Combined Inhibition of MAP Kinase and KIT Signaling Synergistically Destabilizes ETV1 and Suppresses GIST Tumor Growth

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ABSTRACT

Gastrointestinal stromal tumor (GIST), originating from the interstitial cells of Cajal (ICC), is characterized by frequent activating mutations of the KIT receptor tyrosine kinase. Despite the clinical success of imatinib, which targets KIT, most patients with advanced GIST develop resistance and eventually die of the disease. The ETS family transcription factor ETV1 is a master regulator of the ICC lineage. Using mouse models of Kit activation and Etv1 ablation, we demonstrate that ETV1 is required for GIST initiation and proliferation in vivo, validating it as a therapeutic target. We further uncover a positive feedback circuit where MAP kinase activation downstream of KIT stabilizes the ETV1 protein, and ETV1 positively regulates KIT expression. Combined targeting of ETV1 stability by imatinib and MEK162 resulted in increased growth suppression in vitro and complete tumor regression in vivo. The combination strategy to target ETV1 may provide an effective therapeutic strategy in GIST clinical management.

SIGNIFICANCE: ETV1 is a lineage-specific oncogenic transcription factor required for the growth and survival of GIST. We describe a novel strategy of targeting ETV1 protein stability by the combination of MEK and KIT inhibitors that synergistically suppress tumor growth. This strategy has the potential to change first-line therapy in GIST clinical management.

INTRODUCTION

Gastrointestinal stromal tumor (GIST) represents one of the most common subtypes of human sarcoma, with approximately 5,000 cases a year in the United States. GIST arises from the interstitial cells of Cajal (ICC) that depend on high-level KIT expression for lineage specification and survival (1, 2). Families with germline-activating KIT mutations develop diffuse hyperplasia of ICCs that progresses to GIST (3–6). The majority of sporadic GISTs harbor activating
mutations in KIT and to a lesser extent in PDGFRA and BRAF (2, 7–9). These mutations are thought to function as oncogenic “drivers” required for growth and survival of GISTs. These observations have provided the scientific rationale for clinically targeting these mutations in GIST.

Imatinib mesylate (Gleevec), a multitargeted tyrosine kinase inhibitor (TKI) that targets KIT/PDGFR, is the standard first-line therapy in advanced GIST, with a radiographic response rate of approximately 50% and disease stabilization in another 25% to 30% of patients (10–13). Despite the early clinical success, the median progression-free survival is only 20 to 24 months, and the majority of patients develop resistance to imatinib within 2 years of treatment (11–14). Despite the early clinical success, the median progression-free survival is only 20 to 24 months, and the majority of patients develop resistance to imatinib within 2 years of treatment (11–14). Second- and third-line TKIs that target subsets of imatinib-resistant KIT mutations have only limited efficacy, and patients with advanced GIST eventually die of their disease (14–18). Imatinib resistance remains the greatest challenge in the management of advanced GISTs. Because of the vast heterogeneity of resistance mechanisms both between patients and within individual patients, it is challenging to develop next-generation therapies that can address the majority of, if not all, resistance mechanisms (17, 19, 20).

Clinically, complete responses with first-line imatinib therapy are rare. The residual disease represents a significant repository that can adapt, evolve, and eventually break through imatinib therapy through a variety of resistance mechanisms. Moreover, the potential existence of a KIT low and intrinsically imatinib-resistant GIST stem/progenitor population (20) makes it conceivably impossible to eradicate the disease with imatinib alone. We reason that one of the strategies to overcome imatinib resistance is to develop novel therapeutics that are more effective than imatinib alone and can potentially target the GIST stem/progenitor population and therefore prevent the development of imatinib resistance.

We have previously uncovered that ETV1, an ETS family transcription factor, is a master regulator of the normal lineage specification and development of the GIST precursor ICCs. ETV1 is highly expressed in GISTs and is required for the growth and survival of imatinib-sensitive and imatinib-resistant GIST cell lines. ETV1 is a highly unstable protein, and its stability is enhanced by active MAP kinase signaling, and represents an essential effector of mutant KIT/PDGFRA-mediated pathogenesis in GIST (21). These observations point to ETV1 as a novel therapeutic target. However, the in vivo requirement of ETV1 in GIST pathogenesis has not been defined. More importantly, an effective therapeutic strategy to target ETV1, a transcription factor, has not been developed. Here, using genetically engineered mouse (GEM) models, we demonstrate that Etv1 is required for GIST tumor initiation and proliferation in the physiologic in vivo context. Taking advantage of the unique regulation of ETV1 protein stability, we further describe an effective therapeutic strategy to target ETV1.

RESULTS

Etv1 Is Required for Tumor Initiation and Proliferation

To assess whether Etv1 is required for GIST initiation in vivo, we crossed the germline KitΔ558V/+ knockin mouse model that develops ICC hyperplasia throughout the gastrointestinal tract and GIST-like tumors in the cecum (22, 23) with the Etv1−/− knockout mouse model (24) that is defective in ICC development (21). Because the Etv1+/− mice die at postnatal days 10 to 14 (P10–P14; ref. 24), we examined the GI tract of Etv1+/−;KitΔ558V/+ and Etv1−/−;KitΔ558V/+ littermates at day P10. Consistent with prior observations, all three Etv1+/−;KitΔ558V/+ mice developed ICC hyperplasia in the stomach and large intestines (Fig. 1C and D). In contrast, one of the three Etv1−/−;KitΔ558V/+ mice developed GIST-like masses in the cecum that stain positively for KIT and ETV1 (Fig. 1A and B) and diffuse ICC hyperplasia in the stomach and large intestines (Fig. 1C and D). In contrast, one of the three Etv1−/−;KitΔ558V/+ mice developed ICC hyperplasia in the cecum and none developed cecal GIST-like tumors or ICC hyperplasia of the stomach or large intestine (Fig. 1A–C and E). In addition, IHC against ICC makers KIT and ANO1 showed that
Etv1−/−;KitV558Δ/+ mice exhibited loss of the intramuscular ICCs (ICC-IM) and myenteric ICCs (ICC-MY) with preservation of the submucosal ICCs (ICC-SMP; Fig. 1B; Supplementary Fig. S1), phenocopying the ICC loss in Etv1−/− mice, respectively. Yellow arrows, preserved ICC-SMP with positive KIT immunostaining. Scale bar, 50 μm.

Figure 1. ETV1 is required for GIST tumor initiation in vivo. A, representative hematoxylin and eosin (H&E) staining of the cecal mass and the cecum of Etv1+/−;KitΔ558/+ and Etv1−/−;KitΔ558/+ mice, respectively, showing that ETV1 is required for formation of GIST-like cecal tumors (yellow arrows, malignant cells in tumor). M, mucosa; CM, circular muscle; LM, longitudinal muscle. Scale bar, 100 μm. B, representative immunofluorescence of KIT (red), ETV1 (green), and DAPI (blue) of the cecal tumor or cecum of Etv1−/−;KitΔ558/+ and Etv1−/−;KitΔ558/+ mice, respectively. Yellow arrows, preserved ICC-SMP with positive KIT immunostaining. Scale bar, 50 μm. C, summary of the histologic findings in Etv1−/−;KitΔ558/+ and Etv1−/−;KitΔ558/+ cecum examined at 10 days postnatal. D, representative H&E and KIT IHC images of the large intestine and stomach in Etv1−/−;KitΔ558/+ mice, demonstrating hyperplasia of the ICCs (yellow arrows, KIT-positive ICC hyperplasia) in the large intestine and stomach. Scale bar, 50 μm. E, representative H&E and KIT IHC images of the large intestine and stomach in Etv1−/−;KitΔ558/+ mice, demonstrating the lack of a KIT-positive ICC layer between the longitudinal muscle and the circular muscle layers in Etv1−/−;KitΔ558/+ mice. Scale bar, 50 μm.

Etv1−/−;KitΔ558/+ mice exhibited identical phenotype to the KitΔ558/+ mice, with highly proliferative GIST-like tumors of the cecum and ICC hyperplasia of the large intestine and the stomach (Fig. 2A–C). In contrast, tamoxifen-treated mice exhibited significant reduction of cell proliferation by Ki67 IHC in cecal tumors and ICC hyperplasia (Fig. 2A–C). This level of Ki67 reduction is reminiscent of the imatinib treatment (ref. 27; Fig. 2D). These observations demonstrate that Etv1 ablation by tamoxifen treatment induced significant fibrosis indicated by Masson trichrome stain in the cecal tumors similar to imatinib treatment (ref. 27; Fig. 2D). These observations demonstrate that Etv1 is required for GIST tumor proliferation in vivo.

ETV1 and KIT Form a Positive Feedback Circuit to Regulate Target Genes

We next examined the ETV1-regulated transcriptome by comparing transcriptional profiles between tamoxifen and vehicle treatment of Etv1−/−;KitΔ558/+;Rosa26CreERT2/CreERT2 cecal tumors. The RNA sequencing (RNA-seq) profile of Etv1 transcript shows that tamoxifen-treated tumors had an approximately 3.4-fold decrease in the floxed exon 11 count, implying a 3.4-fold decrease in full-length, functional Etv1 transcript (Supplementary Fig. S2B and S2C). This decrease
is due to (i) a 1.7-fold decrease in *Etv1* overall transcript level and (ii) approximately 50% of the remaining transcripts showing aberrant splicing from exon 10 to 12, skipping the floxed exon 11. The reduction of the overall transcript level with *Etv1* genetic ablation suggests that *Etv1* positively regulates its own transcription. Immunoblot analyses confirmed a decrease in ETV1 protein levels in tamoxifen-treated tumors compared with controls (Supplementary Fig. S2D).

Despite the incomplete ablation of *Etv1*, tamoxifen treatment induced robust transcriptional changes as seen by hierarchical clustering (Fig. 3A; Supplementary Table S1). The RNA transcripts of known ETV1 transcriptional targets, including *Daup6*, *Gpr20*, and *Eda3* (21), were significantly reduced (Fig. 3B). Interestingly, the *Kit* RNA transcript level was reduced by 1.7-fold with *Etv1* ablation (Fig. 3B). Immunoblot, immunofluorescence (IF) and IHC analyses showed a consistent decrease in KIT protein levels in tamoxifen-treated cecal tumors (Supplementary Fig. S2D and Fig. 3C and D). The ICC hyperplasia of the large intestine and stomach also showed a reduction in KIT protein levels with tamoxifen treatment (Fig. 3D; Supplementary Fig. S3).

To determine the biologic processes perturbed by *Etv1* ablation, we performed Gene Set Enrichment Analysis (GSEA) comparing tamoxifen- and corn oil–treated tumors (28). Remarkably, the set of genes most downregulated by imatinib in *Kit*^+558V/+ mouse (*Imatinib DN; ref. 23*) is the most enriched gene set among those downregulated by tamoxifen treatment (Fig. 3E; Supplementary Tables S2 and S3). Likewise, the set of genes most upregulated by imatinib is highly enriched among those upregulated by tamoxifen treatment, suggesting that ETV1 and KIT regulate a common set of core transcriptional program. This is consistent with the model that ETV1 is a major downstream effector of KIT, and also that ETV1 regulates *Kit* expression, which in turn regulates KIT-dependent genes. In addition, multiple cell-cycle–related gene sets, including one of E2F target genes, are enriched in those downregulated by tamoxifen treatment (Fig. 3F; Supplementary Table S2). These data are consistent with the decrease in Ki67 staining after tamoxifen treatment and suggest that ETV1 is required for tumor proliferation and growth in vivo.

To determine whether ETV1 regulates *KIT* transcription in human GIST, we knocked down *ETV1* with shRNA in three GIST cell lines: GIST48, GIST882, and GIST-T1. In each line, there was a modest decrease in *KIT* transcript levels after *ETV1* knockdown (Fig. 4A). CRISPR/Cas9–mediated knockout of *ETV1* in GIST48 cells also resulted in a decrease in both KIT transcript and protein levels (Supplementary Fig. S4A and S4B). We next retrovirally overexpressed *ETV1* in GIST882 and GIST-T1 cells and found a modest upregulation in *KIT* transcript level (Fig. 4B). We performed GSEA of *ETV1* knockdown in each of the three cell lines, and for each cell line the genes most downregulated by imatinib were the most enriched gene set among downregulated genes by *ETV1* knockdown, whereas genes most upregulated by imatinib were the most enriched gene set among upregulated genes by *ETV1* knockdown (Fig. 4C), consistent with our observation in mouse tissues (Fig. 3).
ETV1 positively regulates Kit expression in murine GISTs. A, heatmap of significantly differentially expressed genes between corn oil control– and tamoxifen-treated murine GIST tumors identified by RNA-seq. Clustering was based on the most differentially expressed 228 genes with FDR < 0.05 and fold change > 2.0. Samples are color coded based on treatment status: pink, corn oil–treated; orange, tamoxifen-treated. Scale bar, mean normalized fold change by log2. B, RNA-seq gene expression quantification (FPKM, fragments per kilobase mapped) of Kit and a representative group of ETV1 transcriptional targets in tamoxifen-treated versus corn oil–treated murine GISTs. C, representative IF images of ETV1 (green) and Kit (red) protein in cecal tumors from Etv1fl/fox/fl fox mice treated with tamoxifen or corn oil, demonstrating ETV1 ablation and decreased Kit protein level. Nuclei (DAPI, blue). Scale bar, 50 μm. D, representative Kit IHC images of the cecal tumors and ICC hyperplasia in the large intestines of mice treated as in C. Scale bars, 50 μm. E, GSEA plots of the ranked list of the differentially expressed genes between tamoxifen (Tam)-treated versus corn oil–treated murine GIST tumor samples, using two gene sets, Imatinib UP (imatinib upregulated) and Imatinib DN (imatinib downregulated). F, GSEA plots of the ranked list of the differentially expressed genes between tamoxifen-treated versus corn oil–treated murine GIST tumor samples, using the ISHIDA_E2F_TARGETS gene set. GEMM, genetically engineered mouse model; NES, normalized enrichment score.

Combined Inhibition of the KIT and MAP Kinase Signaling Represents an Effective Strategy to Target ETV1 and Suppress GIST Tumor Growth

The fact that the ETV1 protein stability requires active MAP kinase signaling downstream of active KIT signaling (21) has provided us with the rationale to target ETV1 protein stability by inhibiting the MAP kinase and the KIT signaling pathways. When we treated the imatinib-sensitive GIST882 and GIST-T1 cells with either imatinib (a KIT inhibitor) or MEK162 (a MEK inhibitor), we observed a rapid inhibition of the MAP kinase activity [assayed by phosphorylated ERK (pERK)] accompanied by rapid loss of the ETV1 protein (Fig. 5A). This reduction of the total ETV1 protein level is associated with a reduction of ETV1 binding at the ETV1-regulated gene loci, e.g., DUSP6 and Kit (Fig. 5B) and a reduction of the DUSP6 and KIT transcripts by 8 hours of treatment (Supplementary Fig. S5A-D). Notably, the ability of MEK162 to durably inhibit the MAP kinase pathway and ETV1 protein stability is cell line specific—GIST882 cells displayed sustained inhibition, whereas GIST-T1 cells showed reactivation of the MAP kinase pathway and reaccumulation of ETV1 protein starting at 2 hours after treatment (Fig. 5A). We then evaluated the combined lineage inhibition using MEK162 and imatinib. In vitro, we observed additive effects on growth suppression across a range of doses of MEK162 and imatinib. A synergistic effect on growth suppression was best appreciated at lower doses of each drug, best seen when 0.5 μmol/L MEK162 was combined with low-dose imatinib (62.5 nmol/L in GIST882 and 40 nmol/L in GIST-T1; Fig. 5C and D). To assess whether the synergistic effect is due to the on-target effect of MEK162, we expressed wild-type MEK1/2 (WT) or MEK1/2 mutants (MEK1L115P, MEK2L119P) that are resistant to allosteric MEK inhibitors such as MEK162 due to reduced drug binding (29). GIST-T1 cells expressing either MEK1L115P or MEK2L119P were more resistant to MEK162 alone. Moreover, the combination
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therapy provided more potent and durable inhibition of MAP

ment even at significantly reduced doses of MEK162 (10 mg/ kg) or imatinib (50 mg/kg; Fig. 6A and B). Combination

treatment within 7 days and complete responses with prolonged treat-

Remarkably, the combination of imatinib and MEK162 treat-

in vivo. In the GIST882 xenograft model, single-agent imatinib

of MEK162 and imatinib conferred less synergistic growth

inhibition in the presence of MEK11116 or MEK21116 in GIST-

T1 cells (Fig. 5E). This corresponded to a decreased ability of

Next, we tested the effect of combined MEK162 and imatinib

in vivo. In the GIST882 xenograft model, single-agent imatinib

or MEK162 stabilized tumor growth at the MTDs (Fig. 6A).

Remarkably, the combination of imatinib and MEK162 treat-

ment resulted in a dramatic reduction (>50%) of tumor size

within 7 days and complete responses with prolonged treat-

ment even at significantly reduced doses of MEK162 (10 mg/ kg) or imatinib (50 mg/kg; Fig. 6A and B). Combination

therapy provided more potent and durable inhibition of MAP

kinase signaling (Fig. 6C; Supplementary Fig. S6A). Impor-

tantly, the ETV1 protein level was more potently and dura-

bly inhibited, which was associated with reduction of ETV1

transcriptional targets (e.g., DUSP6 and KIT), than with either

imatinib or MEK162 alone (Fig. 6C; Supplementary Fig. S6B).

When GIST882-xenografted mice were treated from the same
day of cell implantation, only the combination of imatinib and MEK162 successfully prevented xenograft tumor formation,
suggesting that dual lineage inhibition could also inhibit GIST
tumor formation in vivo (Supplementary Fig. S6C).

In the GIST-T1 xenograft model, single-agent imatinib led
to tumor stabilization. However, single-agent MEK162 did not
significantly inhibit tumor growth (Fig. 6D), consistent with
the inability of MEK162 to durably inhibit the MAP kinase

pathway in GIST-T1 cells (Fig. 5A and C). Yet, as in GIST882
 xenografts, the combination of imatinib and MEK162 resulted
in near-complete response in GIST-T1 xenografts within 3
weeks of treatment (Fig. 6D and E). The treatment effects


Figure 4. ETV1 positively regulates KIT expression through direct binding to KIT enhancers in human GIST cells and forms a positive feedback circuit in GIST oncogenesis. A, mRNA expression of KIT in human GIST882, GIST48, and GIST-T1 cells with ETV1-specific shRNA. n=3, mean ± SEM. Two-tailed unpaired t test: *P < 0.05; **P < 0.01; ***P < 0.001. B, mRNA expression of KIT in GIST882 and GIST-T1 cells 48 hours after retroviral transduction of ETV1 expression vector or empty vector control. n=3, mean ± SEM. Two-tailed unpaired t test: *P < 0.05; **P < 0.001. C, GSEA plots of the ranked list of the shETV1-downregulated genes in human GIST cells, using the Imatinib DN (imatinib downregulated) gene set. D, representative of ChiP-seq reads of ETV1, H3K4me1, and H3K4me3 at the KIT transcription start site (H3K4me3) and enhancer regions (H3K4me1 and ETV1) in human GIST48 cells. Pink, red, and yellow colors represent regions selected for ChiP–qPCR studies. E, ChiP–qPCR of ETV1 at the KIT enhancer loci as indicated by color code as in G with siRNA-mediated suppression of ETV1 (siETV1) versus scrambled control siRNA (siSCR) in GIST882 cells. n=3, mean ± SD. F, ChiP–qPCR of ETV1 at the KIT enhancer 2 (red mark in D) in GIST48 cells. n=3, mean ± SD. G, ChiP–qPCR of ETV1 at the KIT enhancer 2 (red mark in D) in GIST-T1 cells. n=3, mean ± SD.
**Figure 5.** Combined inhibition of MAP kinase and KIT signaling destabilizes ETV1 protein and results in enhanced growth suppression of human GIST cells. **A,** immunoblot of ETV1, pKIT, and pERK levels in GIST882 and GIST-T1 cells treated with imatinib (500 nmol/L) or MEK162 (1 µmol/L) for the indicated time points. **B,** ETV1 localization at the target gene loci (i.e., KIT and DUSP6) by ChIP–qPCR in GIST cells treated with imatinib (1 µmol/L) or MEK162 (500 nmol/L) for 8 hours in GIST882 cells, or imatinib (80 nmol/L) or MEK162 (500 nmol/L) for 2 hours in GIST-T1 cells. **C,** immunoblot of ETV1 and KIT, and MAP kinase signaling in GIST-T1 parental cells, GIST-T1 cells expressing MEK1 WT, MEK1 L115P, MEK2 WT, and MEK2 L119P. **D,** cell viability by Alamar Blue of GIST882 and GIST-T1 cells treated with various doses of imatinib and MEK162 as indicated for 7 days. n = 3, mean ± SEM. **E,** cell viability by Alamar Blue of GIST-T1 cell expressing different MEK constructs treated with various doses of imatinib and MEK162 as indicated for 7 days. n = 3, mean ± SEM. **F,** immunoblot of ETV1, KIT, and MAP kinase signaling in GIST-T1 parental cells, GIST-T1 cells expressing MEK1 WT, MEK1 L115P, MEK2 WT, and MEK2 L119P. Cells were treated for 1 hour as indicated. V, DMSO; I, imatinib (500 nmol/L); M, MEK162 (1,000 nmol/L).
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Figure 6. Combined inhibition of the MAP kinase and KIT signaling synergistically suppresses tumor growth in in vivo GIST xenograft mouse models. A, treatment response of GIST882 xenografts in SCID mice. The treatment cohorts are as follows: (i) Vehicle (blue): water; (ii) imatinib (green): 100 mg/kg twice a day; (iii) MEK162 (red): 30 mg/kg twice a day; (iv) imatinib + MEK162 (dose 1; magenta): imatinib (100 mg/kg twice a day) + MEK162 (10 mg/kg, twice a day); (v) imatinib + MEK162 (dose 2; yellow): imatinib (50 mg/kg twice a day) + MEK162 (30 mg/kg twice a day); (vi) imatinib + MEK162 (dose 3; black): imatinib (100 mg/kg twice a day) + MEK162 (30 mg/kg twice a day; dose 3, black). n = 6–8, mean ± SEM. Two-tailed unpaired t test: *P < 0.0001; **P < 0.0001; ***P < 0.0001. B, representative H&E images of GIST882 xenografts after 14 days of drug treatment by oral gavage as indicated. Vehicle: water; imatinib: 100 mg/kg twice a day; MEK162: 30 mg/kg twice a day; imatinib (100 mg/kg twice a day) + MEK162 (30 mg/kg twice a day). Scale bar, 50 μm. C, immunoblots of three representative GIST882 xenograft tumors explanted after 2 days of drug treatment by oral gavage as indicated. Vehicle: water; imatinib: 100 mg/kg twice a day; MEK162: 30 mg/kg twice a day; imatinib (100 mg/kg twice a day) + MEK162 (30 mg/kg twice a day). D, treatment response of GIST-T1 xenografts in SCID mice as indicated by oral gavage. The treatment cohorts are as follows: (i) Vehicle: water; (ii) imatinib: 80 mg/kg twice a day; MEK162: 30 mg/kg twice a day; (iii) MEK162: 30 mg/kg twice a day; (iv) imatinib (80 mg/kg twice a day) + MEK162 (30 mg/kg twice a day); (v) imatinib (80 mg/kg twice a day) + MEK162 (30 mg/kg twice a day). n = 10, mean ± SEM. Two-tailed unpaired t test: *P < 0.0001; **P < 0.0001; ***P < 0.0001. E, representative H&E images of GIST-T1 xenografts after 21 days of drug treatment by oral gavage as indicated. Vehicle: water; imatinib: 80 mg/kg twice a day; MEK162: 30 mg/kg twice a day; imatinib (80 mg/kg twice a day) + MEK162 (30 mg/kg twice a day). Scale bar, 50 μm. F, immunoblots of three representative GIST-T1 xenograft tumors explanted after 2 days of drug treatment by oral gavage as indicated. Vehicle: water; imatinib: 80 mg/kg twice a day; MEK162: 30 mg/kg twice a day; imatinib (80 mg/kg twice a day) + MEK162 (30 mg/kg twice a day).

correlated with KIT and MAP kinase signaling pathway inhibition, ETV1 protein destabilization, and downregulation of ETV1 target genes (i.e., DUSP6 and KIT; Fig. 6F; Supplementary Fig. S6D and S6E). These observations demonstrated a clear synergistic growth-inhibitory effect of imatinib and MEK162 in GIST tumor growth in vivo. It is notable that the synergy of combination treatment is more apparent in in vivo human GIST xenograft studies than in in vitro cell line assays.

We next examined the combination-targeting strategy in the genetically engineered KG20S8R6+ GIST mouse model that is partially sensitive to imatinib treatment (26). Treatment with single-agent MEK162 or imatinib for 5 days resulted in a reduction of tumor proliferation by Ki67 and increased tumor fibrosis by trichrome staining (Fig. 7A–D). The combination treatment of imatinib and MEK162 led to increased tumor fibrosis and significantly greater reduction of Ki67 than either single agent (Fig. 7A and B). Moreover, the combination treatment had significantly reduced tumor weight compared with either single agent alone or with vehicle (Fig. 7C). These treatment effects of the combination therapy were accompanied by increased inhibition of the KIT and MAP kinase signaling pathways, decreased ETV1 protein, and reduced expression of its downstream target Dusp6 (Fig. 7D). The treatment data in both xenografted human GIST models and genetically engineered GIST mouse models indicate that the combination therapy of imatinib and MEK162 is a more effective treatment for imatinib-sensitive GIST than either single agent alone in vivo.

DISCUSSION

Using GEM models, we have demonstrated the in vivo requirement of the lineage-specific master regulator, ETV1, in GIST initiation and proliferation. We have further demonstrated that ETV1 positively regulates expression level by direct binding to the KIT enhancer regions, and it forms a positive feedback circuit to cooperate with mutant KIT in GIST oncogenesis. These observations posit ETV1 as a relevant therapeutic target for the treatment of GISTs. In addition, because ETV1 is required for the survival of GIST precursor ICCs and is required for GIST tumor initiation in vivo, it may also represent a therapeutic target for the KIT+ GIST progenitor/stem cell population. Importantly, target-
ing ETV1 will help break the positive feedback circuit and indirectly target KIT expression independent of KIT mutational status.

Although it is challenging to therapeutically target nonligand-dependent transcription factors, the unique MAP kinase signaling–dependent regulation of ETV1 protein stability has allowed us to target ETV1 protein stability in GIST. The acquisition of KIT-activating mutations during GIST tumorigenesis activates downstream MAP kinase signaling and augments stability of ETV1 protein (21). Our data in two imatinib-sensitive GIST cell lines suggest that mutant KIT is the principal driver of MAP kinase activation, as imatinib treatment significantly inhibited MAP kinase activation, ETV1 protein stability, and ETV1-mediated transcription. In vitro, MEK162 synergized with lower doses of imatinib, but higher doses of imatinib alone can maximally suppress MAP kinase activity and cell proliferation (Fig. 5). However, in both xenograft systems and GEM models in vivo, MTDs of imatinib cannot adequately and durably suppress MAP kinase activity and ETV1 protein levels. This may be due to either the inability to attain sufficient drug levels to fully inhibit KIT (27) or the presence of paracrine signals that activate the MAP kinase pathway bypassing KIT (26). The survival signals that bypass KIT may be heterogeneous, dependent on the tumor contexts. Here, the addition of even low doses of MEK162 led to durable destabilization of ETV1 protein and dramatically augmented tumor response, resulting in complete responses.

The response to single-agent imatinib in our model systems mirrors that of patients undergoing first-line imatinib treatment. Although the majority of patients attain clinical benefits with imatinib treatment, the RECIST response rate is only approximately 50%, and radiographic or pathologic complete responses rarely occur. Our data suggest that the combination therapy represents a significantly more effective strategy than imatinib alone in GIST clinical management and may prevent the development of imatinib resistance in advanced GIST if used up front.

METHODS

Generation of Compound Genetically Engineered GIST Models

All mouse studies are approved by Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee under protocol 11-12-029. The Kit<sup>558V/Δ558</sup> knockin mouse was a generous gift from Dr. Peter Besmer (Memorial Sloan Kettering Cancer Center; ref. 30), the Etv<sup>1−/−</sup> mice were a generous gift from Dr. Thomas Jessell (Columbia University; ref. 24), the Etv<sup>558V/+</sup> mice were a generous gift from Dr. David Ladle (Wright State University; ref. 25), and the Rosa<sup>26</sup>CreERT<sup>2</sup>/CreERT<sup>2</sup> mice were a generous gift from Dr. Andrea Ventura (Memorial Sloan Kettering Cancer Center; ref. 31). The Etv<sup>1−/−</sup>;Kit<sup>558V/Δ558</sup>, Etv<sup>558V/+</sup>;Kit<sup>558V/Δ558</sup>, Etv<sup>1−/−</sup>;Rosa<sup>26</sup>CreERT<sup>2</sup>/CreERT<sup>2</sup>, and Etv<sup>558V/+</sup>;Rosa<sup>26</sup>CreERT<sup>2</sup>/CreERT<sup>2</sup> mice were generated through standard mouse breeding within the MSKCC animal facility.

Cell Lines, Antibodies, and Reagents

The GIST48 and GIST882 cell lines were obtained from Dr. Jonathan A. Fletcher (Dana-Farber Cancer Institute) and were maintained as previously described (21). The GIST-T1 cell line was obtained from Dr. Takahiro Taguchi (Kochi University, ref. 32). The GIST-T1 cell line harbored a 57-nucleotide (V570-Y578) in-frame deletion in KIT exon 11 and was maintained in RPMI supplemented with 10% FBS and 10 mmol/L HEPES (pH 7.5). All GIST cell lines have been authenticated previously and are authenticated by the American Type Culture Collection. All GIST cell lines were maintained under the same drug treatment conditions as indicated in A for 1.5 days. Two cecal tumors from two different mice for each treatment conditions. DUSP6 is one of the transcriptional targets of ETV1. GEMM, genetically engineered mouse model.
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was dissolved in 20 mg/mL corn oil and injected intraperitoneally to 6-week-old mice at a dose of 4 mg every other day for three doses. Mice were euthanized 2 weeks after the first tamoxifen dose.

For drug treatment studies in Kit+/+ mice, approximately 8-to-10-week-old Kit+/+ mice were treated in four cohorts by oral gavage: (i) vehicle: water; (ii) imatinib: 50 mg/kg twice a day; (iii) MEK162: 30 mg/kg twice a day; (iv) imatinib + MEK162: imatinib 50 mg/kg twice a day + MEK162 30 mg/kg twice a day. Cecal tumors were isolated and weighed after 5 days of treatment and subjected to paraffin embedding and analyzed by hematoxylin and eosin (H&E), Trichrome stain, and IHC for Ki67. For short-term treatment, the protein and RNA were isolated from cecal tumors after 1.5-day treatment for immunoblots and qRT-PCR analyses, respectively. To generate lysates for Western blots, tissue was homogenized in SDS lysis buffer using the FastPrep-24 system with Lysing Matrix A (MP Biomedicals).

For xenograft studies, 5 × 10^6 GIST882 or GIST-T1 cells resuspended in 100 μL of 1:1 mix of growth media and Matrigel (BD Biosciences) were subcutaneously injected into CB17-SCID mice (Taconic). Tumor sizes were measured weekly starting 2 weeks after xenografting. For short-term treatment, xenografts were explanted after 2 days of drug treatment for histology analysis; protein and RNA were isolated for immunoblots and qRT-PCR analyses, respectively. For long-term treatment, xenografts were treated twice daily until the end of the experiments. For treatment from the same day of implantation, GIST882 cells expressing firefly luciferase were grafted. Tumor growth was monitored by bioluminescence imaging of anesthetized mice with a Genesis Spectrum Xenogen machine (Caliper Life Science). To generate lysates for Western blotting, tissue was homogenized in SDS lysis buffer using the FastPrep-24 system with Lysing Matrix A (MP Biomedicals).

IF, IHC, and Histology

For IF of cryostat sections of the mouse gastrointestinal tract, mouse stomach, small intestine, cecum, and large intestine were dissected and fixed in 4% paraformaldehyde for 2 hours followed by an overnight incubation in 30% sucrose. They were then embedded in optimal cutting temperature compound, flash-frozen, and cut into 5-μm sections using a cryostat. Tissue samples were ground in 1,000 μL L Trizol (Invitrogen) using a PowerGen homogenizer (Fisher Scientific), followed by the addition of 200 μL chloroform. The samples were then centrifuged at 10,000 g for 15 minutes. The upper phase was mixed with an equal volume of 70% ethanol, and the RNA was further purified using the E.Z.N.A total RNA Kit (Omega).
For qRT-PCR, RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (ABI), and PCR was run using Power SYBR Master Mix (ABI) on a Realp machine (Eppendorf). Expression was normalized to the ribosomal protein RPL27. The following primer pairs were used (21):

**ETV1-Eaxon67F**: CTTACCCCTGAGACCACAGATTT, R: CTTAAG CCTGTGGGTGGGAAG

**KIT**: F: GGGATTTCTCTCGGCCTGCT, R: GATGGATGGATGAGGAGAC

**DUSP6**: F: TGCCGGGCCTTCTACCGGGA, R: GGCGAGCTGTGCCTACAGCA

**RPL27**: F: CATGGCAGAAGAAGAGATCG, R: TCCAAAGGAGGATATCCACAGA.

### Cell Viability

GIST882 and GIST-T1 cells were plated at 4 × 10^4 and 1 × 10^4 cells retrospectively per well in a 96-well plate on day 0 and treated with drugs after 12 hours to allow cell attachment. Triplicate wells were cultured retrospectively per well in a 96-well plate on day 0 and treated with drugs after 12 hours to allow cell attachment. Triplicate wells were cultured until day 7. Viability was assessed using Alamar Blue (R&D) for survival.

### Chromatin Immunoprecipitation and Sequencing

Chromatin isolation from GIST882, GIST48, and GIST-T1 cells was performed as previously described (21). For **ETV1** knockdown experiments, chromatin was isolated 72 hours after siRNA transfection with either Scramble (Dharmacon) or ETV1-specific siRNA (siETV1; Dharmacon). For drug treatment experiment, GIST882 chromatin was isolated 8 hours after treatment, and GIST-T1 chromatin was isolated 2 hours after treatment.

The human ChIP–qPCR primer pairs were as follows:

**KIT enhancer1**: F: GAAGCAAAACCCACAGCTGTA, R: TTTGCC AACTGTTGCTTGCC;

**KIT enhancer2**: F: GGGGAAGACAGAAAAACACC, R: TCGAAGA CTTGTTCTCCCTG

**KIT enhancer3**: F: TGGTTTTCCTCGTCACAGATCC, R: GGGAAGA AAGCAGCAGGGA;

**PSA promoter**: F: TGGGCGGTGCTCTTCACAGATCC, R: CTCGGATGC ACCAGGGCC.

HSK4me1 and HSK4me3 ChIP sequencing was performed in GIST48 cells (GSE64609). Next-generation sequencing was performed on either an Illumina Genome Analyzer II or a HiSeq2000 with 50-bp single reads. Reads were aligned to the human genome (hg19) using the Bowtie alignment software within the Illumina Analysis Pipeline, and duplicate reads were eliminated for subsequent analysis. Peak calling was performed using MACS 1.4 comparing immunoprecipitated chromatin with input chromatin. On the basis of ReSeq gene annotation, the resultant peaks were separated into promoter peaks (located within ±2 kb of a transcription start site), promoter distal peaks (located from ~50 kb of a transcription start to +5 kb of a transcription end), and otherwise intergenic peaks. The ChIP-seq profiles presented were generated using Integrated Genome Browser software of SGR format files.

### Gene Expression Analysis

We have also performed at least three sets of independent **ETV1** shRNA knockdown experiments in GIST882, GIST48, and GIST-T1 cells, assayed the effects of **ETV1** suppression on **KIT** expression by qRT-PCR, and pooled all experiments for analysis.

To determine the transcriptional effect of Cre-mediated **Etv1** exon 11 excision in murine cecal tumors, we performed RNA-seq (GSE64608). The isolated RNA was processed using the TruSeq RNA sample Prep Kit (#15026495; Illumina) according to the manufacturer’s protocol. Briefly, the RNA was Poly-A selected and reverse transcribed, and the obtained cDNA underwent end-repair, A-tailing, ligation of the indexes and adapters, and PCR enrichment. The libraries were sequenced on an Illumina HiSeq-2500 platform with 51 bp paired-end reads to obtain a minimum yield of 40 million reads per sample. The sequence data were processed and mapped to the human reference genome (hg19) using STAR v2.3.30 (33). Gene expression was quantified using the Cuffdiff (34). Hierarchical clustering was performed using Partek Genomics Suite. GSEA was performed using JAVA GSEA 2.0 program (28). The gene sets used were the Broad Molecular Signatures Database gene sets c2 (curated gene sets), c5 (gene ontology gene sets), c6 (oncogenic signatures), c7 (immunologic signatures) as well as additional sets “Imatinib UP” and “Imatinib DN” composed of genes upregulated and downregulated by 2-fold with FDR < 0.05 in cecal tumors of Kit^dn/dn^ mice, respectively (26).

### Statistical Analysis

All statistical comparisons between two groups were performed by Graphpad Prism software using a two-tailed unpaired t test.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Targeting ETV1 in GIST

REFERENCES


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