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Mutant SETBP1 enhances NRAS-driven MAPK pathway activation to promote aggressive leukemia

Sarah A. Carratt[1], **Theodore P. Braun**[1], **Cody Coblentz**[1], **Zachary Schonrock**[1], **Rowan Callahan**[1], **Brittany M. Curtiss**[1], **Lauren Maloney**[1], **Amy C. Foley**[1], **Julia E. Maxson**[1]

[1]Oregon Health & Science University, Portland, OR

Abstract

Mutations in SET binding protein 1 (SETBP1) are associated with poor outcomes in myeloid leukemias. In the Ras-driven leukemia, juvenile myelomonocytic leukemia, SETBP1 mutations are enriched in relapsed disease. While some mechanisms for SETBP1-driven oncogenesis have been established, it remains unclear how SETBP1 specifically modulates the biology of Ras-driven leukemias. In this study, we found that when co-expressed with Ras pathway mutations, SETBP1 promoted oncogenic transformation of murine bone marrow *in vitro* and aggressive myeloid leukemia *in vivo*. We demonstrate that SETBP1 enhances the NRAS gene expression signature, driving upregulation of mitogen-activated protein kinase (MAPK) signaling and downregulation of differentiation pathways. SETBP1 also enhances NRAS-driven phosphorylation of MAPK proteins. Cells expressing NRAS and SETBP1 are sensitive to inhibitors of the MAPK pathway, and treatment with the MEK inhibitor trametinib conferred a survival benefit in a mouse model of NRAS/SETBP1-mutant disease. Our data demonstrate that despite driving enhanced MAPK signaling, SETBP1-mutant cells remain susceptible to trametinib *in vitro* and *in vivo*, providing encouraging pre-clinical data for the use of trametinib in SETBP1-mutant disease.

To the Editor,

SET binding protein 1 (SETBP1) mutations are associated with relapsed disease and reduced survival in juvenile myelomonocytic leukemia (JMML), a rare form of early childhood leukemia driven by Ras pathway mutations (NF1, NRAS, KRAS, PTPN11 and CBL)[1]. SETBP1 mutations occur in as many as 30% of JMML patients and reduce the five-year event-free survival rate from 51% to 18%[2, 3]. Although some mechanisms of oncogenesis have been established for SETBP1 mutations[4–7], it remains unclear why they are associated with poor prognosis in this context. The goal of this study was to understand how SETBP1 modulates the biology of Ras-driven leukemias and to determine whether there are therapeutic vulnerabilities that can be exploited.

Correspondence: Julia E. Maxson, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, KR-HEM, Portland, OR 97239, maxsonj@ohsu.edu.

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SETBP1 mutations occur across the spectrum of myeloproliferative and myelodysplastic disorders[8]. In JMML, SETBP1 mutations are secondary mutations that confer poor prognosis[3, 8] and are present in a large portion of patients who relapse[2]. Subclonal SETBP1 mutations are often present at diagnosis (35% of cases)[2]. Mutations in SETBP1 are localized within its degron motif and lead to SETBP1 overexpression at the protein level[4]. SETBP1 overexpression promotes oncogenesis by binding SET, protecting the SET protein from protease cleavage[5]. Stabilization of SET leads to the inhibition of the tumor suppressor PP2A protein through the formation of a SETBP1-SET-PP2A complex[5]. SETBP1 also acts as a transcriptional regulator, and its mutation perturbs transcription of RUNX1[6], HOXA9 and HOXA10[7], which are regulators of hematopoiesis. To address our central question of how SETBP1 mutations modulate Ras-driven leukemia, we first set out to determine whether mutant SETBP1 promotes the growth of hematopoietic progenitors with a Ras pathway mutation. As there are no models of endogenous SETBP1 mutation, we leveraged retroviral vectors to express SETBP1, mimicking protein overexpression driven by the endogenous mutation. The models developed for this study can be leveraged for future drug development efforts and mechanistic studies of SETBP1 mutant disease. Additional methodological information is in the supplement along with key resources (Table S1) and software (Table S2).

SETBP1 mutations are known to co-occur with both PTPN11 and NRAS mutations (such as $PTPN11^{E76K}$ and $NRAS^{G12D}$)[1, 2, 9]. To evaluate synergy between SETBP1 and Ras pathway mutations, we transduced murine bone marrow with either a Ras pathway mutation ($PTPN11^{E76K}$ or $NRAS^{G12D}$), $SETBP1^{D868N}$, BOTH oncogenes or empty vector controls. Cells positive for the mutations of interest were sorted into MethoCult M3234 and evaluated for proliferative potential through a hematopoietic colony forming unit (CFU) assay. We find that, in the absence of exogenous cytokines, both $PTPN11^{E76K}$ (Fig.1AB) and $NRAS^{G12D}$ (Fig.1CD) formed a modest number of murine colony units. The addition of $SETBP1^{D868N}$ significantly augmented colony number with either Ras pathway mutation. To determine whether the addition of a SETBP1 mutation enhanced self-renewal, we performed a serial replating assay with cytokine-free methylcellulose. $PTPN11^{E76K}$ and $SETBP1^{D868N}$ confer replating potential out to the third plating (Fig.1B). The combination of $NRAS^{G12D}$ and $SETBP1^{D868N}$ confer robust serial replating out to at least the fourth plating (Fig.1D), indicating that the $SETBP1^{D868N}$ enhances both oncogenic transformation and self-renewal.

To understand whether mutant SETBP1 augments the oncogenicity of NRAS *in vivo*, we performed a murine bone marrow transplant experiment. For this study, 5,000 lineage-depleted murine hematopoietic cells expressing either $NRAS^{G12D}$, $SETBP1^{D868N}$, BOTH oncogenes or empty vectors, were transplanted into lethally irradiated Balb/c mice (2×4.5Gy) with 200,000 carrier cells. $NRAS^{G12D}$/$SETBP1^{D868N}$ mice developed an aggressive myeloid leukemia with a median survival of 20 days (Fig.1E). In contrast, the mice transplanted with $SETBP1^{D868N}$ or $NRAS^{G12D}$ alone developed disease with a much longer latency (Fig.1E). The $NRAS^{G12D}$/$SETBP1^{D868N}$ mutant leukemia was marked by an expansion of the myeloid compartment (Fig.1F, Fig.S1A), high peripheral leukocytosis (Fig.1G), and splenomegaly (Fig.S1B).

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To understand how $NRAS^{G12D}$ and $SETBP1^{D868N}$ cooperate to produce this aggressive phenotype, we performed RNAseq on lineage-depleted murine bone marrow cells, transduced in triplicate. The impact of $SETBP1^{D868N}$ alone is subtle, with only nine differentially expressed genes for $SETBP1^{D868N}$ alone relative to control. In contrast, we found that cells expressing both $NRAS^{G12D}$ and $SETBP1^{D868N}$ have 803 differentially expressed genes relative to the empty vector control, of which approximately half are also significant in the $NRAS^{G12D}$ alone condition, indicating that their altered expression is primarily driven by $NRAS^{G12D}$ (Fig.1H). We identified 399 differentially expressed genes in the $NRAS^{G12D}$ plus $SETBP1^{D868N}$ condition (BOTH) that are *not* differentially expressed with either oncogene alone. KEGG mouse pathway analysis of these 399 genes suggests that they are involved with MAPK activation and regulation of hematopoietic differentiation.

To understand this phenomenon, we performed clustering on all genes that were differentially expressed between any of the conditions (Fig.1I). As expected, a majority of the signaling changes relative to control could be attributed to $NRAS^{G12D}$ (Clusters 2, 5). However, we identified two clusters in which there were larger gene expression changes when $SETBP1^{D868N}$ was co-expressed with $NRAS^{G12D}$ compared to with either oncogene alone (Clusters 1, 4). Cluster 1 is defined by genes that are upregulated to a small degree by each NRAS and SETBP1 individually, with the greatest upregulation in the combination. Cluster 1 has a strong MAPK signature (Fig.1I). Cluster 4 is defined by pro-differentiation pathways that have the most downregulation with the combination. Additional characterization of the RNAseq dataset using Gene Set Enrichment Analysis (GSEA) is provided in Fig.S2.

To facilitate further mechanistic studies, we generated a $NRAS^{G12D}$/$SETBP1^{D868N}$ model by culturing transduced murine hematopoietic progenitors harvested from the CFU assay (Fig.1CD) in IMDM with 10% serum (Fig.S3AB). This combination of $NRAS^{G12D}$ and $SETBP1^{D868N}$ mutations enables cells to proliferate in culture over multiple passages. Relative to normal bone marrow, these expanded hematopoietic progenitors have increased MAPK and mTOR pathway signaling (Fig.2A). To identify dependencies in SETBP1-transformed cells, we performed a chemical screen on these $NRAS^{G12D}$/$SETBP1^{D868N}$ hematopoietic progenitors (Fig.2B). Screening of these cells against a panel of drugs targeting cell growth and survival pathways revealed a unique sensitivity to Raf/MEK/ERK and mTOR/AKT/PI3K inhibitors when compared with all other samples previously screened on the BeatAML platform[10]. The BeatAML database includes well over 600 blood and bone marrow specimens. The IC50 in our cell line for each inhibitor was divided by the median IC50 for the same inhibitor in the BeatAML cohort.

This evidence of sensitivity to MAPK inhibitors prompted us to investigate whether the enhanced transcription of MAPK-associated genes by SETBP1 was accompanied by an increase in MAPK phosphorylation and activation at the protein level. We transiently transfected 293T17 cells with $NRAS^{G12D}$ and/or $SETBP1^{D868N}$ and found that $SETBP1^{D868N}$ alone does not increase signaling in the MAPK pathway over baseline expression (Fig.2C). However, in the context of a $NRAS^{G12D}$ mutation, $SETBP1^{D868N}$ augments phosphorylation of ERK and MEK, two key MAPK pathway proteins. SETBP1 is known to inhibit activity of the tumor suppressor PP2A, a regulator of the Ras/MAPK

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pathway, through the stabilization of SET[5, 11]. Interestingly, we find that deletion of the entire SET binding domain from SETBP1^{D868N} (previously mapped to AA1238–1434[12], corresponding to AA1292–1488 in our tagged construct) does not abrogate the enhancement of MAPK activation by SETBP1 (Fig.2C). FTY720, which activates PP2A in leukemia in part by destabilizing the inhibitory SET and PP2Ac complex[13], has no effect on the NRAS^{G12D}/SETBP1^{D868N}-mutant cell line at 72h in the sub-micromolar range, with 90% viability at 1000nM (Fig.2F).

A subset of the MAPK and mTOR inhibitors from the chemical screen were validated individually (Fig.2DE), with most efficacious of these drugs being rapamycin and trametinib. Of note, trametinib is a MEK inhibitor that is currently in clinical trial for relapsed and refractory JMML and has an IC50 of 0.42 nM in these cells (Fig.S4A). Trametinib and rapamycin have additive, but not synergistic, efficacy in this cell line (Fig.S4B). Rigosertib, a RAS mimetic that has the potential to block RAS-RAF-MEK-ERK signaling, has an IC50 of approximately 333nM in our NRAS^{G12D}/SETBP1^{D868N}-mutant cell line (Fig.2F). Rigosertib is well tolerated and has been shown to have biological and clinical activity in a phase 1/2 study of patients with myelodysplastic syndromes and acute myeloid leukemia[14].

The two candidates with sub-nanomolar efficacy, trametinib and rapamycin, were evaluated *in vivo*. 100,000 cells from the novel NRAS^{G12D}/SETBP1^{D868N} leukemia cell line were retro-orbitally transplanted into C57BL/6 mice without irradiation (Fig.S3A). Optimization of model is reported in the supplement (Fig.S3C–F). Trametinib treatment significantly increased the median survival in this model from 19.5 days with DMSO to 42 days (Fig.2G). At day 21, when the last of the vehicle-treated mice succumbed to disease, the mean peripheral WBC count in the vehicle-treated mice was 387,000/mm^3 compared to 8,600/mm^3 in the trametinib-treated group (Fig.2H). Rapamycin neither improved survival over DMSO or significantly reduced leukocytosis beyond day 21 (Fig.2HI, Fig.S4C).

To assess the translational relevance of our findings, two chronic myelomonocytic leukemia (CMML) samples with SETBP1 and NRAS mutations were plated in a CFU assay with cytokines and treated with trametinib at 0, 50, 100, and 200nM (Fig.2J, Table S3). Both CMML and JMML are rare, mixed myelodysplastic/myeloproliferative diseases with high peripheral blood monocytes[8]. Where JMML is most commonly diagnosed in young children, CMML is an adult disease. Proliferation in both of the NRAS/SETBP1-mutant CMML samples was reduced by more than 2-fold with 50nM trametinib (Fig.2J). Notably, an equal number of live peripheral white blood cells were plated per well for each CMML sample, proliferation was markedly higher in Patient #2, yet these aggressively proliferating cells were highly sensitive to trametinib.

Targeting the Ras pathway has been of great interest for patients with JMML[15], where additional therapeutic options are needed. A clinical trial is currently underway targeting signaling downstream of Ras with the MEK/ERK inhibitor trametinib in patients with relapsed and refractory JMML (ClinicalTrials.gov Identifier: NCT03190915). It was previously unclear whether the presence of a SETBP1 mutation would alter the efficacy of trametinib. A previous study showed that the allelic fraction of mutant SETBP1 can

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increase over the course of treatment, suggesting SETBP1-mutant cells may be resistant to conventional therapies[2]. With regard to trametinib specifically, inactivation of PP2A by the PP2A Aα R183W point mutation has been shown to drive resistance to MEK inhibitors through the potentiation of Ras signaling and ERK phosphorylation[16]. Since SETBP1 is an inhibitor of PP2A *and* increases MEK/ERK phosphorylation in our models, SETBP1 mutations could theoretically reduce sensitivity to MEK inhibitors. However, in our study, we find that SETBP1-mutant human and murine cells are still highly sensitive to inhibitors of the RAS/ERK/MAPK pathway. Trametinib doubles overall survival in our murine model of NRAS/SETBP1-mutant leukemia and inhibits proliferation of human NRAS/SETBP1-mutant blood cells, providing encouraging pre-clinical data for the use of trametinib in SETBP1-mutant leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests:

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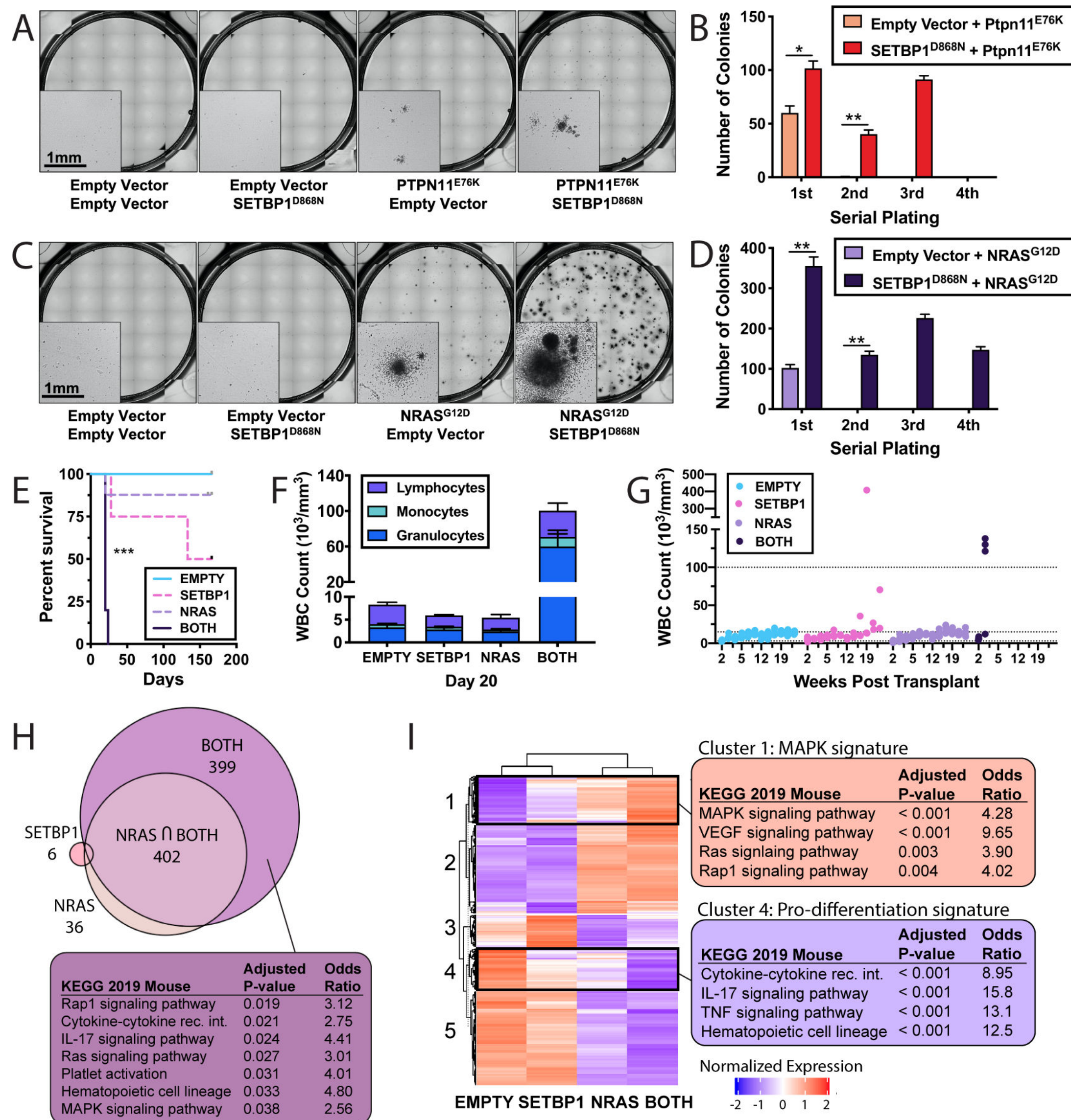

Figure 1. SETBP1^{D868N} enhances the proliferation of NRAS^{G12D} and PTPN11^{E76K} hematopoietic progenitors.

(A) Representative images of colony forming unit (CFU) assays show that SETBP1^{D868N} enhances the colony forming capacity of PTPN11^{E76K}. **(B)** Quantification showing that PTPN11^{E76K} synergizes with SETBP1^{D868N} to produce significantly more colonies than with PTPN11^{E76K} alone. Serial replating of cells co-transfected with PTPN11^{E76K} and SETBP1^{D868N}. 5,000 cells were sorted into methocult for the first plating and 10,000 cells were harvested, washed and re-plated for the subsequent platings. **(C)** Representative images show an enhancement of colony forming potential in cells transduced with both NRAS^{G12D} and SETBP1^{D868N} mutations. While NRAS^{G12D} alone produces some small dense colonies, the combination of NRAS^{G12D} and SETBP1^{D868N} results in an increased number of colonies that were generally larger than with NRAS^{G12D} alone. **(D)** Quantification of colony number showing that when combined with SETBP1^{D868N} mutation, the NRAS^{G12D} mutation produces significantly more colonies than with NRAS^{G12D} alone. For this assay,

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1,500 cells were sorted into methocult for the first plating and 10,000 cells were harvested, washed and re-plated for the subsequent platings. Enhanced serial replating of $NRAS^{G12D}$ and $SETBP1^{D868N}$ expressing progenitors relative to those expressing $NRAS^{G12D}$ alone. Statistical significance is represented as *p<0.05, **p<0.01. **(E)** 5,000 lineage-depleted mouse bone marrow cells expressing $SETBP1^{D868N}$ and/or $NRAS^{G12D}$ with the appropriate retroviral control vectors were transplanted into lethally irradiated mice with 200,000 carrier bone marrow cells. The median survival in mice with BOTH oncogenes ($SETBP1^{D868N}$/ $NRAS^{G12D}$) was 20 days, compared to 149.5 days with SETBP1 alone ($SETBP1^{D868N}$/ Empty). Mice receiving cells expressing NRAS alone (Empty/$NRAS^{G12D}$) did not reach their median survival by 165 days. Significance was determined by logrank (Mantel-Cox test), with the threshold for significance of p-value < 0.0083. Number of mice per group were as follows (N_{EMPTY}=6; N_{NRAS}=7; N_{SETBP1}=4; N_{BOTH}=5). **(F)** At 20 days post-transplant (the median survival time for mice with BOTH mutations together ($SETBP1^{D868N}$/ $NRAS^{G12D}$)), a marked elevation of white blood cells is seen relative to all other groups. **(G)** Peripheral complete blood counts were monitored over time. Mice expressing mice with BOTH oncogenes ($SETBP1^{D868N}$/ $NRAS^{G12D}$) developed high WBC counts in the first three weeks, while mice with SETBP1 only ($SETBP1^{D868N}$/Empty) began to develop high WBC counts after 17 weeks. **(H)** RNAseq differential expression analyses were performed on transduced lineage depleted murine bone marrow cells expressing empty vector (Empty/Empty), SETBP1 ($SETBP1^{D868N}$/Empty), NRAS (Empty/ $NRAS^{G12D}$) or BOTH oncogenes ($SETBP1^{D868N}$/$NRAS^{G12D}$). The Venn diagram shows the number of genes that are differentially expressed relative to the empty vector control for SETBP1, NRAS and BOTH (logFC +/− 1.5, adj p-value < 0.05). There were 402 differentially genes at the intersection of NRAS and BOTH, and 399 genes that were differentially expressed only in the BOTH group. Enrichr analysis of these genes that are only differentially expressed with BOTH oncogenes showed upregulation of inflammatory and Ras/MAPK pathways. **(I)** Unsupervised clustering of differentially-expressed genes (logFC +/− 1.5, adj p-value < 0.05). Cluster 1 shows a strong MAPK signature in genes upregulated by both SETBP1 and NRAS. Cluster 4 is enriched for KEGG pathways associated with myeloid differentiation.

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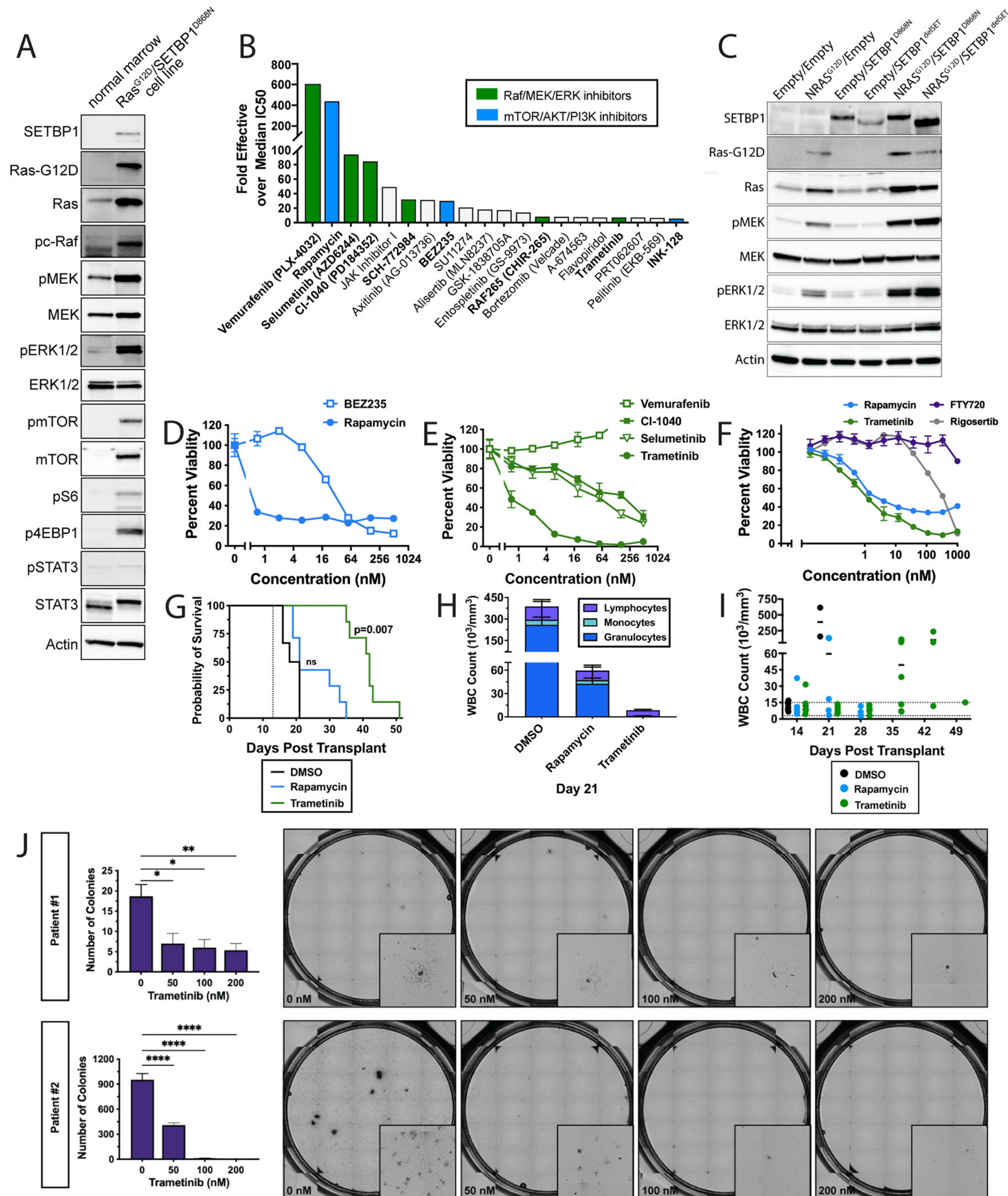

Figure 2. SETBP1^{D868N} enhances MAPK signaling driven by NRAS^{G12D}.

(A) Immunoblot analysis of NRAS^{G12D}/ SETBP1^{D868N} expanded hematopoietic progenitors reveals increased activation of MAPK and mTOR signaling relative to a normal marrow control. **(B)** A chemical screen with commercially available inhibitors was performed on our novel NRAS^{G12D}/SETBP1^{D868N} cell line to identify essential cell growth and survival pathways, and the cells were found to be highly sensitive to Raf/MEK/ERK inhibitors (black). **(C)** Immunoblot analysis of 293T17 cells transiently transfected with and empty vector alone, NRAS^{G12D}, SETBP1^{D868N} or the combination of both genes.

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An empty vector control is used to control for the total amount of plasmid transfected. Co-transfection with $NRAS^{G12D}$ and $SETBP1^{D868N}$ increases the phosphorylation of ERK and MEK above $NRAS^{G12D}$ alone. Deletion of the SET-binding domain from $SETBP1^{D868N}$ ($SETBP1^{delSET}$) does not reduce MEK/ERK activation relative to full length $SETBP1^{D868N}$. **(D)** To validate the efficacy of identified inhibitors against the $NRAS^{G12D}$/$SETBP1^{D868N}$ cells, a 7-point dose response curve (0–500nM) with a 1:3 fixed molar ratio of each of the top agents was performed, and a percent viability calculated relative to untreated cells after 72 hours. Rapamycin had sub-nanomolar efficacy. (**E**) Trametinib had sub-nanomolar efficacy. **(F)** The efficacy of FTY720 and Rigosertib was evaluated in our cell line using an 11-point curve (0–1000nM) with a 1:3 fixed molar ratio. **(G)** To evaluate the efficacy of trametinib *in vivo*, 100,000 $NRAS^{G12D}$/$SETBP1^{D868N}$-mutant cells were retro-orbitally injected into C75BL/6J mice without irradiation. Beginning at day 13 (dotted vertical line), mice were given once-daily treatment of either DMSO (N=6), 10 mg/kg rapamycin (N=7) or 1 mg/kg trametinib (N=7). Median survival in mice receiving the DMSO control treatment was 19.5-days post-transplant compared 42 days with trametinib ($p=0.0007$). Significance was determined by logrank (Mantel-Cox test). Mice treated with rapamycin had a median survival of 21 days. **(H)** At Day 21, the disease burden was markedly higher in the peripheral blood of DMSO-treated mice relative to trametinib-treated mice. (**I**) WBC count over time as measured by automated CBC. **(J)** The efficacy of trametinib in human blood cells was evaluated using two CMML patient samples with $NRAS^{G12D}$/$SETBP1^{G870S\ or\ I871S}$. 100,000 blood cells per well were plated in triplicate in a CFU assay with increasing doses of trametinib (50, 100, 200nM).