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Enhanced MAPK signaling is essential for CSF3R induced leukemia

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Abstract

Both membrane-proximal and truncation mutations in *CSF3R* have recently been reported to drive the onset of chronic neutrophilic leukemia (CNL). Here we show that although truncation mutation alone can not induce leukemia, both proximal and compound mutations (proximal and truncation mutations on same allele) are leukemogenic with a disease latency of 90 and 23 days, respectively. Comparative whole-genome expression profiling and biochemical experiments revealed that induced expression of Mapk adaptor protein *Ksr1* and enhanced Mapk signaling are crucial to leukemogenesis by *CSF3R* proximal and compound mutants. Moreover, inhibition of Mek1/2 by trametinib alone is sufficient to suppress leukemia induced by both *CSF3R* proximal and ruxolitinib-resistant compound mutations. Together, these findings elucidate a Mapk-dependent mechanism of *CSF3R-induced* pathogenesis, and they establish the rationale for clinical evaluation of MEK1/2 inhibition in CNL.

Introduction

The discovery that constitutive kinase activity of BCR-ABL is sufficient and necessary to induce chronic myeloid leukemia (CML) laid the foundation for the development of anti-kinase therapy(1, 2). Subsequently, the clinical success of kinase inhibitors in treating CML not only revolutionized anti-kinase therapy in cancer but also invigorated efforts to identify driver oncogenes in other malignancies for therapeutic targeting(3, 4). In 2013, Maxson and

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colleagues reported mutations in colony-stimulating factor 3-receptor (CSF3R) from 60-80% of chronic neutrophilic leukemia (CNL) and BCR-ABL negative atypical-CML (aCML) patients(5, 6). These mutations are clustered into two different regions in CSF3R: the membrane proximal region (proximal mutations), and frame-shift or nonsense mutations in the cytoplasmic tail resulting in premature truncation of the receptor (truncation mutations).

Mutations in CSF3R (truncation mutations) were first observed in patients with severe congenital neutropenia (SCN) and were suggested to be sufficient to induce SCN (7, 8). Truncated receptors exhibit increased and prolonged activation of downstream signaling, which induces a hyper-proliferative response and clonal expansion, an essential step in leukemic progression(9-18). However, studies using knock-in mice expressing truncated CSF3R did not develop leukemia despite prolonged GCSF treatment(19-22). Thus, CSF3R truncation mutation is not sufficient for leukemia, but represents an early event in leukemogenesis. In contrast, membrane proximal mutations prevent *O*-glycosylation of CSF3R that increases active dimeric receptor configuration, resulting in constitutive activation(23). Expression of CSF3R proximal mutants is sufficient to recapitulate the clinical features of CNL in mice(5, 24).

Analysis of a large cohort of CNL patients revealed that a significant majority of CSF3R mutant cases of CNL (33%) harbor both membrane proximal and truncation mutations on the same allele (compound mutation)(25). It is currently not clear whether compound mutants are sufficient to induce leukemia. Previous studies analyzing proximal and truncation CSF3R mutants showed their dependence on JAK2 and SRC kinase signaling, respectively. Given that the compound mutations include both of these genetic lesions, it is formally possible that compound mutations could be sensitive to JAK2 and/or SRC kinase inhibition. Besides, both CNL and aCML lack established standard of care and are associated with a poor prognosis, it is imperative to molecularly characterize CSF3R compound mutations to develop treatment options for these high-risk CNL patients.”

Because truncation mutations cannot induce leukemia, we reasoned that a comparative *in vivo* characterization and molecular analysis of CSF3R mutants on an isogenic background would uncover the mechanistic underpinnings of disease development and identify critical drug targets for effective treatments. Here we show that both proximal and compound-CSF3R-mutation induced leukemia is dependent on enhanced MAPK signaling. Non-leukemic truncation mutations cannot activate Mek and MAPK pathway due to reduced expression of Mapk adaptor protein Ksr1 (kinase suppressor of Ras 1). Surprisingly, Mek1/2 activation is restored in compound mutation, which induces aggressive leukemia with a disease latency of 3-4 weeks (similar to BCR-ABL-induced CML). In support of clinical observations, mice transplanted with compound mutations do not respond to ruxolitinib treatment. As a proof of concept, we demonstrated that therapeutic targeting of Mek1/2 by trametinib alone suppressed leukemia development in mice expressing either proximal or compound mutations. These results underscore a central role of enhanced Mapk signaling in CSF3R-induced leukemia and provide a proof-of-principle for therapeutic intervention for high risk CNL with compound CSF3R mutations.

Materials and Methods

Plasmids and Constructs

Human CSF3R cloned in pMCV-Sport6 vector was purchased from PlasmID, Harvard Medical School. An HA-tag for protein isolation and immunoblotting was attached at the c-terminus of the receptor by site directed mutagenesis using primers described in supplementary methods and supplementary Table1.

Chemical reagents and cytokines

JAK2 kinase inhibitors ruxolitinib, AZD1840, CYT-387, fedratinib were purchased from Chemitek (Indianapolis, Indiana). Mek inhibitors PD039512 and Trametinib were purchased from LC laboratories (Woburn, MA). Recombinant human GCSF was purchased from Peprotech (Rockyhill, NJ).

Cell proliferation assays and Immunoblotting

1×10^4 cells were seeded in 96 well plates in 100 μl of media with or without GCSF (50ng/ml) and appropriate drug concentrations. The cells were incubated for 60 hours. Cell viability and immunoblottings were performed as described earlier (26).

RNAseq analyses of Kit$^+$ cells from wild type CSF3R mutants

Total RNA was isolated from four to five millions of BM-derived kit$^+$ cells expressing CSF3R variants isolated by FACS using GFP as a asurrogate marker. Total RNA was used to perform RNA-seq (20 million reads with paired ends) at DNA sequencing core of Cincinnati Children's Hospital. Difference in the expression of every gene was measured by t-test between the samples from the non-leukemic (truncation mutations) and leukemic (proximal and compound mutations). Up- or down-regulated genes were chosen based on a p-value cut-off of 0.05. For networks, down- or up-regulated genes were used as seeds to build a coherent network using Gene-Connector functionality in NetWalker suite(27).

Real-Time Q-PCR Analysis

Two μg of total RNA was used to synthesize cDNA using Superscript III first strand synthesis kit (Life technologies). Q-PCRs were performed using the SYBR green method in StepOnePlus real PCR system (ABI). All PCRs were performed in triplicates and the real time data was normalized to β-actin or GAPDH expression.

Mice

Eight to ten weeks old C57Bl/6 mice were purchased from Jackson laboratories and housed in the barrier facility at Cincinnati Children's Hospital. All mouse experiments were performed under an IACUC-approved protocol of the Cincinnati Children's Hospital in accordance with accepted national standards and guidelines.

Results

CSF3R compound mutations are resistant to Jak2 inhibitors

Because CSF3R proximal and truncation mutants were shown to be sensitive to either SRC or JAK2 inhibition suggesting CSF3R compound mutation can be targeted by both SRC and JAK2 inhibitors either alone or in combination. In this regard, a dual JAK2/SRC inhibitor may be an ideal choice to target CNL. To test these possibilities, an unbiased in vitro dose response cell proliferation analysis was performed using the kinase inhibitors ruxolitinib, dasatinib, and fedratinib (a dual JAK2/SRC inhibitor) on BaF3 cells expressing proximal mutation (CSF3R-T618I), truncation mutations representing two extremities of mutational spots (CSF3R-Q741* and CSF3R-W791*), and compound mutation (CSF3R-T618I/Q741* and CSF3R-T618I/W791*), as shown in Figure 1 and Figure S1. As reported earlier, the expression of these mutants transforms BaF3 cells, rendering them independent of growth factor, but differed in growth kinetics and transformation potential, as follows: compound mutations>proximal mutation>truncation mutation>wild-type (Figure S2). In contrast to the previous report, both proximal and truncation mutations showed sensitivity to Jak2 inhibition tested with ruxolitinib and AZD1480 (Figure 1 and Figure S3 a and b), and conferred resistance to the SRC inhibitor dasatinib (Figure S3 c and d). Likewise, both proximal and truncation mutations displayed equal sensitivity to fedratinib (a dual Jak2/Src inhibitor), suggesting truncation mutations are not dependent on SRC family kinase, TNK2 (data not shown). Interestingly, CSF3R compound mutations displayed resistance to both JAK2 and SRC inhibitors (Figure 1 and Figure S3), suggesting these mutants may pose formidable clinical challenges but may also explain the reason for poor prognoses.

CSF3R compound mutations induce aggressive lethal leukemia

While BaF3 cellular transformation assay is a useful surrogate model system for the assessment of the oncogenic potential of a given oncogene, it fails to fully recapitulate in vivo disease development. To determine whether a compound mutation is sufficient to induce leukemia, bone marrow retroviral transduction and transplantation assays were performed using BM-derived hematopoietic kit^+ cells from C57Bl/6 mice transduced with retroviruses expressing proximal, truncated, and compound mutations of CSF3R. Five irradiated mice for each mutation were transplanted with 100,000 GFP-positive cells (a surrogate marker for oncogene expression). Mice were monitored weekly for disease development and leukemic progression using peripheral blood to determine the levels of WBCs, granulocytes, monocytes, B and T cells by FACS. As expected, CSF3R-T618I induced lethal granulocytic leukemia with a disease latency of 90–120 days (Figure 2b). In contrast, mice transplanted with truncation mutations failed to develop leukemia, as leukocyte levels in these mice never exceeded beyond the normal range of WBCs (Figure 2c, and Figure S4). Strikingly, compound mutation CSF3R-T618I/Q741* induces aggressive leukemia with a disease latency of 3 weeks comparable to BCR-ABL induced CML. Whereas mice transplanted with CSF3R-W791/T618I showed very high levels of leukocytes (100–200 k/μl) but displayed a disease latency similar to proximal mutation, 80–100 days, (Figure 2b).

Up-regulation of MAPK network genes in CSF3R membrane-proximal and compound mutants

Because truncation mutations are unable to induce leukemia development despite persistent granulocytic hyperplasia, it is here reasoned that a whole genome expression studies using primary cells might illuminate critical gene/s or pathways essential for disease development under CSF3R pathogenesis. To test this, expression studies were performed using RNA-seq and total RNA from BM-derived kit^+ cells expressing wild-type and mutant CSF3R proteins (Figure S5 a). A comparative RNAseq expression analysis, normalized to kit^+ cells expressing vector (kit^+ cells from C57Bl6 mouse transduced with vector, pMSCV-Ires-GFP) showed altered expression of 5334 genes (data not presented). However, a comparison of CSF3R proximal and compound mutations with truncation mutations displayed differential expression of only 498 genes (Figure S5b). Differences in the expression of every gene were measured by t-test between the samples from the truncation mutations (CSF3R- Q741* and CSF3R-W791*) and leukemic variants (CSF3R-T618I and CSF3R-T618I/Q741*). Up- and down-regulated genes were chosen based on a *P*-value cut-off of 0.05. Further analyses of these differentially expressed genes using Netwalker revealed changes in expression of 23 genes in Mapk pathway (Figure 3a and Figure S5b and c). Most prominent among these are Mapk adaptor protein Ksr1 (kinase suppressor of Ras 1), Pak6, and Bcl2l1 (Figure 3b). Quantitative expression analysis by q-PCR revealed that cells expressing truncation mutations display suboptimal Ksr1 expression in comparison to proximal and compound mutations, while expression of Pak6 and Bcl2l1 are significantly reduced in comparison to both CSF3R-WT and its mutants (Figure 3b). Together these data suggest that failure of truncation mutations to induced leukemia is likely due to inefficient MAPK signaling.

Enhanced MAPK activation in cells expressing CSF3R membrane-proximal and compound mutants

KSR1 is a scaffold protein in RAS/RAF/MEK/MAPK pathway, which regulates the intensity and duration of MAPK cascade activation. KSR1 is a cytosolic protein and constitutively bound with MEK. However, during signal transduction, it translocates to the cell membrane, where it interacts with RAF. In this way, it facilitates the interaction of RAF with its substrate MEK1/2, which in turn activates ERK1/2 to relay the signals (Figure 3c). This indicates that Ksr1 deficiency could result in defective MAPK signaling due to reduced Mek1/2 and Erk1/2 activation. To assess this, immunoblot analysis was performed to detect the levels of activated Mek1/2, Erk1/2, Stat5, Stat3, and Src using phospho-specific antibodies. Expression of CSF3R proteins were detected by anti-HA antibody and normalized to total GFP expression, as they were expressed using bicistronic vector pMSCV-Ires-GFP. Quantitative analysis of CSF3R expression revealed the overexpression (~6-10 fold) of CSF3R truncation and compound mutations in comparison to wild-type CSF3R and membrane proximal mutation (Figure 4 a and b), which is presumably due to the loss of endocytic degradation motifs (14). Further analyses revealed that both CSF3R membrane-proximal and compound mutants show increased activation of Stat3 and Stat5 at baseline in comparison to CSF3R-WT and vector control (Figure 4a). In contrast, truncation mutations displayed reduced activation of STAT3 and STAT5 where reduction in CSF3R-Q741*>CSF3R-W791* (Figure 4 a and b). Interestingly, GCSF treatment restored full activation of STAT3 and STAT5 in cells expressing truncation mutations (Figure 4 a and b).

Likewise, both CSF3R membrane-proximal and compound mutants displayed enhanced activation of Mek and Erk at baseline and after GCSF stimulation (Figure 4a and b). However, while cells expressing truncation mutations lack baseline activation of Mek and Erk, GCSF treatment induced modest Mek and Erk activation (Figure 4a and b). As in previous reports, activation of Src family kinase activation by CSF3R-Q741* was here observed. However, CSF3R proximal mutation (T618I) and truncation mutation (W791*) do not activate Src family kinases (Figure 4a). Paradoxically, compound mutations exhibited restored Mek and Erk activation and enhanced activation of Stat3 and Src family kinases (Figure 4a).

Reduced expression of Ksr1 and failure to fully activate the Mek1/2 in truncation mutations suggest that CSF3R-induced leukemia, such as Ras driven-malignancies, is critically dependent on enhanced MAPK signaling. Therefore, we hypothesize that targeting Mek1/2 alone will be effective against both CSF3R proximal mutations and ruxolitinib-resistant compound mutations. To assess this, dose-response analysis of CSF3R mutants against PD032590 (Mek1/2 inhibitor) was performed and in combination with increasing amount of ruxolitinib. No difference in cell proliferation was observed in response to Mek inhibitor alone (data not shown). However, a combination of PD032590 (200 nM) with ruxolitinib sensitized both proximal and compound mutations. Notably, all CSF3R mutations showed 2–3 fold reductions in IC50 values in comparison to single ruxolitinib treatment (Figure S6). Next, hematopoietic colony forming unit (CFU) assays were performed using mouse bone-marrow-derived kit^+ cells expressing different CSF3R mutants (Figure 5a). Although it is not a perfect system for the evaluation of the efficacy of drugs, nonetheless, this assay allows the user to quickly determine the cytotoxic effect of a given chemical agents and provides a range of effective drug concentrations for in vivo evaluations. CSF3R-expressing kit^+ cells plated with 5 nM of PD032590 alone suppressed CSF3R proximal (40-20%), truncation (50-60 %) and compound mutations (40-25%), Figure 5b. Likewise, treatment with ruxolitinib alone [1 μM] showed 50–60% reduction in CFU formation. Strikingly, cells treated with a combination of PD032590 [5 nM] and ruxolitinib [1 μM], showed potent response against CSF3R truncation and compound mutations (Figure 5b). Altogether, these data suggest that Mek1/2 inhibition alone could be effective against CSF3R expressing cells while a combination of Mek1/2 and Jak2 inhibitors perhaps would be more effective in targeting the ruxolitinib-resistant compound mutations.

Trametinib treatment alone suppresses *CSF3R-induced* leukemia

Next, we determined the in vivo efficacy of Mek1/2 and Jak2 inhibitors in leukemic mice induced by CSF3R proximal and ruxolitinib resistant compound mutation, T618I/Q741*. Bone marrow kit^+ cells were transduced with CSF3R-T618I-Ires GFP and CSF3R-T618I/Q741*-Ires-GFP retroviruses. Mice were transplanted with 100,000 GFP-positive cells mixed with 0.3 million RBC-depleted bone marrow cells from the wild-type mouse (Figure 6a). Mice were treated with a clinically approved Mek1/2 inhibitor (trametinib, 10 mg/kg twice daily) or ruxolitinib (50 mg/kg twice daily) and a combination of ruxolitinib +trametinib (50+10 mg/kg twice daily) after two weeks of transplantation. As shown earlier, mice recipients of proximal mutation responded to ruxolitinib treatment, with prolonged survival and reduction in WBC count but not to a normal level (Figure 6b). In contrast,

ruxolitinib treatments were ineffective against compound mutation and all mice in this cohort succumbed to disease within 7–8 weeks (Figure 6c). Strikingly, trametinib treatment alone induced complete hematological response and protected mice in both groups from developing leukemia (Figure 7 a and b). However, both trametinib alone and the combination of trametinib+ruxolitinib treatment did not show any significant change in leukemic allele burden monitored by changes in the level of circulating GFP^+ cells (Figure 7 a and b, bottom panels). Together these data provide evidence for the necessity of enhanced MAPK signaling in CSF3R-induced pathogenesis, and trametinib treatment alone is sufficient to suppress both proximal and ruxolitinib resistant compound mutation.

Discussion

The normal function of CSF3R signaling is to promote growth and survival of neutrophils; mice lacking CSF3R develop neutropenia(28). Likewise, during infection, the innate immune response increases the level of GCSF rapidly, which increases neutrophil levels to combat infection(29). Under normal conditions, activation of wild type recpetor activates both Jak (JAK2 and JAK1)(30, 31), and Src family kinases (Lyn and Syk)(32), which results in activation of STAT5 and STAT3(33-36). However, oncogenic CSF3R mutants preferentially signal either through JAK2 (proximal mutation) or TNK2 (truncation mutation), consequentially display differential sensitivity to inhibition by tyrosine kinase inhibitors, ruxolitinib, and dasatinib, respectively. In contrast, we observed that both proximal and truncation mutations are sensitive to ruxolitinib but resistant to the Src inhibitor dasatinib. Notably, truncation mutations are more sensitive to Jak2 inhibition than proximal mutation (Figure 1). This apparent difference between our results and that of Maxson et al. might lie in the patient-derived cells they used for drug screening (5). Perhaps some unknown mutation in the patient samples, either alone or in the context of CSF3R truncation mutation, conferred sensitivity to SRC inhibition. It would be very interesting to determine the genetic context for dasatinib sensitivity in CNL, perhaps a whole genome sequencing coupled with expression profiling studies may inform the underlying mechanism.

Patients harboring compound CSF3R mutations were presumed to be sensitive to either ruxolitinib or dasatinib. Moreover, given that truncation mutations alone are non-leukemogenic, compound mutations (which include truncation) could by extension be either non-leukemic or have weakened leukemia. Our results showed that compound mutations induce aggressive leukemia, although they did so with variable disease latency, which seemingly depend on the length of truncation. For example, compound mutations with shorter CSF3R due to truncation at Q741* displayed a disease latency similar to BCR-ABL in CML, while truncation at W791* resulted in disease latency similar to proximal mutation suggesting that the truncation site has a profound impact on disease progression. This additive or synergistic effect of mutations (displayed by compound mutations) could be due to the altered interaction of positive and negative regulators, which results into the restoration of Mek/Erk activation and enhanced Stat3 and Src family kinase activations. While ruxolitinib treatment alone displayed significant therapeutic response against proximal mutation(24), it is ineffective against compound mutation, which suggests that Jak2 is not a consistent target in CSF3R-induced leukemia.

Using a comparative expression profiling of leukemic (CSF3R-proximal and compound mutation) and non-leukemic (CSF3R truncation mutation) cells, we found that the expression of Mapk adaptor protein Ksr1 was observed in cells expressing leukemic CSF3R mutants. Accordingly, reduced Ksr1 expression in cells expressing truncation mutations showed low Mek or Erk activation. Even higher concentrations of GCSF failed to fully activate Mek/Erk via the truncated receptor, supporting the notion that enhanced Mapk signaling constitutes a critical node for transformation in CSF3R-induced leukemia, which is modulated by the Mapk adaptor protein Ksr1. Ksr1 is a scaffolding protein that orchestrates the assembly of the mitogen-activated protein kinase (Mapk) cascade to promote activation of Mek and Erk. Mice lacking Ksr1 revealed that it is not required for normal cellular function; however, its depletion resulted in loss of the high-molecular-weight Raf/Mek/Erk complex and decreased Mapk signaling (37). Later studies demonstrated that the Ksr1 is essential where enhanced Mapk output is needed. For instance, Ksr1 deficient cells are resistant to transformation by oncogenic Ras (38),(39). Together these studies revealed that Ksr1 is not essential to basal Mek/Erk activation but crucial to enhanced Mapk signaling and that it constitutes non-oncogene dependence under conditions in which enhanced Mapk output is necessary, such as in Ras-driven cancers. Our data clearly show that cells expressing CSF3R truncation mutation are defective in Mek/Erk activation, which provides one explanation why these mutations are unable to induce leukemia. Conversely, these results suggest that enhanced MAPK signaling is crucial to CSF3R-induced leukemia. As envisioned, Mek inhibition by trametinib is equally effective and sufficient to completely suppress leukemia in both proximal and compound mutations. While we observed ruxolitinib treatment to be effective against CSF3R proximal mutation, it failed to suppress compound-mutation-induced leukemia, suggesting that patients harboring compound mutations (37%) will be refractory to Jak2 inhibitors.

In sum, these data provide evidence that Mapk signaling constitutes non-oncogene dependence in CSF3R-mutant CNL; providing a new therapeutic option for the 37% of CNL patients with compound mutations. These findings elucidate a novel paradigm of CNL pathogenesis and explain how CSF3R mutations drive the development of the disease. Our study provides a proof-of-concept for the therapeutic targeting of Mek and establishes the rationale for clinical evaluation of this concept.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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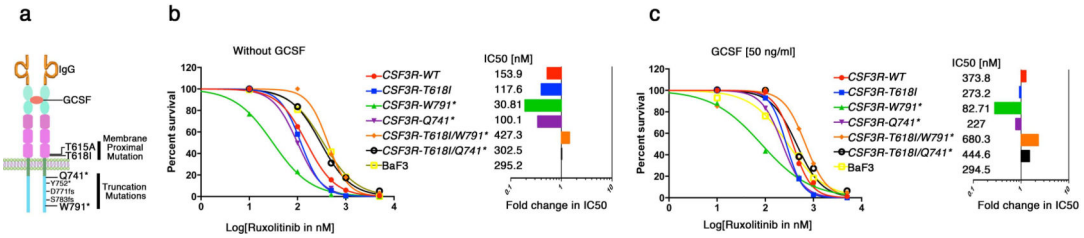

Figure 1. CSF3R compound mutations are resistant to Jak2 inhibitors

a. A cartoon depiction of CSF3R receptor showing the location of *CSF3R* mutations. **b and c.** Showing dose–response sigmoidal curve of BaF3 cells for ruxolitinib determined without GCSF (b) and with 50 ng/ml of GCSF (c). IC_{50} values are indicated in parenthesis and relative fold differences in the IC_{50} values are shown in bar graph. Plate assays were repeated three times with similar results.

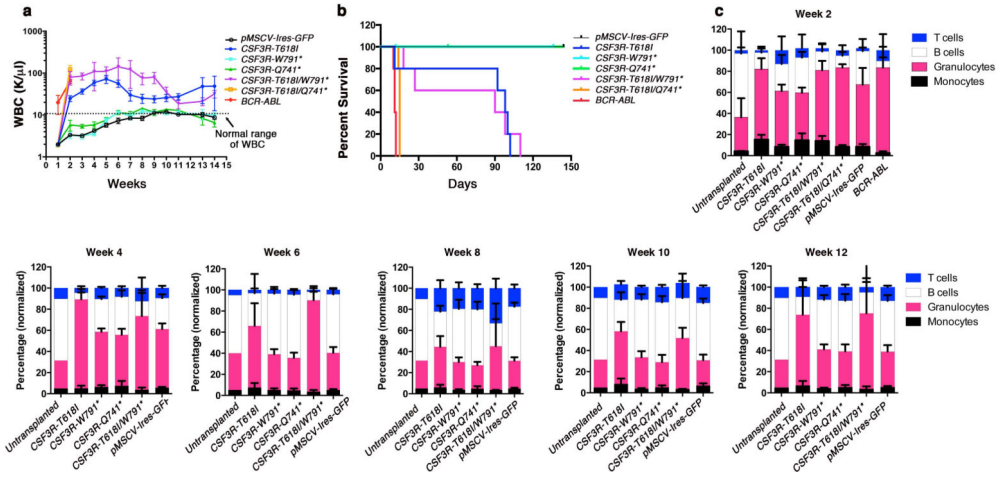

Figure 2. CSF3R compound mutations induce aggressive neutrophilic leukemia

a. Graph showing the total WBC levels from the peripheral blood. CSF3R proximal and compound mutations induce leukocytosis while truncation mutations display normal range of white blood cells (WBCs). **b.** Survival curve of mice showing CSF3R proximal and compound mutations induce lethal leukemia with a disease latency of 30 and 90 days, respectively. **c.** Bar graphs showing the levels of granulocytes, monocytes, B and T cells. Representative data are from three independent transplants.

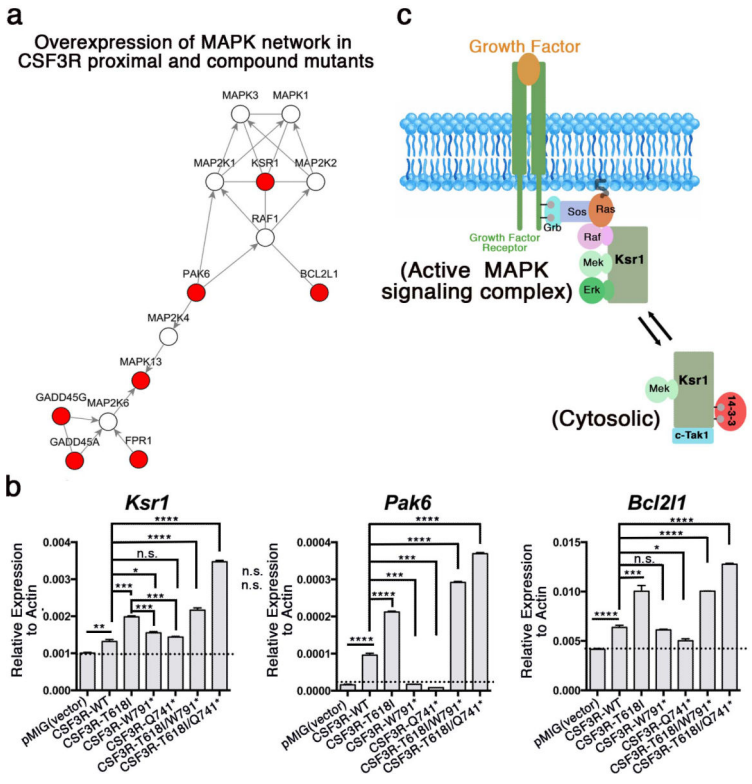

Figure 3. Induction of MAPK network genes in CSF3R membrane-proximal and compound mutants

a.Differential modulation of 23 MAPK pathway genes between leukemic and non-leukemic CSF3R variants. Overexpressed genes are shown as red circles in the network. **b.** Bar graphs showing the relative expression of Ksr1, Pak6 and, Bcl2l1. Representative data shown are the mean of two independent qPCR analysis performed in triplicate ± S.D. (* = $p < 0.05$, ** = $p < 0.01$, ***=$p<0.001$, and ****=$p<0.0001$). **c.** A cartoon depiction of Ksr1 in steady state (cytosolic) and membrane bound during active growth factor signaling.

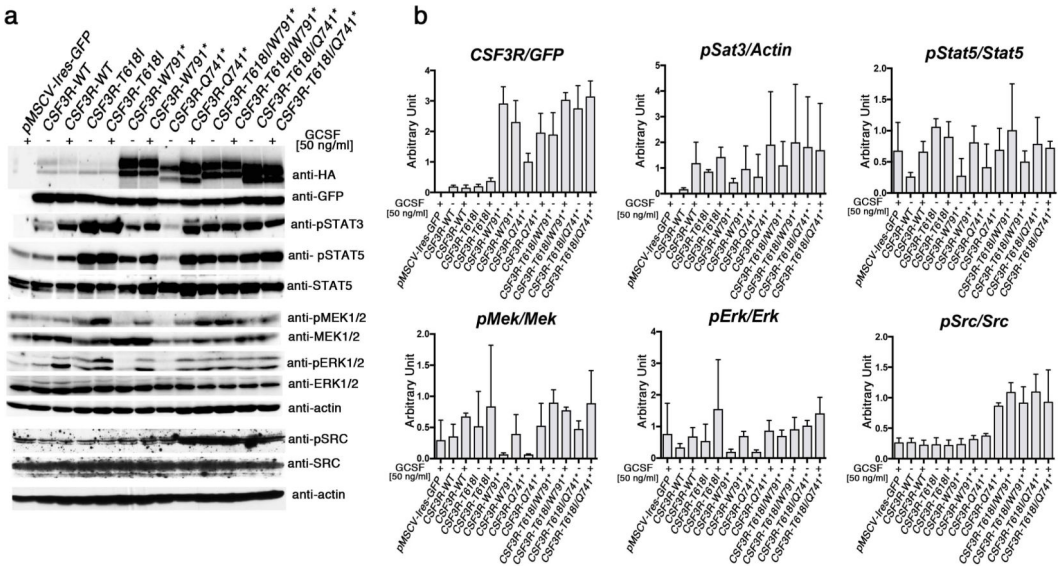

Figure 4. Enhanced MAPK signaling in membrane proximal and compound CSF3R Variants

a. Immunoblots from the total cell extracts of BaF3 cells expressing CSF3R variants probed with anti-HA to determine the expression CSF3R. Signaling proteins were determined by phospho-specific antibodies (indicated on the right side of the blot) normalized to total poteins or actin. Representative immunoblots are from two independent experiments. **b**. Bar graphs showing levels of CSF3R and phosphoproteins (pSTAT3, pSTAT5, pMek1/2, pErk1/2 and pSRC) normalized to respective total protein in CSF3R expressing cells. Representative data shown are the mean values ± SEM from two independent experiments.

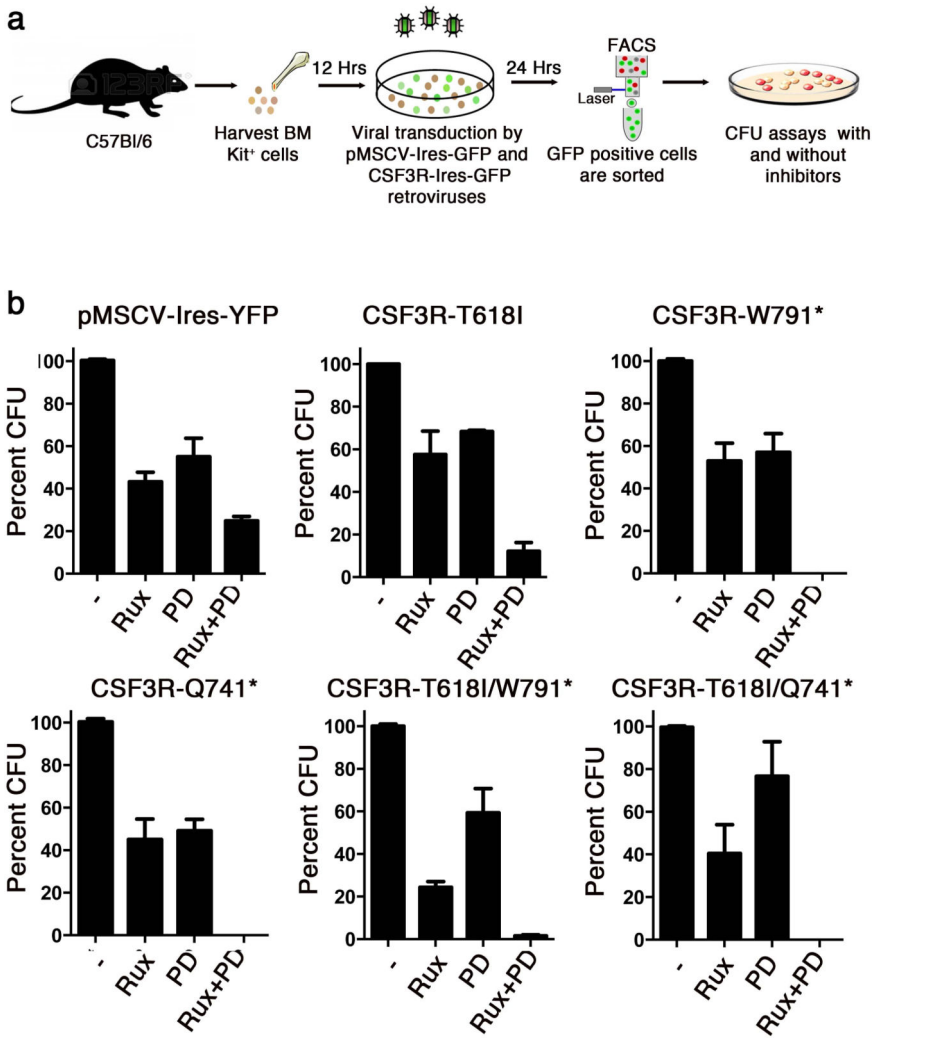

Figure 5. A combined Mek1/2 and Jak2 inhibition completely suppressed the CFU formation in BM derived Kit$^+$ cells expressing CSF3R truncation and compound mutations

a. Schematic representation of *in vitro* CFU assays. **b.** Bar graphs showing percent CFU treated with PD329501 [5 nM], ruxolitinib [1μM] and PD329501 [5 nM]+Ruxolitinib [1μM]. Representative data shown are the percent mean CFU ± SEM. (n=3) from two independent experiments.

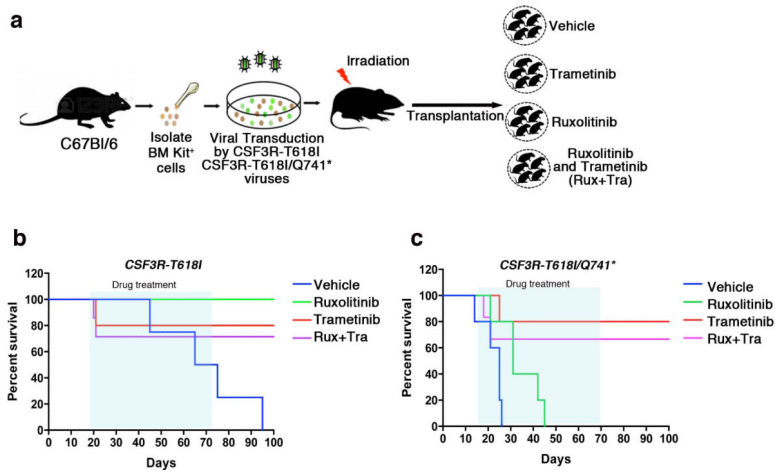

Figure 6. Trametinib treatment alone suppresses leukemia induced by both proximal and compound CSF3R mutants

a. Schematic representation of *in vivo* experiment to determine the efficacy of Mek1/2 and Jak2 inhibitors. **b**. Twenty mice were transplanted with CSF3R-T618I expressing Kit^+ cells. After two weeks of transplantation, mice were treated by vehicle (n=4), Ruxolitinib (n=5), Trametinib (n=5) and Ruxolitinib+Trametinib (n=6). **c**. Twenty-one mice were transplanted with CSF3R-T618I/Q741* expressing Kit^+ cells. After two weeks of transplantation, mice were treated by vehicle (n=5), Ruxolitinib (n=5), Trametinib (n=5) and Ruxolitinib +Trametinib (n=6). Representative data are from two independent transplant experiments.

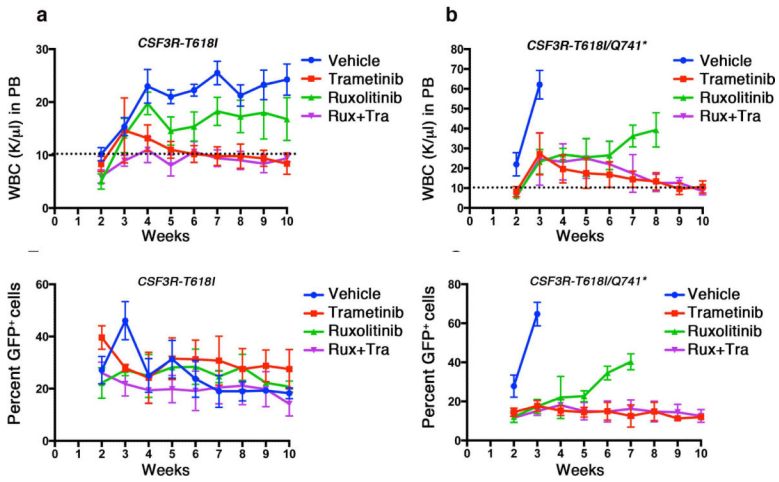

Figure 7. Trametinib treatment alone suppresses leukemia induced by both proximal and compound CSF3R mutants

a and b. Graphs showing the total WBC levels (top panel) and GFP positive cells as a surrogate leukemic burden (bottom panel) from the peripheral blood of mice transplanted with CSF3R-T618I (**a**) and CSF3R-T618I/Q741* (**b**). Dotted lines represent normal WBC levels. Representative data are from two independent transplant experiments.