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A novel germline variant in CSF3R reduces N-glycosylation and exerts potent oncogenic effects in leukemia

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Abstract

Mutations in the colony stimulating factor 3 receptor (CSF3R) have been identified in the vast majority of patients with chronic neutrophilic leukemia and are present in other kinds of leukemia, such as AML. Here we studied the function of novel germline variants in CSF3R at amino acid N610. These N610 substitutions were potently oncogenic and activated the receptor independently of its ligand GCSF. These mutations activated the JAK-STAT signaling pathway and conferred sensitivity to JAK inhibitors. Mass spectrometry revealed that the N610 residue is part of a consensus N-linked glycosylation motif in the receptor, usually linked to complex glycans. N610 was also the primary site of sialylation of the receptor. Membrane-proximal N-linked glycosylation was critical for maintaining the ligand dependence of the receptor. Mutation of the N610 site prevented membrane-proximal N-glycosylation of CSF3R, which then drove ligand-independent cellular expansion. Kinase inhibitors blocked growth of cells with an N610 mutation. This study expands the repertoire of oncogenic mutations in CSF3R that are therapeutically targetable and provides insight into the function of glycans in receptor regulation.

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Keywords

CSF3R; GCSFR; myeloid leukemia; glycosylation; receptor activation

Introduction

Mutations in CSF3R, also known as granulocyte colony-stimulating factor receptor (GCSFR), occur in the majority of chronic neutrophilic leukemia (CNL) patients (1,2) and also found more rarely, in acute myeloid leukemia (AML) patients (3–7). Truncation mutations that lead to a premature stop in the cytoplasmic domain are found in CNL (1) and result in increased expression of CSF3R on the cell surface (8). The most common CSF3R mutation in CNL is T618I (T595I), a point mutation in the membrane-proximal extracellular domain that causes ligand independence (Figure 1A) (9). There are two numbering conventions; the second historical one we will put in parentheses does not include the 23 amino acid signal peptide. Recently, the presence of a CSF3R T618I mutation became a part of the World Health Organization (WHO) criteria for diagnosis of CNL (10).

Although CSF3R T618I is the most common mutation in CNL, there are other rarer variants that also cause profound receptor activation, such as T615A (T592A), a mutation seen in the membrane proximal domain (1). In the transmembrane domain, a point mutation at T640N (T617N), was reported in a family with congenital neutrophilia, as well as in a few patients with CNL (11,12). This T640N mutation is predicted to cause intramolecular hydrogen-bonding and has been experimentally shown to increase ligand-independent dimerization (11,12). While study of transmembrane and transmembrane proximal mutations has greatly improved our ability to diagnosis and treat the disease, there are still rare variants for which their significance is not clear. Herein, we study the mechanism of action of a rare germline CSF3R N610H mutation. This patient had a condition most consistent with primary myelofibrosis with mild leukocytosis. Because of N610's proximity to other more common oncogenic CSF3R point mutations found in CNL, we were interested in understanding the functional consequences and therapeutic implications of N610 substitutions.

There is a substantive body of literature that correlates changes in glycosylation with cancer progression (13). Previously, we reported that T618I is at a site of O-glycosylation and changes in glycosylation at that site have been linked to oncogenesis (14). It is therefore critical to understand the post-translational modifications on CSF3R. The Asn residue at 610 is part of an N-linked glycosylation consensus N-X-(S/T) motif (15) and N-glycosylated proteins typically migrate to the extracellular space. N-Glycans help to fold, traffic, and thermodynamically stabilize the protein (16).

In this study, we confirm the identity of the glycans on N610 by mass spectrometry (MS) analysis. We determined that N610 is occupied with a sialylated hybrid N-glycan. Furthermore, we identify the oncogenic pathway for these mutations. Both the N610H substitution and a second germline mutation identified (N610S) highly activate CSF3R, causing cytokine-independent growth in Ba/F3 cells. Like the common T618I mutation, these mutations render the receptor ligand-independent. Downstream, N610H and N610S activate the JAK/STAT pathway as demonstrated by an increase in the levels of phospho-

STAT3. The loss of N-glycosylation in the membrane-proximal region of CSF3R promotes ligand-independent receptor activation and oncogenesis. Rare human mutations can provide significant insight into the relationship between receptor structure and function.

Materials and Methods

Sequencing

Genomic DNA from skin biopsy and peripheral blood were sequenced. Exon 14 of CSF3R was amplified using the following M13F and M13R tagged primers (F-(GTAAAACGACGGCCAGTCCACGGAGGCAGCTTTAC and R-CAGGAAACAGCTATGACCAAATCAGCATCCTTTGGGTG), purified on an Amicon Ultra 0.5 mL Centrifugal Filter (Millipore) followed by Sanger Sequencing (Eurofins genomics) using M13F and M13R primers. The N610S mutation was identified through a custom RainDance Thunderbolt Sequencing panel run at Memorial Sloan Kettering on both blood and nail clippings. The genes sequenced on this panel are as follows: *ASXL1, BCOR, BCORL1, BRAF, CALR, CBL, CBLB, CEBPA, CSF3R, DNMT3A, ETV6, EZH2, FLT3, GATA1, GATA2, GNAS, HRAS, IDH1, IDH2, JAK1, JAK2, JAK3, KDM6A, KIT, KRAS, MAP2K1, MPL, MYD88, NOTCH1, NPM1, NRAS, PHF6, PML, PTEN, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2.* Patient samples were obtained with written informed consent in accordance with the Declaration of Helsinki and institutional review boards of Memorial Sloan Kettering and Washington University of St. Louis.

Plasmid Construction

MSCV-IRES-GFP (MigR1) was made compatible for Gateway cloning using the Gateway Vector Conversion Kit (Invitrogen). A gateway pDONR vector for CSF3R transcript variant 1 (NM_00760.2) was purchased (GeneCopoeia). CSF3R was mutagenized as described previously for the CSF3R T618I mutation(1) or using the Quikchange II XL Site Directed Mutatgenesis Kit (Agilent Genomics). The following primers were used for site directed mutagenesis: N610H (F-ggctggggccacccacagtacagtcct, R-aggactgtactgtgggtggccccagcc), N610Q (F-gtgaggactgtactctgggtggccccagcct, R-aggctggggccacccagagtacagtcctcac), N610S (F-gaggactgtactgctggtggccccagc, R-gctggggccaccagcagtacagtcctc). After sequence conformation of the mutagenesis, CSF3R mutants were subcloned into Gateway-MSCV-IRES-GFP using LR Clonease II, a recombination based strategy (Invitrogen).

Cell Culture

Ba/F3 cells were obtained from Brian Druker at OHSU grown in RPMI 1640 with 10% fetal bovine serum (FBS, HyClone), Pen/Strep, 15% WEHI conditioned medium (which contains IL3) and L-glutamine. BaF3 cells were not allowed to exceed passage 20. 293T17 cells (obtained from ATCC CRL-11268) were grown in DMEM containing GlutaMAX (Gibco) with 10% FBS and Pen/Strep. After obtaining cells from ATCC, cells were frozen at low passage (p3-p5) and then thawed and used at passage 9 or lower. Cell line authentication was not performed for these studies. 293T17 cells were transfected with Fugene 6 (Promega) at a 5:1 ratio of lipid to DNA. Retrovirus was made by co-transfection of MigR1 constructs with pEcopac. Cell stocks were mycoplasma tested prior to freezing or upon thaw, in addition to

monthly testing of cells in culture. Mycoplasma testing was performed using the MycoAlert Mycoplasma Detection Kit (Lonza).

Ba/F3 cytokine independent growth assays

Viral supernatants were filtered using 0.45 micron filters and then Ba/F3 cells or mouse bone marrow were spinoculated in the presence of polybrene and HEPES buffer. Cells were spun at 2500 r.p.m., for 90 min at 30 °C (brake turned off). GFP+ cells were sorted on a BD FACSAria II sorter, and then sorted cells were allowed to expand for 2–4 days. GFP+ Ba/F3 cells expressing CSF3R mutants or controls were washed three times, and plated at 5×10^5 cells/mL in the absence of cytokine support (RPMI 1640 media, with 10% FBS, L-glutamine and Pen/Strep). Cell viability and number were monitored on a Bio-Rad TC20 cell counter. For drug treatment studies, Mig empty (control vector) and WT CSF3R were grown in the presence of IL3 (WEHI conditioned medium) and CSF3R mutant constructs were grown in the absence of IL3. Cells in 96 well plate format were treated with increasing doses of Ruxolitinib (Selleckchem) or Trametinib (Selleckchem). GCSF-independence assays were performed with GFP-sorted cells maintained in IL3-containing media. Cells were washed three times in media without IL3 and then grown in 96 well format with increasing doses of GCSF.

Mouse bone marrow colony assays

Mouse bone marrow was harvested from 6–10 week old C57/BL6 mice. Marrow was cultured overnight in the presence of SCF, IL6 and IL3. Virus was prepared as described in the cell culture section and then filtered using 0.45 micron filters. Mouse bone marrow (1×10^6 cells) were spinoculated with viral supernatant, HEPES buffer and polybrene on two subsequent days. For the spinoculation, cells were spun at 2500 r.p.m, for 90 min at 30 °C (brake turned off). One day after the second sorting, GFP percentage was assessed by flow cytometry to determine that all vectors had infected the bone marrow cell. Cells were plated in triplicate with 10,000 cells in 1 mL mouse methylcellulose without added cytokines (MethoCult M3234, Stemcell Technologies). Cells were imaged using STEMvision (Stemcell Technologies) at day 14, blinded, and then manually counted. All animal work was performed in an AAALAC accredited facility with prior approval from the Oregon Health & Science University Institutional Animal Care and Use Committed, under protocol IP00000482.

Immunoblot analysis

Transfected 293T17 cells with the indicated CSF3R-MigR1, constructed as described under cell culture, were lysed 48 hours post-transfection in Cell Lysis Buffer (Cell Signaling Technologies) containing Complete Protease Inhibitor Cocktail Tablets (Roche) and Phosphatase Inhibitor Cocktail II (Sigma). Lysates were centrifuged at 14,000 r.p.m. for 10 min at 4 °C. Supernatant was transferred to a new tube then mixed with 3x SDS sample buffer (75 mM Tris pH 6.8, 3% SCS, 15% glycerol, 8% beta-mercaptoethanol, 0.1% bromophenol blue) and then heated for 5 min at 95 °C. Lysates were run on 4–15% criterion TGX Precast Protein Gels (Bio-Rad). Gels were transferred using the Trans-Blot Turbo Transfer System (Bio-Rad). After blocking in TBST with 5% milk, blots were probed in anti-GCSFR (38643, R&D Systems), Rb anti-pSTAT3 (9131, Cell Signaling Technologies),

Rb anti-Stat3 (9132, Cell Signaling Technologies), or GAPDH (#25778, Santa Cruz Biotechnology). Blots were washed with TBST and then an appropriate HRP-conjugated secondary, followed by incubation with a chemiluminescent substrate (Thermo Scientific) and imaged with a ChemiDoc Gel Imaging System (Bio-rad).

Immunoprecipitation

Protein purification was performed as previously described (14). In brief, 293T17 cells from American Type Culture Collection (ATCC) were maintained at 37 °C and 5% CO2 incubator in DMEM with 10% FBS and penicillin/streptomycin. 293T cells were transfected using FuGENE 6 (Promega) in Optimem. Transfected cells were lysed using cell lysis buffer (Cell Signaling Technologies) containing complete protease inhibitor 3 (CalBioChem), spun at 10,000 r.p.m. for 10 min to pellet cell debris and supernatant collected. FLAG-tagged constructs were immunoprecipitated from cell lysates by incubation with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 hour at 4 °C on a rotator. Beads were washed with cell lysis buffer. Proteins were disassociated from beads by incubating with FLAG peptide at room temperature for 1 hour and then subjected to immunoblotting analysis.

Detailed mass spectrometry (MS) protocols are provided in Supporting Information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD011118

Results

Identification of a novel germline mutation in CSF3R

The CSF3R N610H mutation was initially identified in a patient with a myeloproliferative neoplasm through next-generation sequencing using a custom AML/MDS mutation hotspot panel for 42 genes that have biological importance in myeloid malignancies. This sequencing of the patient's bone marrow revealed a mutation at N610H in CSF3R at a 50% mutant allele frequency. This myeloproliferative neoplasm was most consistent with a JAK2, CALR, MPL mutation-negative primary myelofibrosis. This patient had a history of mild leukocytosis for several years with the most recent white blood cell counts between 13.3 and 15.3 × 10^3/μL with granulocytic left shift. A bone marrow biopsy revealed 90% cellularity with a mild increase in reticulin fibrosis, increased myeloid to erythroid ratio, no overt dysplasia, and less than 5% blasts. The bone marrow cells were karyotypically normal, but 59% of interphase nuclei carried a micro-deletion of the 3' end of PDGFRB (5q) (identified by FISH). At the time of diagnosis, the patient had minimal symptoms with no anemia or thrombocytopenia, and was being monitored, but not receiving any specific treatment.

The near 50% allele fraction of the N610H mutation prompted us to determine whether the mutation was germline or somatic. Sanger sequencing confirmed the presence of a heterozygous N610H mutation in a sample of blood as well as in a skin biopsy (Fig. 1B); confirming the presence of a novel CSF3R N610H germline mutation in this patient with an unusual myeloproliferative neoplasm. The patient has no known family history of hematologic malignancies or neutrophilia.

Subsequent to the mutational analysis, the patient developed severe anemia and became transfusion dependent. His hemoglobin was 7.3 grams per deciliter, with a red blood cell count of $1.9 \times 10^3/\mu L$, and a hematocrit of 21.9 percent. At this time, he continued to have moderate leukocytosis with a white blood cell count of $14.6 \times 10^3/\mu L$. Sequencing of a bone marrow biopsy at this time revealed the acquisition of an ASXL1 Q1448Pfs*8 mutation at 33% variant allele frequency. ASXL1 is a chromatin modifying protein that is part of the polycomb repressor complex 2 (17). ASXL1 mutations are commonly found in myeloid malignancies and are often associated with poor prognosis (18–21). ASXL1 mutations can also occur alongside CSF3R mutations in CNL (22,23). Due to increasing transfusion dependence, decitabine was initiated and a bone marrow transplant is being considered.

The CSF3R N610H mutation is activating and causes ligand-independence

Because of N610H's proximity to the most common, T618I CSF3R mutation found in CNL, we were interested in understanding whether this novel mutation might be important for the disease pathology. To test the oncogenic capacity of this novel mutation we used a cytokine-independent growth assay. In this assay, the murine pro-B cell line, Ba/F3, was transduced with a retrovirus expressing the WT or mutant CSF3R using a vector containing an IRES-GFP. GFP positive cells expressing the gene of interest were sorted, allowed to recover, washed to remove cytokine support and then cultured in the absence of exogenous cytokines. Native Ba/F3 cells and those expressing WT CSF3R depend on cytokines for growth and die when the cytokines are removed. We found that the N610H mutation, (which results in a positive charge) and also a more conservative N610Q substitution, are both highly activating in CSF3R. Both mutations promote cytokine-independent growth in the murine Ba/F3 cell line after 3 days, whereas cells expressing WT CSF3R are unable to proliferate (Fig. 2A). Glutamine (Q) was chosen as the more conservative substitution because glutamine is structurally similar asparagine (N) and also uncharged.

One feature of the CSF3R T618I mutation is robust activation of the JAK/STAT pathway. We assessed the ability of the N610H and N610Q mutations to activate this pathway using phosphorylated STAT3 as a marker. Both of these mutations lead to robust phosphorylation of STAT3 above and beyond the increase in signaling with WT CSF3R (Fig. 2B).

The CSF3R T618I mutation is the most common in CNL, and confers ligand-independent receptor activation. To test whether the N610H mutation also conferred ligand-independent growth, we grew Ba/F3 cells transduced with either WT or mutant forms of CSF3R in decreasing concentrations of GCSF, the ligand for CSF3R. Like the T618I mutant, both the N610H and N610Q mutations exhibit ligand independence, while WT CSF3R cell viability has a dose dependent relationship with the GCSF concentration (Fig. 2C).

N610 is a site of N-linked glycosylation in CSF3R

We previously reported that the CSF3R T618 site is O-glycosylated. The common T618I mutation causes a loss of glycosylation leading to ligand independence and neutrophil expansion (14). Interestingly, N610 is part of an N-X-T motif, which is a consensus sequence for N-linked glycosylation. Haniu, et al., demonstrated that N610 is one of eight CSF3R sites that results in a lower molecular weight gel shift when treated with a cocktail of

deglycosylation enzymes, indicating that N610 is likely a site of N-glycosylation (24). We set out to confirm the glycosite utilization and identifying the glycan structures at N610. We began by performing free glycan analysis of CSF3R. In brief, this was achieved by first digesting WT CSF3R-purified proteins with trypsin, and next releasing N-glycans enzymatically from the crude mixture with PNGase F. Released N-glycans are then permethylated with methyl iodide and finally analyzed by MS (Fig 3A). Interestingly, we observed complex, biantennary, and high mannose glycan structures (Fig. 3B). We confirmed the identity of these glycan structures by electrospray ionization mass spectrometry (ESI-MS). Although this analysis accurately assigns glycan structure, this type of MS analysis does not assign glycans to particular sites on the protein. We therefore performed additional studies to identify the glycan at the N610 site. Consistent with the previous study by Haniu et al(25), MS-based glycoproteomic analysis revealed that the N610 site is occupied by a glycan (Figure 4). Additionally, we identified N-linked high mannose glycosylation at asparagine residues N389 and N474 and transient occupancy at site N51 (full mass spectrometry data has been uploaded to the EMBL-EBI PRIDE database).

Sialylation at N610

Free glycan analysis revealed several glycan structures on native CSF3R with sialic acid, also known as *N*-acetylneuraminic acid (Neu5Ac). Aberrant expression of sialic acid on proteins has been observed in many types of cancer (26). To confirm that WT CSF3R is indeed sialylated at N610, we performed a metabolic glycosylation assay. As previously described, sialoglycoproteins are targeted specifically by metabolically labeling cells (Fig. 4A) with a modified peracetylated *N*-acetylmannosamine (Ac_4ManNAc) analog, such as peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz, **1,** Fig. 4) that incorporates into sialic acid glycoproteins (27,28). The azide-modified sugar passively diffuses through the cell and is converted by the Roseman-Warren pathway into the corresponding azidosialic acid (29), which allows for the site-specific labeling with a biotin-conjugated reagent, such as a biotin-alkyne probe (**2,** Fig. 4A) under copper-catalyzed azide–alkyne [3+2] cycloaddition (CuAAC) conditions (30). HEK 293 cells were transfected with WT, N610H or T618I FLAG-tagged constructs and incubated with 50 μM Ac_4ManNAz or DMSO, as a control. The FLAG-tagged constructs were then immunoprecipitated from whole cell lysates and biotin-labeled. Western blot analysis revealed robust labeling of WT CSF3R and T618I, whereas we did not observe labeling in the N610H mutant, suggesting that N610 is the only site of sialylation in CSF3R (Fig.4B). N-glycan structures are diverse and can contain a variety of different sugars, including sialic acid. Our data suggests that while there are multiple N-glycosylated sites in CSF3R, N610 is the primary site of sialylation. Thus raising the possibility that this charged modification may have important structural or functional roles in this critical region of the receptor.

MS-based glycoproteomic analysis reveals N610 glycan structure

To confirm glycosylation at the N610 site, we performed a glycoproteomic experiment on the digested purified protein. We were not able to observe by MS the peptide containing the N610 site, in samples digested with trypsin, as the asparagine sits in a rather large 71 amino acid residue peptide, and although possible, peptides of this size are challenging to analyze

by mass-spectrometry based methods. However, an alternative digestion with chymotrypsin (31), which cleaves on the N-terminal side of hydrophobic amino acid residues (tyrosine, tryptophan, and phenylalanineleucine and leucine) did prove successful. FLAG-purified WT and N610H constructs allowed us to identify by MS in vitro several hybrid glycoform structures (Fig. 4C-D). We observed several diagnostic oxonium ions from HexNAc *m/z* at 138, 168, 186 and 204, Neu5Ac oxonium ions at 274 and 292 and the intensity of HexHexNAc oxonium ion at *m/z* 366. This site-directed mass spectrometry analysis confirms that N610 is occupied by sialylated glycans.

Identification of a germline CSF3R N610S mutation in a patient with CML

Subsequent to these initial studies, a CSF3R N610S mutation was detected in a 54-year-old female patient with chronic phase CML. The patient's initial white blood cell count was 13,000, with a hemoglobin of 15 grams per deciliter and a platelet count of 428,000. Cytogenetic analysis revealed an atypical 9;22 translocation as well as t11;17(p11.2;p13). She was initially treated with imatinib and achieved complete hematologic remission and complete cytogenetic response but plateaued short of major molecular remission. She was then switched to nilotinib. This patient was in deep molecular remission after therapy with nilotinib, with minimally evident (>4 log reduction from untreated International Standard baseline) or undetectable BCR-ABL sequentially, who developed increasing thrombocytosis. Evaluation for typical myeloproliferative drivers was unrevealing and bone marrow pathology noted mild myeloid hyperplasia and megakaryocytic MPN-like changes (clustering, increased nuclear-cytoplasmic ratio). Molecular analysis by MSKC IMPACT sequencing of bone marrow and fingernail DNA revealed a germline CSF3R N610S variant and a somatic DNMT3A mutation. At this time point the DNMT3A R882H mutation (c. 2645 G>A) was found at a VAF of 6.4% in the marrow. The CSF3R N610S mutation (c. 1829 A>G) mutations was found at 48% in the bone marrow and 53% in fingernail DNA (Figure 5A). The patient has no known family history of hematologic malignancies. Together these data suggests that N610 mutations represent a novel leukemia predisposition variant.

To test whether the serine substitution was also transforming at the 610 position we ran a cytokine independent growth assay as described above. In this assay, the N610S mutation was robustly transforming, and allowed for a similar growth capacity as the N610H and T618I mutations in CSF3R (Fig. 5B). To confirm the transforming potential of this mutation and assess ligand independence we performed a colony forming unit (CFU) assay. In this assay, mouse bone marrow is transduced with a retroviral vector expressing WT CSF3R, mutant CSF3R, or an empty vector control (mig empty). The cells are then plated in methylcellulose without any added cytokine support. WT CSF3R produces very few colonies but the CSF3R T618I mutant, which is able to signal in the absence of ligand produces abundant colonies. Both N610H and N610S were able to induce colony formation at similar levels as T618I, indicating that they are robustly oncogenic (Fig. 5C). Furthermore, the N610 mutations allow for enhanced replating of hematopoietic progenitors in CFU assays, similarly to the T618I mutation (Fig. 5D). Together these data indicate that CSF3R N610H and N610S mutations are oncogenic.

Therapeutic relevance of CSF3R N610 substitutions

We previously identified the JAK kinase inhibitor, ruxolitinib, as a potential therapeutic strategy for patients with CSF3R mutations (32). A recent study identified the MEK inhibitor, trametinib, as being efficacious in a mouse bone marrow transplant model of the CSF3R T618I mutation (33). We tested the ability of both ruxolitinib and trametinib to inhibit the viability of Ba/F3 cells expressing the N610H and N610S mutations and found both mutations to be as sensitive to the two inhibitors as the common T618I mutation (Fig. 5E-F). The WT CSF3R and Mig empty vector control cells are grown in medium containing IL3 for the drug studies (as they would die in its absence), which is known to exert its pro-survival effects on Ba/F3 cells through the JAK/STAT pathway. The growth inhibition of these cells by ruxolitinib is an on-target effect. Taken together, the similar properties of N610 and T618 substitutions, along with similar drug sensitivity, indicate that the CSF3R N610H and N610S variants, although rare, are clinically targetable mutations.

Discussion

In the age of widespread sequencing of samples from patients with hematologic malignancies, a lack of functional annotation for less common variants represents a major challenge. In this study, we employ functional and biochemical analysis of rare germline variants of CSF3R to show that a loss of glycosylation at N610 accompanies activation of the receptor. Furthermore, this study provides evidence that these clinically-identified N610 mutations, although rare, are likely therapeutically targetable. Mechanistically, these substitutions highlight the importance of membrane-proximal N-glycosylation for regulation of CSF3R activity. CSF3R mutations are the first example of a cancer-associated mutation that alters glycosylation in a site-specific manner to cause oncogenic transformation.

CSF3R mutations are the defining genetic feature of CNL (2,32), but can also occur in other hematologic malignancies. CSF3R mutations have been previously implicated in the development of severe congenital neutropenia associated acute myeloid leukemia (SCN-AML)(34–37). CSF3R mutations are also found at lower frequency (0.5–8%) in de novo or secondary acute myeloid leukemia (AML) (3,5,6,32,38). In CNL, SCN-AML and AML, the main classes of CSF3R mutations are membrane-proximal point mutations (e.g. T618I and T615A) and truncation mutations in the cytoplasmic domain (e.g. Q741 and S783fs), both of which are thought to be somatic mutations. We were therefore surprised to identify a germline N610H CSF3R mutation in a patient with unusual myelofibrosis. We subsequently identified another patient with a different germline substitution (N610S) at this same site. It is essential to understand whether these mutational events in CSF3R are oncogenic and have therapeutic relevance. The N610H, and N610S mutations are highly transformative in both cell line studies (Fig. 2) and in primary murine bone marrow (Fig. 5). The germline nature of the N610H/S mutations identified in this study, and the familial germline T640N mutations identified in a family with neutrophilia (11), suggest that additional mutations are likely necessary for the full clinical phenotype of CNL.

Emerging evidence suggests that protein glycosylation can affect signaling, especially during oncogenesis. Takahasi and coworkers described the role of glycans in the function of epidermal growth factor receptor (EGFR) (39). Proper N-glycosylation is required for

ligand-binding (40); once EGFR is glycosylated, it binds specific N-glycan ligands. For example, on N420 of EGFR are involved in dimerization (41) and highlights that changes in glycosylation can have profound effects on the signaling capacity and activation of receptors. For CSF3R, WT protein treated with N-glycosidases results in a 17kDA band shift, indicating glycosylation in a significant portion of the protein (24). When CSF3R is mutated, as with T618I, it loses O-linked glycosylation in the membrane-proximal region of CSF3R, increases ligand-independent receptor activation, and leads to oncogenesis. Although interplay is known between neighboring phosphorylation sites (42), this has not yet been studied in proximal N- and O- linked glycosylation sites. Mass-spectrometry analysis of the CSF3R T618I mutant protein showed that the N610 site was still occupied. One of the glycan structures was the same between the mutant and WT. Although, this result does not rule out subtle differences in N-linked glycosylation at this site, it does indicate that the T618I mutation does not grossly alter the occupancy of the nearby glycosite.

In this study, we identify CSF3R N610H as a rare therapeutically relevant germline mutation in myeloid leukemia. The germline nature of these mutations suggests that they may represent leukemia predisposition variants. Further studies will be needed to determine whether CSF3R N610 mutations are recurrent in familial leukemia. CSF3R N610 mutations are highly activating and cause ligand-independence with increased phosphorylation of STAT3. We verified that both ruxolitinib and trametinib inhibit the viability of Ba/F3 cells expressing the CSF3R N610H and N610S mutations and found them to be as sensitive to both compounds as the well-characterized CSF3R T618I mutation. Furthermore, these mutations reveal the critical importance of membrane-proximal N-linked glycosylation for the maintenance of ligand dependency. This study highlights how careful investigation of cancer-associated mutations can provide critical insight into the relationship between protein structure and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

This study reveals the critical importance of membrane-proximal N-linked glycosylation of CSF3R for the maintenance of ligand dependency in leukemia

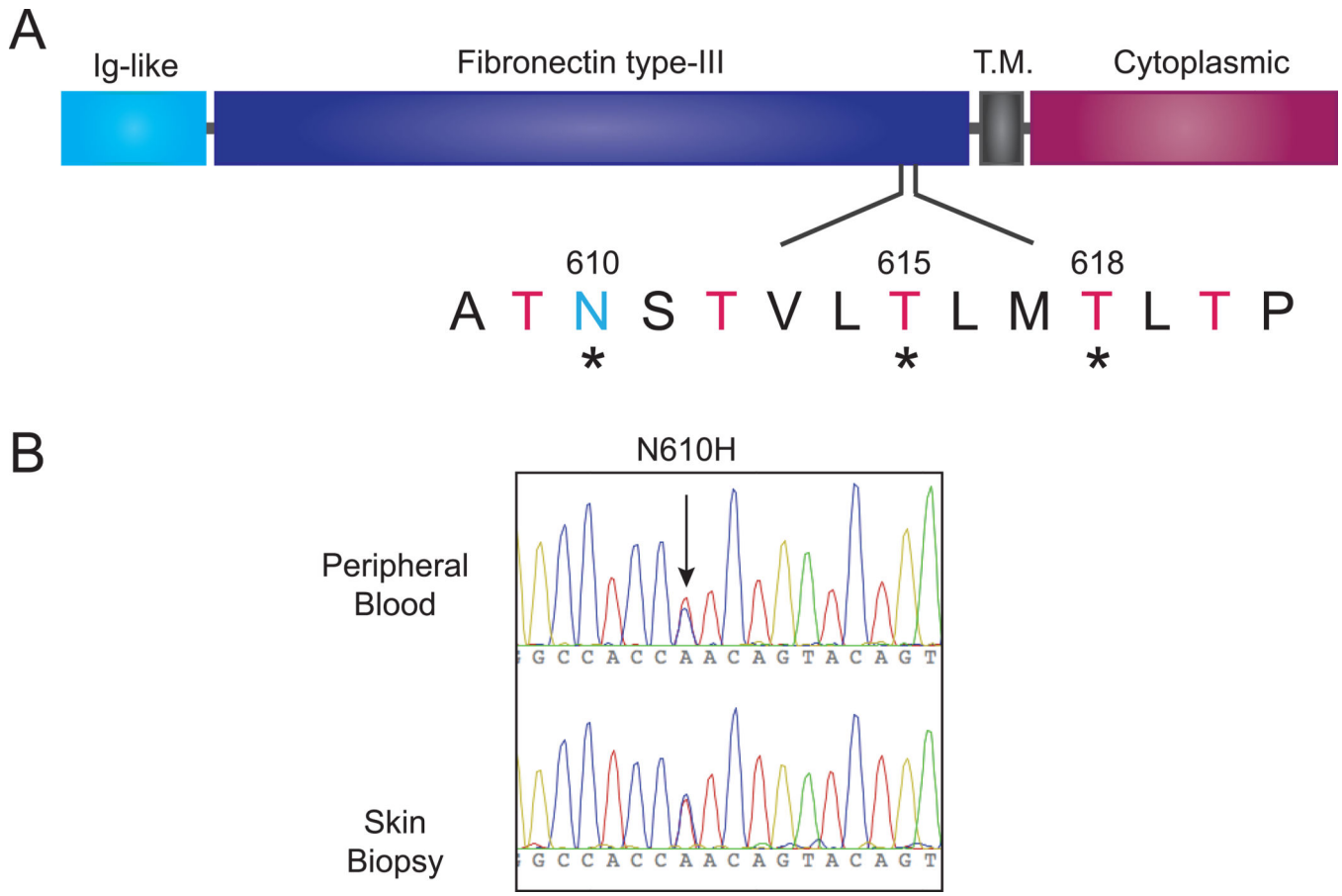

Figure 1. A novel germline mutation in CSF3R.

A. Schematic of the CSF3R membrane-proximal location of the N610H mutation and nearby leukemia-associated T615A and T618I mutations. B. Sanger sequencing of CSF3R exon 14 confirms the presence of the CSF3R N610H mutation in both the peripheral blood and skin biopsy from a patient with a myeloproliferative neoplasm. T.M. refers to transmembrane domain

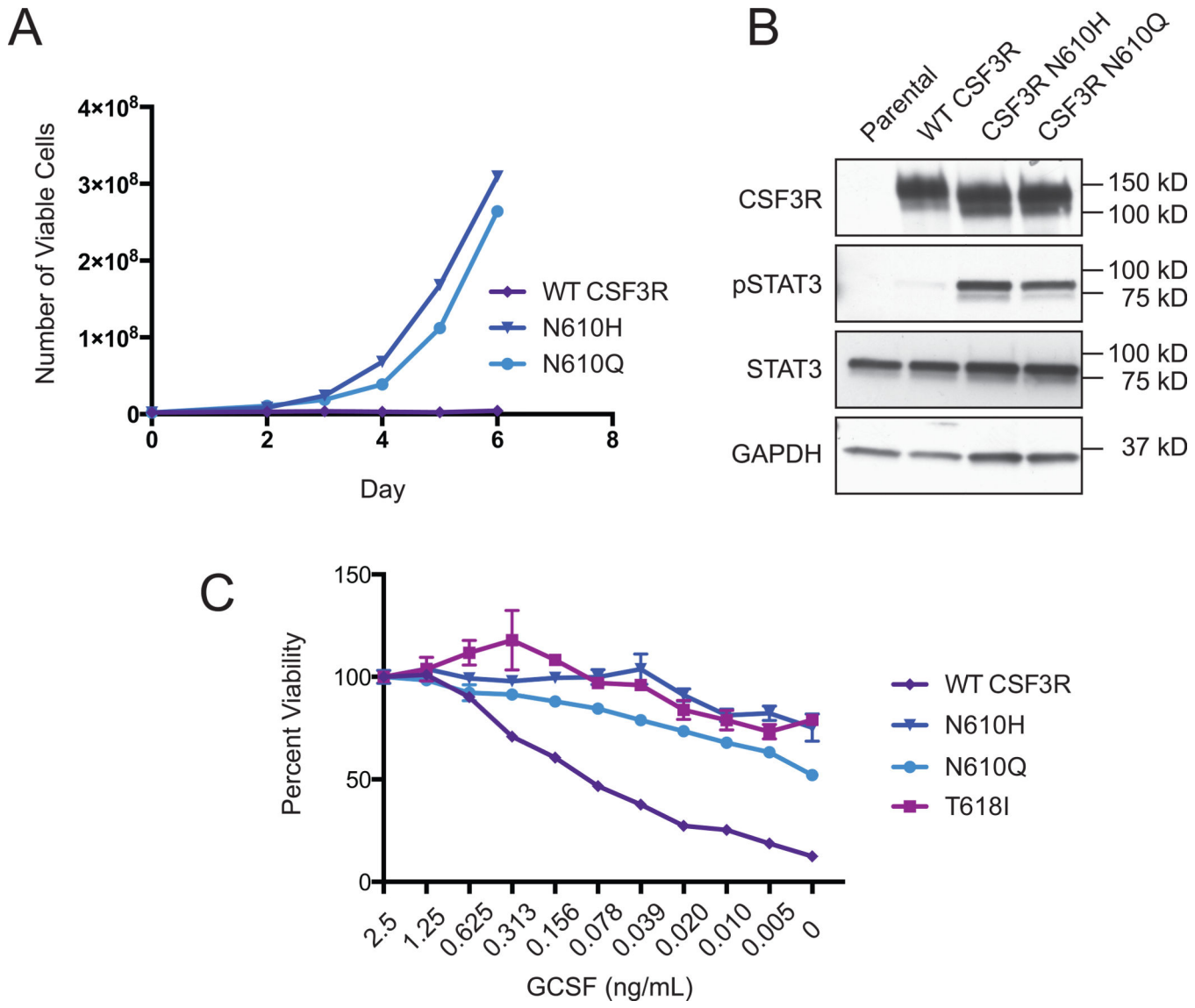

Figure 2. The CSF3R N610H mutation is transforming and activates the JAK-STAT pathway in a ligand-independent manner.

A. Transforming capacity of the CSF3R N610H (and a conservative N610Q) mutations in the murine Ba/F3 cytokine-independent growth assays. B. The CSF3R N610H mutation activates the JAK/STAT pathway as measured by immunoblotting of phosphorylated-STAT3 (pSTAT3) in 293T17 cells transiently transfected with WT or CSF3R mutants. N610H (the CSF3R N610H mutation), N610Q (CSF3R N610Q mutation, a conservative substitution) and T618I (the CSF3R T618I mutation found commonly in CNL). C. The CSF3R N610H, N610Q and T618I mutation confers ligand-independence to the receptor as measured by a titration of GCSF in Ba/F3 cells expressing WT CSF3R. Cell lines were plated in decreasing concentrations of GCSF and then cell viability was measured after 72 hours using a tetrazolamine based assay (CellTiter Aqueous one solution cell proliferation MTS assay). Total protein loading was confirmed by GAPDH.

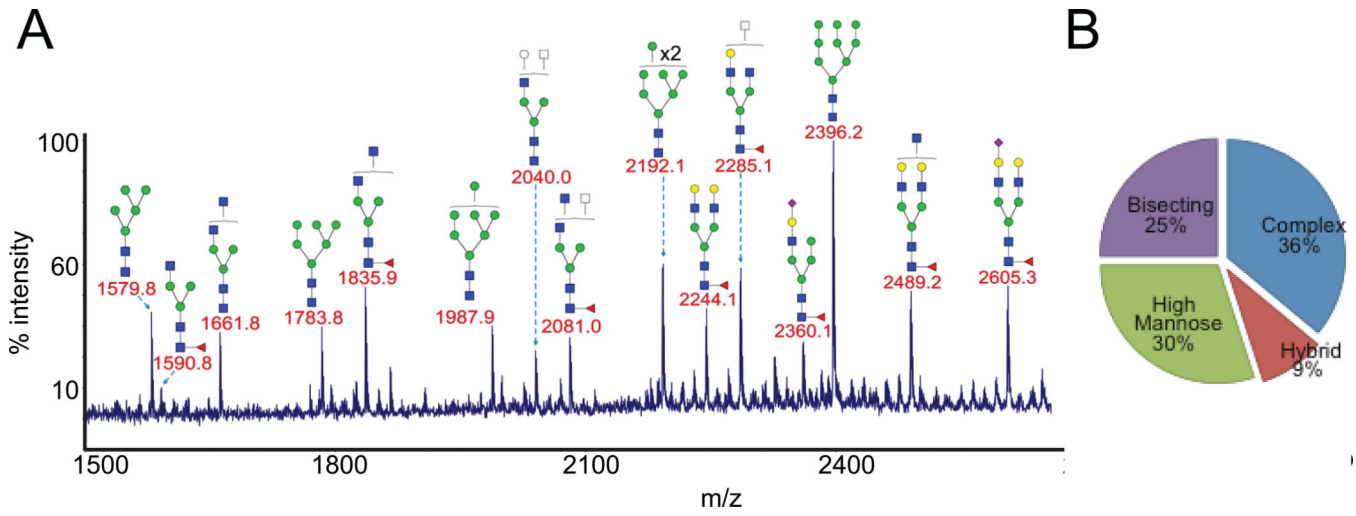

Figure 3. Annotated MALDI-TOF MS spectra of permethylated N-glycans (m/z 1500–3000) from wildtype CSF3R.

A. Released permethylated N-glycan analysis by MALDI. Glycan structures identified were confirmed by electrospray ionization mass spectrometry (ESI-MS/MS). Putative structures are based on the molecular weight and N-glycan biosynthesis pathway. B. The ratio indicates a comparison of the glycan relative abundance to those identified.

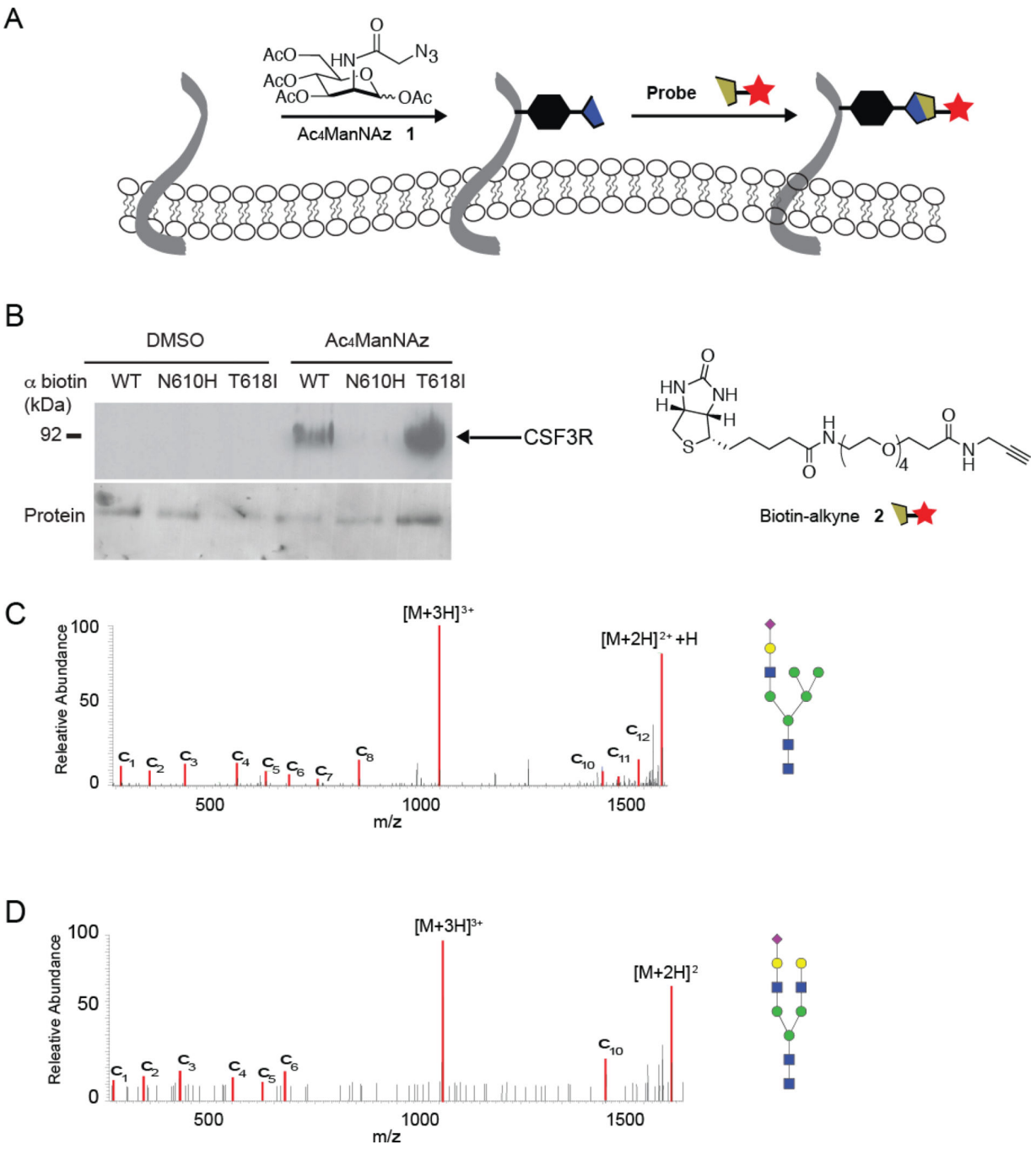

Figure 4. Identification of the glycan at N610.

Schematic of labeling of sialic acid glycoproteins with an azide-functionalized sugar (i.e. $Ac_4ManNAz$, **1**). B. Wild-type, N610H or T618I CSF3R were transfected in 293T cells and then incubated with either $Ac_4ManNAz$ or DMSO, as a control. After incubation, cells were lysed and reacted with a DIBCAC-functionalized biotin probe (Biotin-alkyne, **2**). Sialoglycoproteins (biotinylated) were immunoprecipated using an avidin resin and visualized with an HRP-conjugated anti-FLAG. Equal avidin resin was loaded. C-D. The MS/MS spectra of electron transfer dissociation (ETD) fragmented N610 peptide

"AASQAGATNSTVL". C. The most intense peak corresponds to the peptide with a $HexNAc_3Hexose_6NeuAc$. D. Spectrum corresponds to glycan structure $HexNAc_3Hexose_6NeuAc$. The illustrated glycans depict possible structures for each glycan composition GlcNAc; yellow square: GalNAc; white square: HexNAc; yellow circle: Glycoworkbench (43,44) was used for creating the glycan structure figures. Galatose; green circle: mannose; white circle: Hexose; purple diamond: N-Acetylneuraminic acid (Neu5Ac).

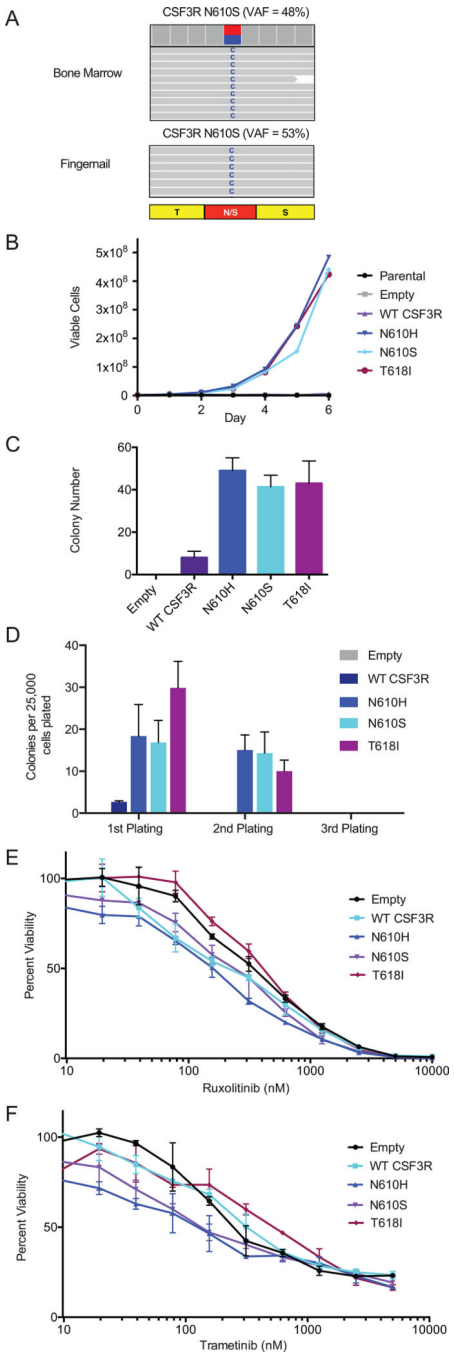

Figure 5. A germline N610S mutation identified in a patient with CML is transforming and therapeutically targetable.

A. Sequencing of bone marrow and fingernail on the MSKCC IMPACT panel reveal a germline CSF3R N610S mutation in a patient with chronic phase CML. B. The CSF3R N610S mutation is transforming in the Ba/F3 cytokine independent growth assay. C. The CSF3R N610H and N610S mutations confer cytokine-independent growth in a mouse bone marrow colony assay. D. N610H and N610S confer serial replating capacity in the absence of cytokines. E. Sensitivity of CSF3R expressing Ba/F3 cells to the JAK kinase inhibitor ruxolitinib. Ba/F3 cells were grown in the presence (Mig empty and WT CSF3R) and

absence (N610H, N610S and T618I) of the cytokine, IL3 in 96 well format. Cell lines were plated in triplicate and were treated with a dose curve of ruxolitinib. After 72 hours, cell viability/proliferation was measured using a tetrazolium based CellTiter Aqueous One Solution and read on a plate reader. Viability is represented as a percentage of the untreated control. F. Sensitivity of cells expressing CSF3R mutants to the MEK inhibitor trametinib as outlined in panel E. Parental (untransformed cells), Mig empty (empty vector control), N610H (the CSF3R N610H mutation), N610S (the CSF3R N610S mutation) and T618I (the CSF3R T618I mutation). VAF = variant allele frequency.