A novel colony stimulating factor 3 receptor activating mutation identified in a patient with chronic neutrophilic leukemia

Activating mutations in the extracellular domain of colony stimulating factor 3 receptor (CSF3R, aka GCSFR) are present in an overwhelming majority of patients with chronic neutrophilic leukemia (CNL). These point mutations have been primarily observed in glycosylation sites located close to the cell membrane. Herein we describe a novel activating mutation in *CSF3R* (N579Y) in a patient with CNL. This mutation disrupts a putative glycosylation site that is significantly more distant from the membrane than those previously documented. We demonstrate that *CSF3R*^{N579Y} results in ligand independent activation and is capable of oncogenic transformation. It also causes enhanced activation of JAK/STAT and MAPK signaling, and is sensitive to JAK inhibition. This study characterizes a novel oncogenic variant of CSF3R that is therapeutically relevant, and suggests that mutations in glycosylation sites further from the known major hotspots may be clinically significant.

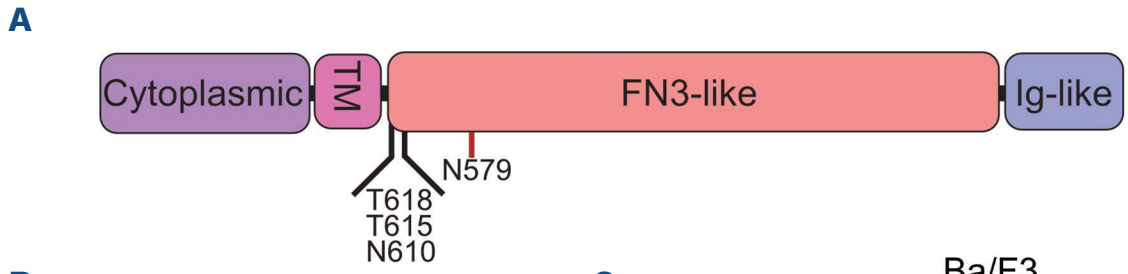
CSF3R activation is critical for neutrophil production. Leukemia-associated *CSF3R* mutations lead to aberrant receptor activation.[1,2] Mutations in *CSF3R* are highly enriched in CNL, where they occur in approximately 90% of patients.[3,4] More rarely, mutations in *CSF3R* can also occur in other myeloid malignancies including atypical chronic myeloid leukemia (aCML), acute myeloid leukemia, chronic myelomonocytic leukemia, and others.[2,3,5,6]

There are two types of CSF3R mutations: those that truncate the cytoplasmic domain of the receptor and those that activate the receptor through point mutations.[1-3] The cytoplasmic truncation mutations lead to a loss of endocytic, degradative, and negative regulatory motifs.[7] The loss of these regions causes ligand hypersensitivity.[7] In contrast, CSF3R point mutations activate the receptor in a ligand-independent manner.[1,3,8] Point mutations either occur in the transmembrane domain (such as T640N), where they promote receptor dimerization through intramolecular interaction of the transmembrane alpha helices,[9] or they occur in the membrane-proximal portion of the extracellular domain. These membrane-proximal mutations (such as T618I, T615A and N610H) occur in sites of either O- or N-linked glycosylation,[8,10,11] resulting in a loss of the glycan at these sites and ligand-independent receptor dimerization.[8,10]

The membrane-proximal point mutation *CSF3R*^{T618I} is the most frequently found mutation in CNL,[3,4] although truncating mutations can also occur. Ligand-independent activation of CSF3R by the T618I mutation leads to strong constitutive signaling downstream through the JAK/STAT and MAPK signaling pathways.[3,12] This results in the expression of pro-proliferation and pro-neutrophil differentiation programs resulting in an overproduction of mature neutrophils, which is one of the characteristics of CNL. Inhibition of CSF3R-driven JAK/STAT signaling has been investigated as a therapeutic strategy for CNL with promising results in trials.[3,13]

The deployment of JAK inhibitors for CNL requires an understanding of the spectrum of *CSF3R* mutations that are responsive to therapy. In this study, we characterize a novel mutation in *CSF3R* identified in a patient with CNL. This mutation, N579Y, is part of a consensus motif for N-glycosylation, but lies outside the membrane-proximal portion of the cytoplasmic domain where N610H, T615A and T618I reside (Figure 1A). In this study, we characterize the signaling dysregulation, oncogenic potential, and drug sensitivity of the CSF3R^{N579Y} mutation.

CSF3R^{N579Y} was identified in a patient presenting with a myeloproliferative neoplasm with neutrophilia and splenomegaly. The patient was a 55-year-old man with a 3-month history of progressive fatigue and a 40-pound weight loss. At the time of his initial presentation, his white blood count was 96.48x10^9/L with a differential of 3% bands, 74% neutrophils, 4% lymphs, 3% monocytes, 6% metameyelocytes, 8% myelocytes, and 2% promyelocytes. Hemoglobin was 10.9 g/dL and platelets were 740x10^9/L. One year prior, his WBC had been normal with a mild monocytosis. Bone marrow (BM) biopsy revealed a markedly hypercellular (90% cellular) BM exhibiting myeloid-predominant maturing trilineage hematopoiesis with left-shifted myeloid maturation, mild megakaryocytic atypia (small, hypolobated forms), mild reticulin fibrosis most consistent with a myeloid neoplasm. Given the myeloid-predominant BM without significant dysgranulopoiesis in the context of peripheral leukocytosis with peripheral neutrophilia, a diagnosis of chronic neutrophilic leukemia (CNL) was favored. However, the left-shifted myeloid maturation and reticulin fibrosis is atypical. CML was excluded given the absence of a *BCR-ABL1* positive fusion transcript, and aCML was excluded due to lack of significant dysgranulopoiesis. The patient had had a history of a seizure substance use disorder and had subsequently been found unresponsive with a large intracranial hemorrhage. He was started on hydroxyurea



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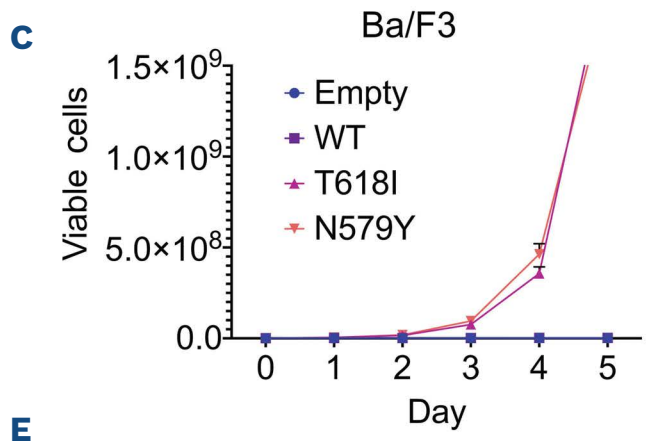
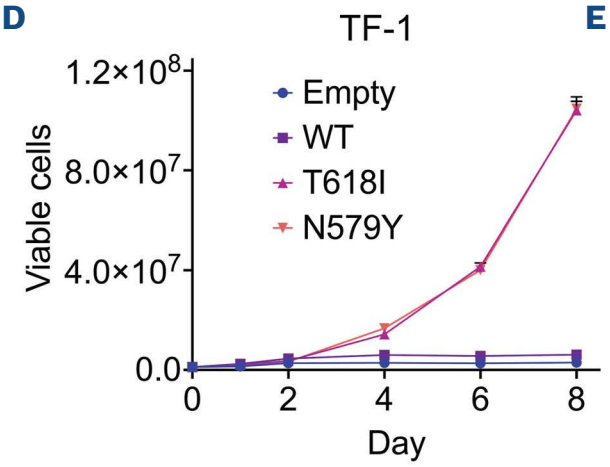
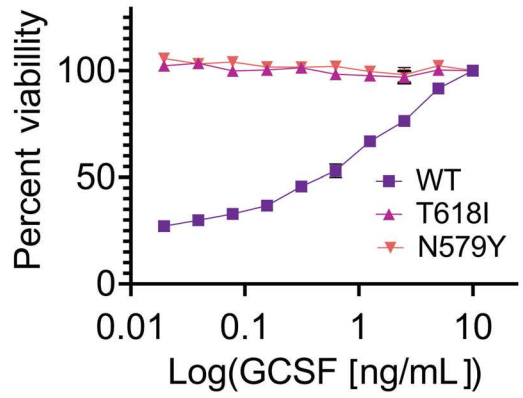
Gene	Mutation	VAF
CSF3R	N579Y	51.8%
CSF3R	739*	25.9%
CSF3R	741*	24.2%
ASXL1	G646Wfs*12	40.4%
ETNK1	H243Q	12.7%
ETV6	E410_p411insP	51.8%







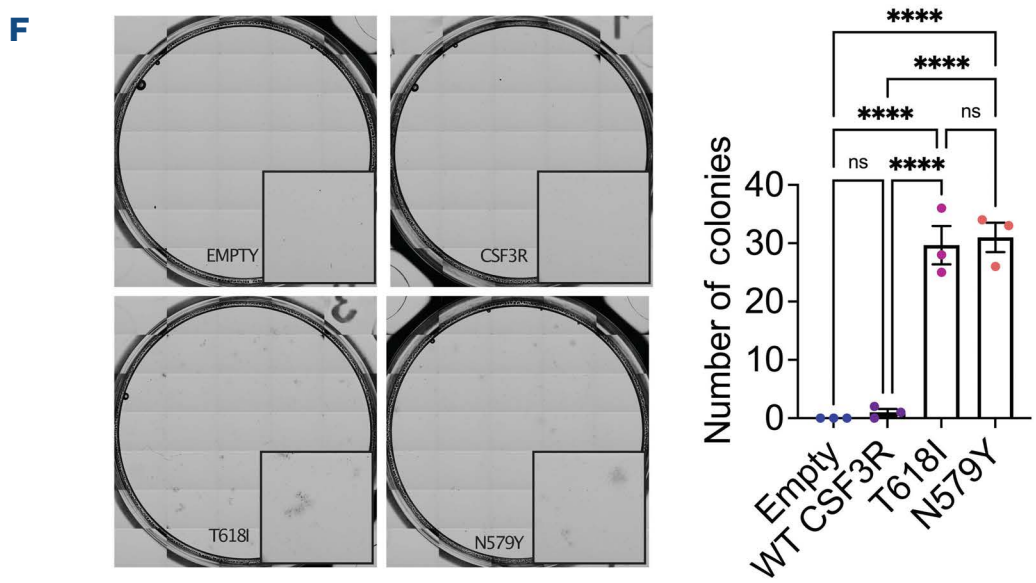


Figure 1. CSF3R^{N579Y} is a ligand independent activating mutation that drives myeloid proliferation. (A) Representation of CSF3R with mutations at predicted glycosylation site mapped. TM: transmembrane domain. (B) Table of mutations identified in patient with a myeloproliferative neoplasm (MPN) containing the *CSF3R^{N579Y}* mutation. Variant allele frequency (VAF) is shown for each mutation. (C) Ba/F3 proliferation assay demonstrating that CSF3R^{N579Y} is capable of driving IL3-independent growth. Ba/F3 cells transduced with an empty vector control, *CSF3R^{WT}*, *CSF3R^{T618I}*, or *CSF3R^{N579Y}* were washed to remove IL3 from the media, growth was measured daily for 7 days on an automated cell counter in triplicate. WT: wild-type. (D) TF-1 proliferation assay demonstrating that CSF3R^{N579Y} is capable of driving granulocyte-macrophage colony-stimulating factor (GM-CSF)-independent growth. TF-1 cells transduced with an empty vector control, *CSF3R^{WT}*, *CSF3R^{T618I}*, or *CSF3R^{N579Y}* were washed to remove GM-CSF from the media, growth was measured daily for 8 days on an automated cell counter in triplicate. (E) Ba/F3 cytokine sensitivity assay demonstrating that CSF3R^{N579Y}-driven growth is insensitive to GCSF concentration. Ba/F3 cells transduced with an *CSF3R^{WT}*, *CSF3R^{T618I}*, or *CSF3R^{N579Y}* were washed out of IL3-containing media and plated in 96-well format with increasing concentrations of GCSF added to the media in triplicate. Plates were analyzed by MTS cell proliferation assay after 72 hours. Standard Error of Mean (SEM) is shown with black error bars. (F) Colony formation assay showing that CSF3R^{N579Y} drives colony formation in the absence of cytokine support. Bone marrow harvested from C57BL/6 mice was transduced with an ecotropic murine retrovirus with empty vector control, *CSF3R^{WT}*, *CSF3R^{T618I}*, *CSF3R^{N579Y}*, sorted by flow cytometry, and plated in methylcellulose (Stem Cell M3234) without added cytokine support in triplicate. Plates were imaged after 7 days, and files were blinded before colonies with at least 50 cells were counted. Representative images of colonies and quantification of colony numbers showing that CSF3R^{N579Y} and CSF3R^{T618I} form significantly more colonies than an empty vector control or CSF3R^{WT}. Statistical significance was determined using an ordinary one-way ANOVA test in GraphPad Prism; ****P<0.0001; ns: not significant.

and transitioned to a palliative approach before succumbing to his disease. It had not been possible to perform germline testing.

Targeted next-generation sequencing analysis was run using the Dana-Farber/Brigham and Women's Cancer Center Rapid Heme Panel. This revealed three mutations in *CSF3R*: Q739* (variant allele frequency [VAF] 26%), Q741* (VAF 24%), and N579Y (VAF 52%). *ASXL1* G646fs (38% VAF) and *ETNK1* H243Q (VAF 12%) mutations were also present. In CNL, when truncating mutations occur, they most frequently occur alongside an activating point mutation.[11,14] Truncations of the cytoplasmic domain are well characterized in this region, and result in enhanced CSF3R half-life and cell surface expression.[7,11] When found in combination with an activating mutation, truncations may provide some proliferative advantage to clones harboring both mutations, over those with a point mutation alone.[11,15] This, combined with the knowledge that N579 was part of a consensus motif for N-linked glycosylation prompted us to assess the oncogenicity of this variant.

To test the oncogenic potential of CSF3R^{N579Y}, we evaluated its ability to transform the Ba/F3 cells (Figure 1C). Ba/F3 is a murine-derived blood cell line that is normally dependent on IL3 for growth and survival. In the absence of IL3, these cells die, but some oncogenes (such as *CSF3R*^{T618I}) can enable IL-3-independent growth of these cells.[3] This makes Ba/F3 cells a simple model to use to assess oncogenic transformation. We used retroviral transduction to express an empty vector control, *CSF3R*^{WT}, *CSF3R*^{T618I}, or *CSF3R*^{N579Y} with an IRES-GFP in the Ba/F3 cells, then selected for expression by flow cytometry. Each line was washed and plated in media (n=3) without IL3. Cell growth was tracked daily using an automated cell counter. Only CSF3R^{N579Y} and CSF3R^{T618I} were able to transform the Ba/F3 cells to IL-3-independent growth. This was replicated in human hematopoietic cell line TF-1 cells dependent on GM-CSF, in which CSF3R^{N579Y} and CSF3R^{T618I} also transformed cells to cytokine-independent growth (Figure 1D). To further assess the ability of CSF3R^{N579Y} to drive cytokine-independent growth, we performed a hematopoietic colony formation assay (CFU) (Figure 1F). Mouse BM is unable to form colonies in CFU assays without added cytokine support or the addition of a proliferative oncogene.[8] We transduced murine BM from young mice with an empty vector, *CSF3R*^{WT}, *CSF3R*^{T618I}, or *CSF3R*^{N579Y} in vectors containing an IRES-GFP. GFP-positive cells were sorted by flow cytometry into methylcellulose medium without added cytokines, then plated (n=3). After 7 days plates were imaged and colonies were counted. CSF3R^{N579Y} and CSF3R^{T618I} were able to form colonies without added cytokine support to a similar degree, while the empty vector and CSF3R^{WT} were unable to form colonies. From these experiments, we conclude that CSF3R^{N579Y} is an oncogene capable of transforming Ba/F3 cells and driving cytokine-independent colony formation.

To assess whether CSF3R^{N579Y} promotes ligand-independent receptor activation, we performed a GCSF titration curve in the Ba/F3 model (Figure 1E). Cells were washed out of the IL3-containing media and plated in 96-well format in increasing concentrations of GCSF. After 72 hours (h) we performed an MTS-based proliferation assay to assess cell viability and growth. We found CSF3R^{T618I} and CSF3R^{N579Y} exhibit robust growth unaffected by GCSF, while the proliferation of CSF3R^{WT}-expressing cells is dependent on high levels of GCSF in the media. These data indicate that N579Y causes ligand-independent receptor activation.

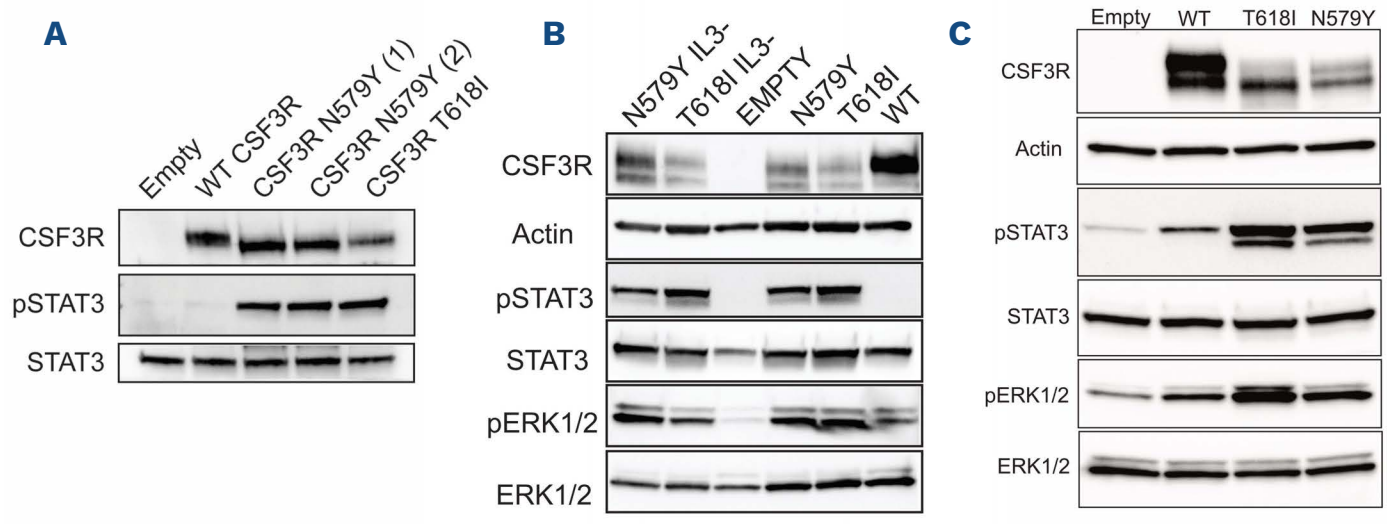
Next, we wanted to test the ability of CSF3R^{N579Y} to enhance the activation of CSF3R-associated signaling pathways. To accomplish this, we transiently transfected HEK293 cells with an empty vector, *CSF3R*^{WT}, *CSF3R*^{T618I}, or *CSF3R*^{N579Y} (Figure 2A). Cells were harvested 48 h after transfection and lysate was analyzed by western blot for STAT3 activation. Cells expressing CSF3R^{N579Y} had enhanced phosphorylation of STAT3 compared to the empty vector or CSF3R^{WT}. This enhancement was comparable in magnitude to CSF3R^{T618I}. To evaluate CSF3R^{N579Y} signaling activation in a hematopoietic cell line, we performed an immunoblot in the Ba/F3 CSF3R-expressing lines described above. We washed IL3 out of cells, and harvested them 24 h later for analysis alongside CSF3R^{N579Y}- and CSF3R^{T618I}-expressing cells that had been cultured without IL3 for an extended period (Figure 2B). We then assessed JAK/STAT and MAPK activation. In this model, CSF3R^{N579Y} and CSF3R^{T618I} demonstrated enhanced STAT3 and ERK1/2 phosphorylation compared to CSF3R^{WT} and the empty vector. This was true both in cells that had acute IL3-withdrawal and those that were proliferating continuously in media without IL3. Enhanced activation of STAT3 was also found in TF-1 cells, further confirming that this signaling enhancement occurs in a human hematopoietic model (Figure 2C). We find CSF3R^{N579Y} robustly enhances both JAK/STAT and MAPK activation, suggesting that it is an activating mutation in the receptor.

Recently, a clinical trial testing the efficacy of ruxolitinib, a JAK1/2 inhibitor, in patients with CNL or aCML showed an overall response rate of 32%, higher in patients with CNL driven by a *CSF3R*^{T618I} mutation.[12] Since CSF3R^{N579Y} exhibits similar transformation capacity as CSF3R^{T618I}, we decided to test the sensitivity of CSF3R^{N579Y} to ruxolitinib. For this we used Ba/F3 cells expressing CSF3R^{N579Y} and CSF3R^{T618I} cultured without IL3 (Figure 2D). Cells were plated in 96-well format with ruxolitinib concentrations ranging from 0 nM to 500 nM. After 72 h cells were analyzed by MTS cell proliferation assay. We found that CSF3R^{N579Y} is sensitive to ruxolitinib and has a half-maximal inhibitory concentration (IC_{50}) closely matching

CSF3R^{T618I}. This was replicated in TF-1 cells, demonstrating that T618I and N579Y both confer sensitivity to ruxolitinib in a human hematopoietic cell line with comparable IC_{50} (Figure 2E). We validated this finding by treatment of primary murine hematopoietic cells expressing CSF3R^{N579Y} or CSF3R^{T618I} in a CFU assay with ruxolitinib (Figure 2F).

To further investigate ruxolitinib sensitivity in human leukemia cells, peripheral blood cells from the patient described above were enriched for CD34 positivity using a MACS separation column, and plated in methylcellulose medium containing 0 nM-10,000 nM of ruxolitinib (Figure 2G and H). Plates were imaged after 10 days and colonies greater than 50 cells were counted. We found that CD34$^+$ cells isolated from the peripheral blood of the patient harboring the *CSF3R^{N579Y}*, *CSF3R^{741*}*, and *CSF3R^{739*}* mutations were sensitive to ruxolitinib.

In this report, we have identified and characterized the novel mutation *CSF3R^{N579Y}* in a patient with CNL. We find that this mutation causes receptor activation in a ligand-independent manner. This mechanism is like other oncogenic *CSF3R* point mutations (such as T618I, T615A and



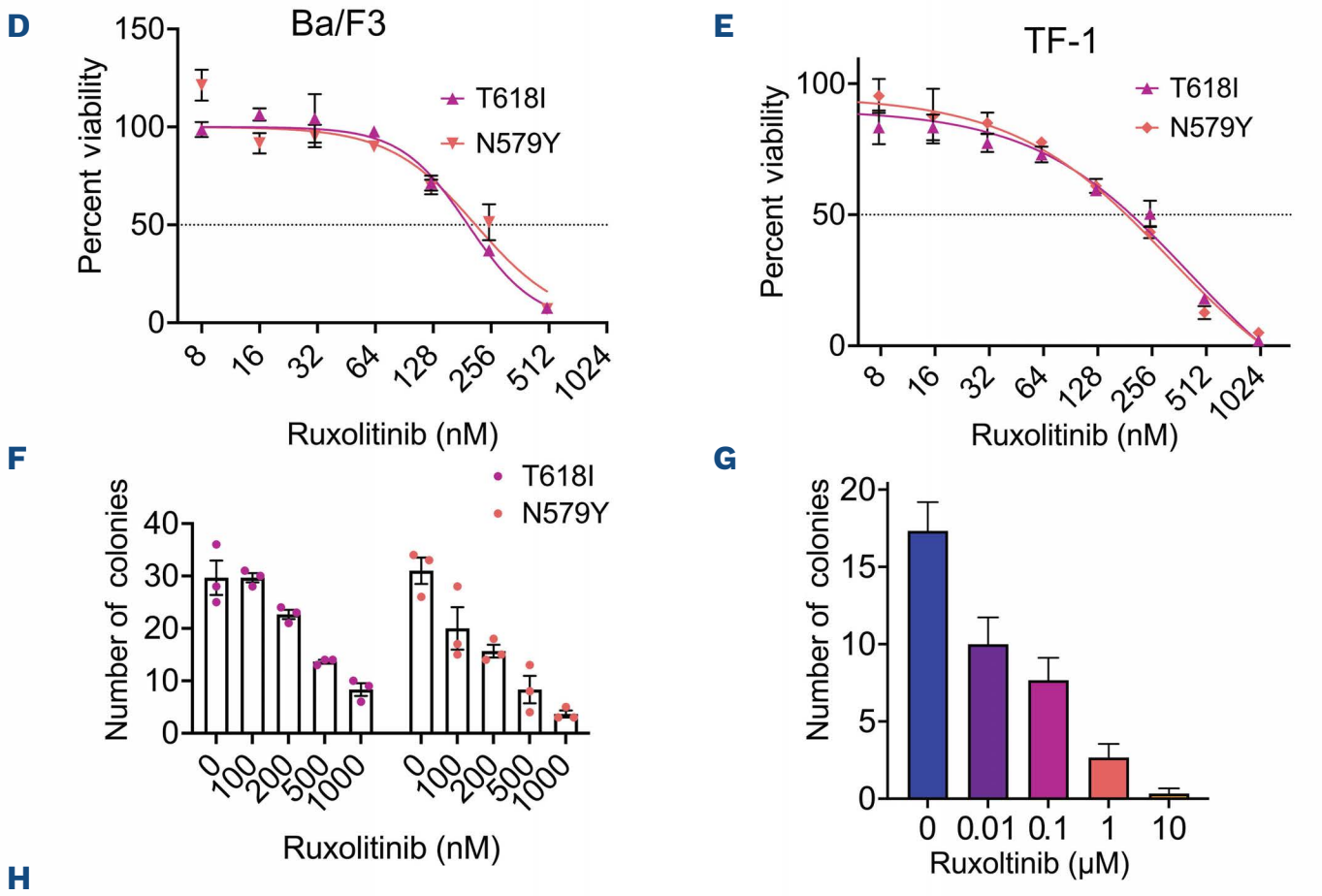


Figure 2. The activating mutation CSF3R^{N579Y} enhances STAT3 and ERK1/2 activation and confers sensitivity to the JAK1/2 inhibitor ruxolitinib. (A) Western blot showing CSF3R^{N579Y} has enhanced activation of STAT3 in HEK293 cells. HEK293 cells were transiently transfected using Fugene-6 with empty vector control, *CSF3R^{WT}*, *CSF3R^{T618I}*, or *CSF3R^{N579Y}*, and harvested 48 hours (h) later for western blot analysis of pSTAT3 activation. (B) CSF3R^{N579Y} has enhanced STAT3 and ERK1/2 activation in Ba/F3 cells. Ba/F3 cells transduced with an empty vector control, *CSF3R^{WT}*, *CSF3R^{T618I}*, or *CSF3R^{N579Y}* were washed of IL3 24 h prior to analysis, or had been cultured without IL3 for an extended period (annotated with IL3-). 5x10^6 cells per condition were harvested and analyzed by western blot for STAT3 and ERK1/2 phosphorylation. (C) CSF3R^{N579Y} has enhanced STAT3 activation in TF-1 cells. TF-1 cells cultured in IL3 were transduced with an empty vector control, *CSF3R^{WT}*, *CSF3R^{T618I}*, or *CSF3R^{N579Y}* were washed of IL3 24 h and serum starved for 4 h prior to harvest for western blot. 4x10^6 cells per condition were harvested and analyzed by western blot for STAT3 and ERK1/2 phosphorylation. (D) CSF3R^{N579Y}-dependent Ba/F3 growth is sensitive to ruxolitinib to a similar extent as CSF3R^{T618I}. Ba/F3 cells transduced with a *CSF3R^{T618I}* or *CSF3R^{N579Y}* were cultured without IL3 for an extended period and plated in 96-well format with increasing concentrations of ruxolitinib between 0-500 nM (n=3). After 72 h plates were analyzed by an MTS-based cell proliferation assay (CellTiter AQueous One). Half-maximal inhibitory concentration (IC_{50}) was calculated for CSF3R^{T618I} (194 nM) and CSF3R^{N579Y} (213nM) using Graphpad Prism. (E) CSF3R^{N579Y}-dependent TF-1 growth is sensitive to ruxolitinib to a similar extent as CSF3R^{T618I}. TF-1 cells transduced with a *CSF3R^{T618I}* or *CSF3R^{N579Y}* were cultured without GM-CSF for an extended period and plated in 96-well format with increasing concentrations of ruxolitinib between 0-500 nM (n=3). After 72 h plates were analyzed by an MTS-based cell proliferation assay (CellTiter AQueous One). IC_{50} was calculated for CSF3R^{T618I} (147nM) and CSF3R^{N579Y} (162nM) using Graphpad Prism. (F) CSF3R^{N579Y}-dependent mouse bone marrow (BM) colony formation is sensitive to ruxolitinib. Murine BM transduced with *CSF3R^{N579Y}* or *CSF3R^{T618I}* was sorted and treated with ruxolitinib at concentrations between 0 nM-1,000 nM and plated in cytokine-free methylcellulose in triplicate. Plates were imaged after 7 days, and files were blinded before counting colonies larger than 50. (G and H) Patient peripheral blood sample harboring CSF3R^{N579Y} is sensitive to ruxolitinib. Peripheral blood was received and immediately enriched for CD34$^+$ cells on a MACS column before plating in methylcellulose treated with ruxolitinib in triplicate. After 10 days, plates were imaged, files blinded, and colonies counted. Representative images of wells from colony assay and quantification of colony numbers are shown.

N610H). However, CSF3R^{N579Y} is much further from the transmembrane domain than those previously identified, suggesting that loss of extracellular glycosylation sites further from the membrane may also confer oncogenic potential. CSF3R^{N579Y} shows enhanced JAK/STAT and MAPK signaling. This mutation is sensitive to the JAK1/2 inhibitor ruxolitinib *in vitro*, which has previously been used to treat *CSF3R*-mutant neoplasms. Collectively, this work characterizes N579Y as a novel activating mutation in *CSF3R* with diagnostic and potential therapeutic relevance.

Disclosures

PS-A provides consulting for Kura Oncology. DJD received funding from Norvartis, Abbvie, Blueprint Medicines, and Glycomimetrics, and has provided consulting for Agios, Amegen, Autolus, Forty-Seven, Incyte, Jazz, Norvartis, Pfizer, Servier, and Takeda. JEM received funding from the NIH, Blueprint Medicines, Gilead Sciences, Kura Oncolology, and has an ongoing collaboration with Ionis Pharmaceuticals. The other authors have no conflicts of interest to disclose.

Contributions

BNM, JC, PSA, DJD and JEM are responsible for data collection and analysis. BNM, JC, DJD and JEM prepared and wrote the manuscript.

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Data-sharing statement

The datasets generated in this study are available from the corresponding author by request.

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