-9T>A			8.70%	5.40%	1
104161895	**Exon 23**	**c.2557C>T**	**p.Arg853***				**1**
104162365	3´UTR	c.*232A>C		41371753	5.50%		1
104162682	3´UTR	c.*549G>A					1

Table S5. Results of *NFKB2* Sequencing in Simplex CVID Affected Individuals. The genomic position and gene position are shown along with the genotype. The protein change, if applicable, is also shown. RS numbers were obtained from the dbSNP database, and frequency information was obtained from the 1000 genomes and 5400 exomes databases. The number of times the allele was detected in the pool is listed. For example, a variant present in one allele corresponds to a heterozygous variant in one individual in the sample pool.

VAAST Rank	Gene	VAAST p-value	VAAST Score
1	*COL4A6*	4.34E-09	43.56
2	*PNMA3*	4.34E-09	43.56
3	*FAAH2*	4.34E-09	43.56
4	*NFKB2*	4.34E-09	41.98

Table S6. VAAST analysis of Family A. VAAST analysis[1] of genes containing variants from all affected individuals in Family A (P1, P2, and P3) not present in A.II.1 are shown along with VAAST rank, p-value, and VAAST score.

VAAST Rank	Gene	VarBin Annotation	VAAST p-value	VAAST Score
1	*NFKB2*	True Variant	1.56E-11	55.75
2	*RP1L1*	False Positive	1.56E-11	52.14
3	*MUC4*	False Positive	1.56E-11	49.48

Table S7. VAAST analysis of Family A and P4. VAAST analysis[1] of genes containing variants from all affected individuals (P1, P2, P3, and P4) not present in A.II.1 are shown along with VAAST rank, p-value, and VAAST score. VarBin annotation (personal communication) of the variant as a False Positive or True Variant is shown.

Peripheral lymphoid organs	T-cells	B-cells	Other
***Nfkb2*$^{-/-}$** **no p100/p52** **(Caamaño et al., 1998)[2]**			
Spleen: disrupted architecture Marginal zone: absent Lymph node: disrupted architecture Germinal center: absent Peyers patches: absent	αCD3, Con A: ↑IL-2 ↑ splenic numbers	LPS, αCD40, αIgD: ↓ Proliferation ↓ Antigen-specific antibodies	
***Nfkb2 Lym1*$^{-/-}$** **Y868X; p52 deficiency** **(Tucker et al., 2007)[3]**			
Spleen: disrupted architecture, enlarged Marginal zone: absent Lymph node: disrupted architecture Germinal center: absent Peyers patches: absent	↑CD4 and CD8 T cells	LPS, αCD40: ↓Proliferation ↓Mature peripheral B cells ↓Antibody levels	Inflammatory lung and liver infiltrates. Nfkb2 Lym1 -/+ with intermediate phenotype.

Table S8. Characteristics of *Nfkb2* mouse models

Individual	Gene	Chr	Position	Variant	Zygosity	Classification	MAF	Coding Change	dbSNP rsID
P4	*LHX3*	9	139092571	C>T	Het	Synonymous	1.67%	c.123G>A	33998096
P4	*LHX4*	1	180235662	C>T	Het	Synonymous	1.09%	c.384C>T	141139762

Table S9. Variants in genes associated with adrenal insufficiency in our cohort.

Observations	***Lym 1/+***	***Lym1/Lym1***
Survival	~75-80% survival at 250 days (compared to ~90% in the wildtype)	0% survival at 250 days
Fertility	Fertile	Reduced fertility with less frequent litters and smaller litter size
Lymph nodes and Peyer's patches	Absence of peripheral LN Mesenteric LN present but reduced in size and cellularity Absence of Peyer's patches	Absence of peripheral LN, mesenteric LN, as well as Peyer's patches
Splenic architecture	Intermediate disruption in spleen architecture compared to Lym1/Lym1 and wildtype	Disorganized spleen architecture
Organ inflammatory infiltrates	Lung/liver inflammatory cell infiltrates with smaller foci of inflammatory cell infiltrates compared to Lym1/Lym1	Lung/liver inflammatory cell infiltrates with extensive, large foci of T cells, B cells and macrophages. Progressive inflammation with age.
Osteoclast formation and osteopetrosis	Reduced osteoclast formation in response to RANKL stimulation. Osteopetrosis via histomorphometric measurements were similar to Lym1/Lym1	Severely reduced (<0.5% compared to wildtype) osteoclast generation after RANKL stimulation. Presence of osteopetrosis.
Western blotting of splenic B cells	Cytoplasm: Very little p52 detected in lysates from untreated or αCD40 stimulated splenic B cells. No reduction or significant accumulation in prescursor p100 levels after stimulation.	Cytoplasm: Mutant p100 protein accumulated in the cytoplasm in response to αCD40 stimulation. No p52 detected.
	Nucleus: minor increases in p52 post stimulation.	Nucleus: no p52 observed

LN = lymph nodes; RANKL = receptor activator of NF-κB ligand

Table S10. Differences within the *Nfkb2*$^{Lym1/+}$ and *Nfkb2*$^{Lym1/Lym1}$ mouse models as described in Tucker et al.[3]

REFERENCES

1. Yandell, M., Huff, C., Hu, H., Singleton, M., Moore, B., Xing, J., Jorde, L.B., and Reese, M.G. (2011). A probabilistic disease-gene finder for personal genomes. Genome Res 21, 1529-1542.
2. Caamano, J.H., Rizzo, C.A., Durham, S.K., Barton, D.S., Raventos-Suarez, C., Snapper, C.M., and Bravo, R. (1998). Nuclear factor (NF)-kappa B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. J Exp Med 187, 185-196.
3. Tucker, E., O'Donnell, K., Fuchsberger, M., Hilton, A.A., Metcalf, D., Greig, K., Sims, N.A., Quinn, J.M., Alexander, W.S., Hilton, D.J., et al. (2007). A novel mutation in the Nfkb2 gene generates an NF-kappa B2 "super repressor". J Immunol 179, 7514-7522.