Supplemental Material

Heterozygous germline *CSF3R* variants as risk alleles for development of hematologic malignancies

Supplemental Methods

Candidate germline predisposition gene identification strategy

Gene variants likely to be of germline origin were prioritized for further study based on their persistence at a VAF $\geq$ 0.4 over multiple tests. This VAF cut-off was chosen based on previous work showing that VAFs > 0.4 on OncoPlus testing of hematologic malignancies is predictive of germline origin.[1]

CSF3R (transcript NM_000760.3) variants were ranked by predicted pathogenicity using standard American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) criteria.[2] Germline variants most likely to exhibit deleterious function, including pathogenic, likely pathogenic, and variants of uncertain significance (VUSs) that fulfilled pre-set criteria, were prioritized and selected for functional testing. The pre-set criteria were designed to aid in identifying which VUSs were most likely to be pathogenic and included: total population variant frequency < 0.001 in gnomAD (v2.1.1), since a minor allele frequency cut-off of 0.001 is recommended for autosomal dominant Mendelian disease gene discovery;[3] a REVEL score[4] ≥ 0.4; and a greater number of damaging compared to benign *in silico* functional predictions among the DANN, DEOGEN2, EIGEN, FATHMM, FATHMM-MKL, FATHMM-XF, GERP, LRT, M-CAP, MutationAssessor, MutationTaster, PROVEAN, PrimateAI, SIFT, and SIFT4G functional prediction algorithms. Individual scores for these functional prediction algorithms were obtained from the VarSome genomic search engine.[5] REVEL is an ensemble method that integrates scores from MutPred, FATHMM, VEST 3.0, PolyPhen-2, SIFT,

PROVEAN, MutationAssessor, MutationTaster, LRT, GERP++, SiPhy, phyloP, and phastCons. Although a cut-off of 0.5 is commonly used for the Revel score, there is no established threshold for individual genes, such as *CSF3R*. In this case, a cut-off of 0.4 was chosen, because p.Thr618Ile, a well-studied and known pathogenic *CSF3R* variant and the most common variant found in CNL has a REVEL score of 0.41.[4] A Revel score cut-off of 0.4 has a sensitivity of 0.82 and specificity of 0.84 (compared to a sensitivity of 0.76 and specificity of 0.89 for a cut-off of 0.5).[4]

Testing for germline status

Skin fibroblasts were cultured in AmnioMAX C-100 containing AmnioMAX C-100 supplement (Gibco). MSCs were cultured in MEM-α containing GlutaMAX (Gibco) with 20% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. PCR products were purified (Genelute PCR clean-up kit; Sigma-Aldrich) and sequenced (Applied Biosystems 3730XL 96-capillary and 3130 16-capillary automated DNA sequencers) to test the germline nature of the variants.

Generation and characterization of CSF3R expressing cell lines

Construct fidelity was confirmed by Sanger sequencing. Ba/F3 cells were transfected by electroporation, grown in G418 (1.0 mg/ml), and CSF3R positive cells sorted by FACS and maintained as previously described.[6] Proliferation of Ba/F3 cells expressing mutant or WT CSF3R in response to varying concentrations of G-CSF was quantified using the CellTitre-Glo Luminescent assay (Promega). Results were normalized to the values obtained in the absence of G-CSF. CSF3R surface expression and internalization were analyzed by flow cytometry as previously described.[7] Receptor expression and ligand induced STAT activation were examined by immunoblot analysis using the following antibodies: CD-114 to detect CSF3R (BD Biosciences #554536), beta-actin (Santa Cruz Biotechnology #sc-81178), Phospho-STAT3 (Cell

Signaling #9131s), STAT3 (Cell Signaling #9139s), Phospho-STAT5 (Cell Signaling #9351s), and STAT5 (Cell Signaling #94205s).

CSF3R mRNA transcript expression in primary cells

Polymorphonuclear cells (PMNs) and monocytes were isolated from whole blood by negative selection using immunomagnetic beads (Stemcell Technologies #19666 and Stemcell Technologies #19059, respectively). For analysis of nonsense mediated decay, monocytes were incubated with or without 50 ug/mL cycloheximide for 3 hours. RNA was extracted using TRIzol and total *CSF3R* transcript levels were quantified by TaqMan qRT-PCR (assay ID: Hs01114427_m1).

Supplemental Data

Supplemental Table 1. Genes included in OncoPlus next-generation sequencing test.

Genes analyzed for SNVs, insertions and deletions	Genes analyzed for CNVs	Genes analyzed for fusions/translocations
ABL1, AKT1, ALK, APC, ARID1A, ARID2, ASXL1, ATM, ATR, ATRX, AXL, B2M, BAP1, BCOR, BCORL1, BIRC3, BLM, BRAF, BRCA1, BRCA2, BTK, CALR, CBL, CBLB, CCND1, CCND2, CCND3, CDH1, CDK4, CDK6*, CDKN2A, CEBPA, CHEK1, CHEK2, CSF1R, CSF3R, CTCF, CTNNA1, CTNNB1, CUX1, CXCR4, DAXX, DDR2, DDX3X, DDX41, DICER1, DNMT3A, EGFR, EP300, EPHA3, EPHA5, ERBB2, ERBB3, ERBB4, ERCC3, ESR1, ETV6, EZH2, FANCA, FAT3, FBXW7, FGFR1, FGFR2, FGFR3, FH, FLT3, FOXL2, GATA1, GATA2, GNA11, GNAQ, GNAS, GRIN2A, H3F3A, HIST1H3B, HIST1H3C, HNF1A, HRAS, IDH1, IDH2, IKZF1, ITPKB, JAK2, KDM6A, KDR, KIT, KMT2A, KRAS, MAP2K1, MAPK1, MDM2*, MET, MLH1, MLH3, MPL, MRE11A, MSH2, MSH6, MTOR, MYC*, MYCN*, MYD88, NBN, NF1, NF2, NFE2L2, NOTCH1, NOTCH2, NPM1, NRAS, PALB2, PBRM1, PDGFRA, PDGFRB, PHF6, PIK3CA, PIK3CB, PIK3R1, PLCG2, POLE, POT1, PPP2R1A, PTCH1, PTEN, PTPN11, RAD21, RAD51, RB1, RET, RUNX1, SDHB, SDHC, SDHD, SETBP1, SF3B1, SMAD4, SMARCB1, SMC1A, SMC3, SMO, SRSF2, STAG2, STAT3*, STAT5B*, STK11, TERT (promoter only), TET2, TP53, TSC1, TSC2, U2AF1, VHL, WT1, ZRSR2*	*ABL1, AKT1, ALK, APC, ARID1A, ARID2, ASXL1, ATM, ATR, AXL, B2M, BAP1, BIRC3, BLM, BRAF, BRCA1, BRCA2, CALR, CBL, CBLB, CCND1, CCND2, CCND3, CDH1, CDK4*, CDK6*, CDKN2A, CEBPA, CHEK1, CHEK2, CSF1R, CSF3R, CTCF, CTNNA1, CTNNB1, CUX1, CXCR4, DAXX, DDR2, DDX41, DICER1, DNMT3A, EGFR, EP300, EPHA3, EPHA5, ERBB2, ERBB3, ERBB4, ERCC3, ESR1, ETV6, EZH2, FANCA, FAT3, FBXW7, FGFR1, FGFR2, FGFR3, FH, FLT3, FOXL2, GATA2, GNA11, GNAQ, GNAS, GRIN2A, H3F3A, HIST1H3B, HIST1H3C, HNF1A, HRAS, IDH1, IDH2, IKZF1, ITPKB, JAK2, KDR, KIT, KMT2A, KRAS, MAP2K1, MAPK1, MDM2*, MET, MLH1, MLH3, MPL, MRE11A, MSH2, MSH6, MTOR, MYC*, MYCN*, MYD88, NBN, NF1, NF2, NFE2L2, NOTCH1, NOTCH2, NPM1, NRAS, PALB2, PBRM1, PDGFRA, PDGFRB, PIK3CA, PIK3CB, PIK3R1, PLCG2, POLE, POT1, PPP2R1A, PTCH1, PTEN, PTPN11, RAD21, RAD51, RB1, RET, RUNX1, SDHB, SDHC, SDHD, SETBP1, SF3B1, SMAD4, SMARCB1, SMC3, SMO, SRSF2, STAT3*, STAT5B*, STK11, TERT, TET2, TP53, TSC1, TSC2, U2AF1, VHL, WT1*	*ALK, RET, ROS1*

CNV, copy number variant; SNV, single nucleotide variant. *Additional genes added to the OncoPlus panel in 2018.

Supplemental Table 2. PCR primers.

Gene	Variant(s)	Forward (5' - 3')	Reverse (5' - 3')	Annealing temperature, ºC
CSF3R	p.Thr420Ala, c.1258A>G	TTGACCTCTGTGCTCTTCTGG	TGTGGTTTCTTGGAGACCCTC	58
CSF3R	p.Gln739* c.2215C>T; p.Pro784Thr, c.2350C>A; p.Glu808Lys c.2422G>A	CAGGAGGGGGAAGTTGAGC	CACAGTGCTGGAGGAGGATG	60
CSF3R	p.Trp547*, c.1640G>A	AGGGGTGTACGGTCAGCATA	GAAGCCACAAGAAGTCCAACC	62
CSF3R	p.Ala119Thr, c.355G>A	TTAGAGCCACACTGCCTCCAT	TCTATCATCACCCTGCCCCAC	62
CSF3R	p.Thr618Ile, c.1853C>T, p.Arg583Cys c.1747C>T	TCTCCTCCCTCCGACCAG	TATGCTGACCGTACACCCCT	58
BRCA2	p.Arg2892Thrfs*14, c.8673_8674del (NM_000059.3)	GGGTGTTTTATGCTTGGTTCT	CATTTCAACATACTCCTTCCTG	55

Supplemental Table 3. Mutagenic oligonucleotides*

***CSF3R* variant**	**Mutagenic forward primer (5' - 3')**
p.Pro784Thr	CTTGGCGGGCCTCACTCCCAGCACCAAGTCCTATGAGAAC
p.Trp547*	CTGGGCACAGCTGGAGTAGGTGCCTGAGCC
p.Ala119Thr	GGTTGAGCTGCGCACAGGCTACCCTCC
p.Thr640Ile	TTCGGCCTCCTGCTGTTGCTCATCTGCCTCTGTGGAACTG
p.Arg583His	ATCCTGAATGCCTCCTCCCATGGCTTTGTCCTCCAT

*Note: A reverse complement primer of the forward primers was also used for each mutagenesis.

Supplemental Table 4. Disease phenotypes of patients with germline or possible germline *CSF3R* variants.

CSF3R variant	Cohort germline status	Disease phenotype
p.Lys49Arg c.146A>G	unconfirmed	AML
p.His94Arg c.281A>G	unconfirmed	Pancytopenia with mild megakaryocytic dysplasia*
p.Ala119Thr c.355G>A	Germline	Multiple myeloma
		B-cell ALL (Ph negative)
p.Gln216His c.648A>C	unconfirmed	Primary myelofibrosis
		CML
p.Thr420Ala c.1258A>G	Germline	Chronic lymphoproliferative disorder of NK cells
p.Thr486Met c.1457C>T	Germline	Primary myelofibrosis
p.Trp547* c.1640G>A	Germline	t-MDS & prior bladder cancer
		Hypoplastic MDS single lineage dysplasia
p.Ala603Ser c.1807G>T	unconfirmed	Lymphoma
p.Thr640Ile c.1919C>T	Germline	AML
		Plasma cell leukemia
	unconfirmed	Polycythemia vera
		Multiple myeloma
		Neutropenia and thrombocytopenia with myeloid maturation arrest
p.Arg698Cys c.2092C>T (NM_156039.3)	unconfirmed	AML
		AML
		Amyloidosis
p.Pro784Thr c.2350C>A	Germline	Multiple myeloma
p.Pro803Ser c.2407C>T	unconfirmed	t-AML & CLL
		t-MDS & prior lymphoma
		Multiple myeloma and colon cancer
		Prefibrotic myelofibrosis
p.Val812Ile c.2434G>A	unconfirmed	CML

Only variants classified by ACMG criteria as pathogenic, likely pathogenic, or VUS are included. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome, t-AML, therapy-related AML; t-MDS, therapy-related MDS. *Did not meet diagnostic criteria for MDS.

Supplemental Table 5. Clinical information for patients with germline *CSF3R* variants selected for assessment of oncogenic potential.

CSF3R variant	Disease	At diagnosis							Treatment history	Family history of malignancy
		Age, yrs	Hgb, g/dL	Platelets, x10^3/uL	WBC, x10^3/uL	ANC, x10^3/uL	Other pathogenic variants on Oncoplus	VUSs on Oncoplus		
p.Trp547* c.1640G>A	t-MDS & prior bladder cancer	76	8.8	87	6.1	4.3	*ASXL1* c.1934dup, p.G646Wfs*12 (NM_015338.5) – VAF: 29.4% *BRCA2* c.8673_8674del, p.R2892Tfs*14 (NM_000059.3) – VAF: 48.6% *CEBPA* c.766_769dup, p.G257Afs*65 (NM_004364.4) – VAF: 33.2% *SRSF2* c.284C>A, p.P95H (NM_003016.4) – VAF: 41.3% *STAG2* c.1997del, p.N666Tfs*26 (NM_001042749.2) – VAF: 71.6% *TET2* c.1968dup, p.S657Lfs*24 (NM_001127208.2) - VAF: 33.1%	*FGFR3* c.1156T>C, p.F386L (NM_001163213.1) – VAF: 48.1% *ITPKB* c.2740G>A, p.V914I (NM_002221.3) – VAF: 49.9% *NBN* c.511A>G, p.I171V (NM_002485.4) – VAF: 43.2% *NOTCH2* c.4996G>A, p.V1666I (NM_024408.3) – VAF: 51.1% *RET* c.2372A>T, p.Y791F (NM_020975.5) – VAF: 51.5%	Neoadjuvant MVAC for bladder ca (2017). Azacitidine for t-MDS (ongoing)	Mother: breast cancer diagnosed in her 70's
p.Trp547* c.1640G>A	Hypoplastic MDS Single Lineage Dysplasia	33	9.4	48	2.86	0.83	nd	nd	initially treated as AA (ATG/CSA/Methylprednisolone with no response). Diagnosis revised to hypoplastic MDS and treated with Azacitidine followed by alloSCT	None
p.Pro784Thr c.350C>A	Multiple myeloma	67	10.4	186	7.9	3.3	*KRAS* c.183A>C, p.Q61H (NM_033360.3) - VAF: 18%	*ATR* c.5739-11_5739-4del, p.? (NM_001184.3) - VAF: 44% *DDX41* c.1706G>A, p.G569E (NM_016222.3) - VAF: 18% *FOXL2* c.469C>G, p.P157A (NM_023067.3) - VAF: 53% *PALB2* c.13C>T, p.P5S (NM_024675.3) - VAF: 46% *PDGFRA* c.3155C>T, p.T1052M (NM_006206.5) - VAF: 48% *POLE* c.2683G>A, p.A895T (NM_006231.3) - VAF: 54%	KRd x 4 cycles, followed by ASCT (2018) & KRd consolidation (ongoing)	None
p.Ala119Thr c.355G>A	Multiple myeloma	57	8.7	287	6	4.1	None	*FGFR2* c.1141A>G, p.I381V (NM_022970.3) – VAF 45.7% *PIK3CA* c.1850G>A, p.R617Q (NM_006218.3) – VAF 52.1%	CyBorD x 1, then KRd x 5 cycles. Followed by VD-PACE x 2, ASCT, and VPd consolidation (ongoing)	Paternal Grandfather: hepatic cancer

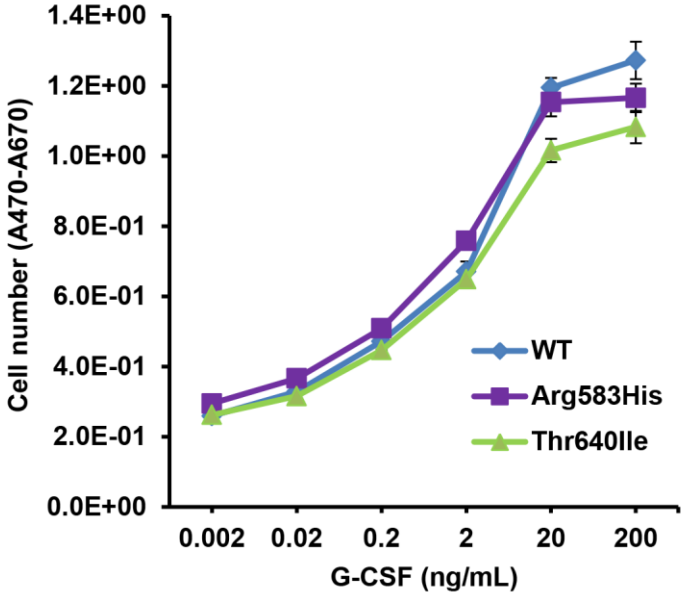
p.Ala119Thr c.355G>A	B-cell ALL (Ph negative)	58	13.1	31	40.2	1.2	*JAK2* c.2047A>G, p.R683G (NM_004972.3) *JAK2* c.2078_2080del, p.P693_F694delinsL (NM_004972.3) – VAF 35.3%	*EGFR* c.1350A>C, p.L450F (NM_005228.3) – VAF 48.4%	HyperCVAD, blinatumomab, attenuated VAD, and CAR-T cells (2016). Monthly IVIG (ongoing)	Unknown

Highlighted variants were confirmed germline in origin through sequencing of cultured skin fibroblasts and/or cultured mesenchymal stromal cells. AA, aplastic anemia; ALL, acute lymphoblastic leukemia; AlloSCT, allogeneic stem cell transplant; ANC, absolute neutrophil count; ASCT, autologous stem cell transplant; ATG, anti-thymocyte globulin; CAR-T, chimeric antigen receptor T cells; CVAD, cyclophosphamide, vincristine, doxorubicin adriamycin, and dexamethasone; CyBorD, cyclophosphamide, bortezomib, and dexamethasone; Hgb, hemoglobin; IVIG, intravenous immunoglobulin; KRd, carfilzomib (Kyprolis), lenalidomide (Revlimid), and dexamethasone; MVAC, methotrexate, vinblastine, adriamycin and cisplatin; nd, not done; t-MDS, therapy-related myelodysplastic syndrome; VAD, vincristine, doxorubicin and dexamethasone; VD-PACE, bortezomib, dexamethasone, cisplatin, doxorubicin (Adriamycin), cyclophosphamide, etoposide; VPd, bortezomib, pomalidomide, dexamethasone; VUS, variant of uncertain significance; WBC, white blood cell count.

Supplemental Table 6. One-hundred sixty genes evaluated as part of an inherited bone marrow failure panel called MarrowSeq.

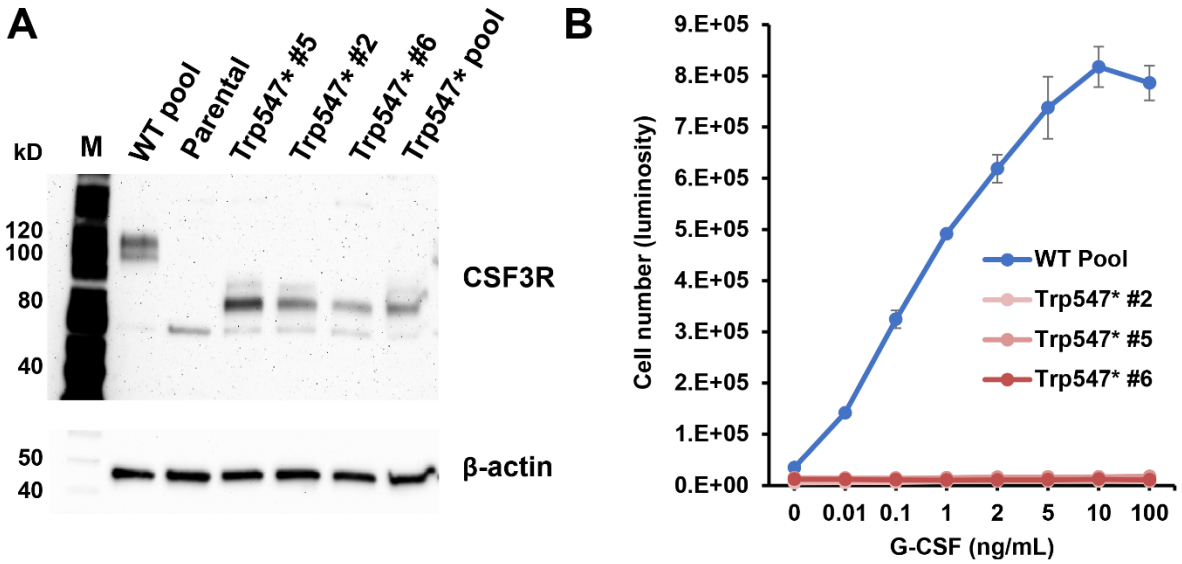
Genes analyzed for SNVs, insertions and deletions
ABL1, AKT1, ALK, APC, ARID1A, ARID2, ASXL1, ATM, ATR, ATRX, AXL, B2M, BAP1, BCOR, BCORL1, BIRC3, BLM, BRAF, BRCA1, BRCA2, BTK, CALR, CBL, CBLB, CCND1, CCND2, CCND3, CDH1, CDK4, CDK6, CDKN2A, CEBPA, CHEK1, CHEK2, CSF1R, CSF3R, CTCF, CTNNA1, CTNNB1, CUX1, CXCR4, DAXX, DDR2, DDX3X, DDX41, DICER1, DNMT3A, EGFR, EP300, EPHA3, EPHA5, ERBB2, ERBB3, ERBB4, ERCC3, ESR1, ETV6, EZH2, FANCA, FAT3, FBXW7, FGFR1, FGFR2, FGFR3, FH, FLT3, FOXL2, GATA1, GATA2, GNA11, GNAQ, GNAS, GRIN2A, H3F3A, HIST1H3B, HIST1H3C, HNF1A, HRAS, IDH1, IDH2, IKZF1, ITPKB, JAK2, KDM6A, KDR, KIT, KMT2A, KRAS, MAP2K1, MAPK1, MDM2, MET, MLH1, MLH3, MPL, MRE11A, MSH2, MSH6, MTOR, MYC, MYCN, MYD88, NBN, NF1, NF2, NFE2L2, NOTCH1, NOTCH2, NPM1, NRAS, PALB2, PBRM1, PDGFRA, PDGFRB, PHF6, PIK3CA, PIK3CB, PIK3R1, PLCG2, POLE, POT1, PPP2R1A, PTCH1, PTEN, PTPN11, RAD21, RAD51, RB1, RET, RUNX1, SDHB, SDHC, SDHD, SETBP1, SF3B1, SMAD4, SMARCB1, SMC1A, SMC3, SMO, SRSF2, STAG2, STAT3, STAT5B, STK11, TERT (promoter only), TET2, TP53, TSC1, TSC2, U2AF1, VHL, WT1, ZRSR2

Supplemental Figure 1.



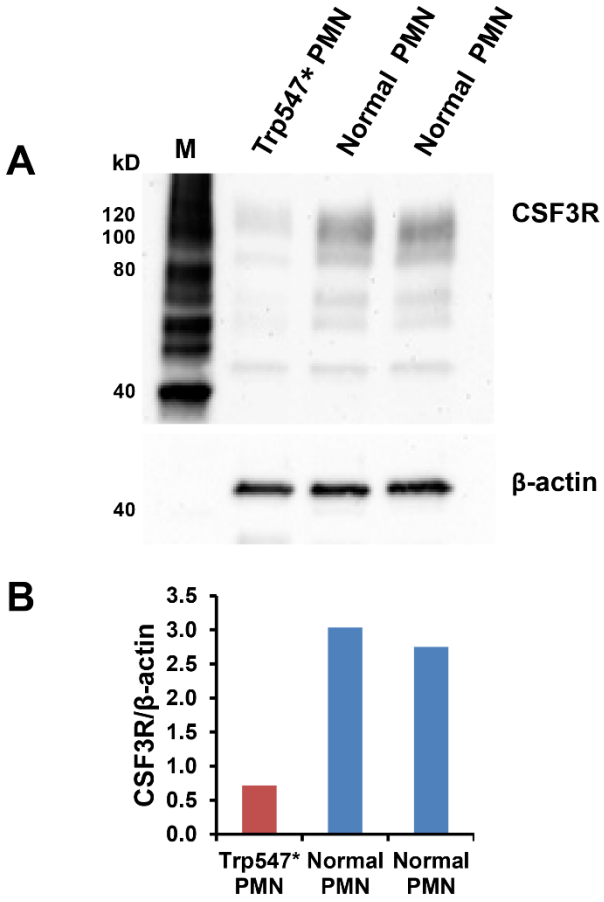
Supplemental Figure 1. Proliferative capacity of the p.Arg583His and p.Thr640Ile *CSF3R* variants. Proliferative responses of Ba/F3 cells expressing wild type (WT), p.Arg583His, and p.Thr640Ile CSF3R in response to varying concentrations of G-CSF. Cell numbers were quantified using an XTT assay at 48 hours after culture in the indicated concentrations of G-CSF. EC50 values calculated by dynamic curve fitting of the data for WT, p.Arg583His, and p.Thr640Ile were 3.19 ± 0.71, 1.65 ± 0.36, and 2.34 ± 0.50, respectively (mean ± SE), with *P* values of 0.126 and 0.556 for p.Arg583His and p.Thr640Ile relative to WT, respectively (determined by one way ANOVA). Data shown are representative of three independent experiments.

Supplemental Figure 2.



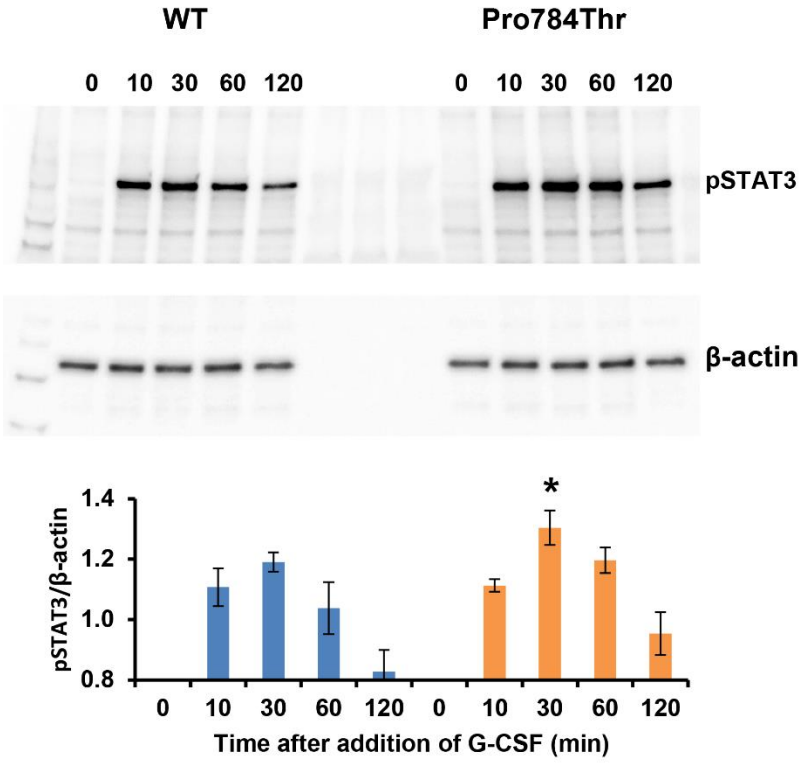
Supplemental Figure 2. Expression and proliferative capacity of single cell Ba/F3 clones expressing the p.Trp547* *CSF3R* variant. (A) Immunoblot analysis of receptor expression in untransfected Ba/F3 cells (parental), Ba/F3 cells expressing wild type CSF3R (WT pool), three Ba/F3 clones expressing the p.Trp547* *CSF3R* variant generated by limiting dilution (Trp547* #2, Trp547* #5, and Trp547* #6), and a pool of unsorted Ba/F3 cells expressing the p.Trp547* *CSF3R* variant. (B) Proliferative responses of cells in (A) in response to varying concentrations of G-CSF. Cell numbers were quantified using a luminescence-based assay at 48 hours after culture in the indicated concentrations of G-CSF. Error bars represent standard error (n=3).

Supplemental Figure 3.



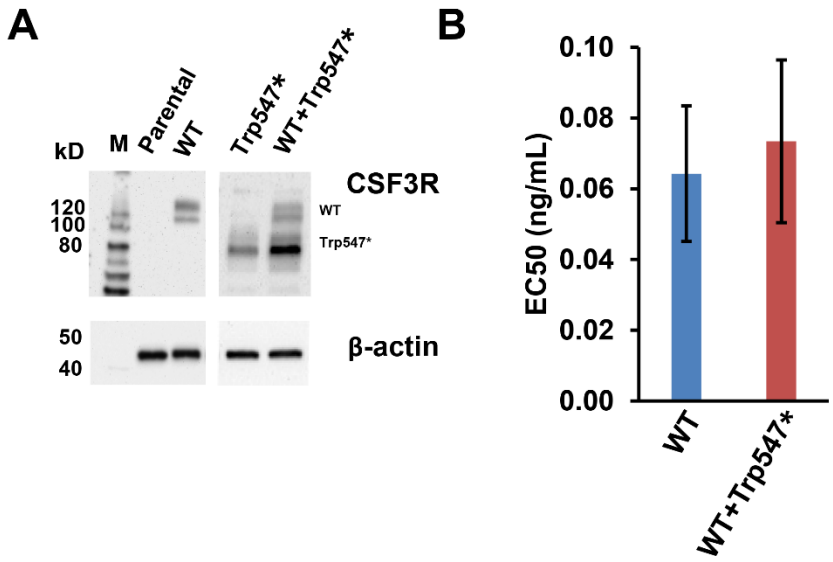
Supplemental Figure 3. Decreased CSF3R protein in neutrophils from a patient with the p.Trp547*allele. Immunoblot analysis of CSF3R expression in peripheral blood neutrophils (PMN) isolated from a patient with the p.Trp547* variant (Trp547* PMN) and from two different normal donors. (A) The upper blot was probed with anti-CSF3R antibody, and the lower blot with anti-β-actin antibody as a control for loading. (B) Densitometric analyses of the immunoblots were performed using ImageJ Software. The data are presented as the ratio of CSF3R/β-actin.

Supplemental Figure 4.



Supplemental Figure 4. p.Pro784Thr *CSF3R* variant shows increased STAT3 activation. Cells expressing p.Pro784Thr CSF3R or wild type (WT) CSF3R were serum and cytokine deprived for 2 hours then stimulated with 1 ng/mL G-CSF for the indicated times in minutes. Whole cell lysates were immunoblotted with antibodies to phospho-STAT3 (pSTAT3) and β-actin. Representative blots of three independent experiments for each *CSF3R* variant are shown. Densitometric analyses of the immunoblots were performed using ImageJ Software. The data are presented as the ratio of phospho-STAT3/β-actin. Error bars show the standard error. *P value < 0.05.

Supplemental Figure 5.



Supplemental Figure 5. Response to G-CSF is unchanged by co-expression of the Trp547* CSF3R with the wild type receptor. (A) Immunoblot analysis of receptor expression in untransfected (Parental) Ba/F3 cells, Ba/F3 cells expressing the wild type (WT) CSF3R, the Trp547* mutant CSF3R (Trp547*), or co-expressing both the wild type and mutant receptors (WT+Trp547*). (B) Half maximal effective concentration (EC50) of proliferative responses to G-CSF of the wild type (WT) and wild-type/mutant co-expressing cells (WT+Trp547*) shown in (A). The mean of two independent experiments is shown. Error bars indicate the standard deviation (*P* value of 0.20, calculated using Student's t-test).

Supplemental References

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