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. Cancer Discov; 5(3); 304–15. © 2015 AACR.

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. Families with germline-activating KIT mutations develop diffuse hyperplasia of IC cells that progresses to GIST. 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/PDGFRA*, 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). 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 repertoire 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 *Kit*Δ558V/+ 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*−/−;*Kit*Δ558V/+ and *Etv1*+/−;*Kit*Δ558V/+ littermates at day P10. Consistent with prior observations, all three *Etv1*+/−;*Kit*Δ558V/+ 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 is required for GIST tumor initiation in vivo. A, representative hematoxylin and eosin (H&E) staining of the cecal mass and the cecum of Etv1Δ558/+;KitΔ558/+ and Etv1Δ558/Δ;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 Etv1Δ558/+;KitΔ558/+ and Etv1Δ558/Δ;KitΔ558/+ mice, respectively. Yellow arrows, preserved ICC-SMP with positive KIT immunostaining. Scale bar, 50 μm. C, summary of the histologic findings in Etv1Δ558/+;KitΔ558/+ and Etv1Δ558/Δ;KitΔ558/+ cecum examined at 10 days postnatal. D, representative H&E and KIT IHC images of the large intestine and stomach in Etv1Δ558/+;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 Etv1Δ558/+;KitΔ558/+ mice, demonstrating the lack of a KIT-positive ICC layer between the longitudinal muscle and the circular muscle layers in Etv1Δ558/Δ;KitΔ558/+ mice. Scale bar, 50 μm.

Etv1Δ558/+;KitΔ558/+ 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 Etv1Δ558/Δ;KitΔ558/+ mice (21). These observations suggest that Etv1 is required for GIST tumor initiation in vivo through its direct regulation of the lineage specification and development of the GIST precursor ICCs.

To evaluate whether Etv1 is required for GIST tumor proliferation, we crossed the Etv1lox/lox conditional knockout mouse model where Etv1 exon 11 that encodes the DNA binding domain has been placed between LoxP sites (25) with the Rosa26CreERT2 mouse that ubiquitously expresses the tamoxifen-responsive CREERT2 domain has been placed between LoxP sites (25) with the Etv1lox/lox conditional knockout mouse model where Etv1 exon 11 that encodes the DNA binding domain has been placed between LoxP sites (25) with the Rosa26CreERT2 mouse that ubiquitously expresses the tamoxifen-responsive CreERT2 to generate a GEM model where Etv1 can be temporally ablated in adult tissues by tamoxifen treatment. Tamoxifen administration in adult Etv1lox/lox;Rosa26CreERT2/CreERT2 mice caused no observable phenotype, suggesting that the degree of Etv1 ablation achieved is compatible with animal survival (data not shown). We next generated Etv1lox/lox;Rosa26CreERT2/CreERT2, CreERT2/+;Rosa26CreERT2 mice and compared the effect of tamoxifen and vehicle (corn oil) treatment in 2-month-old adult mice. In mice treated with tamoxifen, genomic DNA PCR of cecal tumor samples confirmed significant but incomplete excision of Etv1 exon 11 (Supplementary Fig. S2A). Vehicle-treated mice exhibited an identical phenotype to the Kitlox/+ 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 in Kitlox/+ mice (26). Further, 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 Etv1lox/lox;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 Dusp6, 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 KitΔ/Δ mice (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 log. 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/fl;KitV558D/ΔRosa26CreERT2/CreERT2 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–S5D). 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 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 (MEK1ΔL115P, MEK2ΔL119P) that are resistant to allosteric MEK inhibitors such as MEK162 due to reduced drug binding (29). GIST-T1 cells expressing either MEK1ΔL115P or MEK2ΔL119P were more resistant to MEK162 alone. Moreover, the combination...
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therapy provided more potent and durable inhibition of MAP pathway even at significantly reduced doses of MEK162 (10 mg/kg), resulting in a dramatic reduction (Δ) in tumor size. Remarkably, the combination of imatinib and MEK162 resulted in near-complete response in GIST-T1 xenografts within 3 weeks of treatment (Fig. 6D). 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 of MEK162 and imatinib conferred less synergistic growth inhibition in the presence of MEK1111p or MEK2111p in GIST-T1 cells (Fig. 5E). This corresponded to a decreased ability of MEK162 to inhibit ERK phosphorylation and ETV1 protein stability (Fig. 5F). These data indicate that the synergistic effect of MEK162 and imatinib combination treatment is the result of the on-target effect of MEK162.

Next, we tested the effect of combined MEK162 and imatinib in vitro. In the GIST882 xenograph model, single-agent imatinib or MEK162 stabilized tumor growth at the MTDs (Fig. 6A). Remarkably, the combination of imatinib and MEK162 treatment resulted in a dramatic reduction (>50%) of tumor size within 7 days and complete responses with prolonged treatment 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). Importantly, the ETV1 protein level was more potently and durably 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 xenograph 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 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, MAP kinase, and AKT signaling pathways in GIST882 and GIST-T1 cells treated with various doses of imatinib and MEK162 as indicated for 8 hours. 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 MEK1WT, MEK1L115P, MEK2WT, and MEK2L119P. 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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**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 KIT 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 substantially 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 vitro, 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 KitΔV558+/ΔV558+ knockin mouse was a generous gift from Dr. Peter Besmer (Memorial Sloan Kettering Cancer Center; ref. 30), the ETV1−/− mice were a generous gift from Dr. Dr. Jonathan A. Fletcher (Dana-Farber Cancer Institute) and were maintained as previously described (21). The GIST-T1 cell line harbored a 57-nucleotide (V570-Y578) in-frame deletion in KIT previously described (21). The GIST-T1 cell line was obtained from A. Fletcher (Dana-Farber Cancer Institute) and were maintained as described in Methods. 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 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 for KIT mutations by DNA sequencing and have tested negative for mycoplasma infection by the MycoAlert Plus Mycoplasma Detection Kit (Lonza), most recently in February 2014.

Antibodies to the following were used for IHC, IF: Western blotting, and ChIP: rabbit anti-ETV1 (Abcam; 1:100 for IF, 1:500 for Western blotting, 2 µg for ChIP), rabbit anti-AN01 clone SP31 (LSBio; 1:50 for IHC), rabbit anti-KIT (Cell Signaling Technology, #3074; 1:1,000 for Western blotting, 1:100 for IHC), rat anti-mouse KIT (clone ACK4; Cedarlane; CL8936ap; 1:100 for IF, 2 µg for ChIP), rabbit anti-Ki67 (Abcam; ab15580; 1:400 for IHC), rabbit anti-H3K4me1 (for ChIP,
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Lentiviral Knockdown and CRISPR/Cas9-Mediated Knockout

plKO.1 constructs against ETV1 (shETV1: TRCN0000013925, targeting CGACCAGTTAGTACCCACAA in exon 7) were purchased from Open Biosystems, and plKO.1 shScr (targeting CCTAAGGTGACCCAGTGTATGAACACAA in exon 7) were purchased from Addgene. Lentiviruses were generated by cotransfecting the shETV1 hairpin constructs with psPax2 and pVSVG (Addgene) into 293FT cells (Invitrogen) using Lipofectamine 2000 (Invitrogen). GIST-T1 cells were infected with shETV1 shRNA lentiviruses (Invitrogen). GIST882, GIST48, and GIST-T1 cells were infected with shScr or shETV1 lentivirus. RNA was collected 72 hours after infection and analyzed for KIT mRNA by RT-PCR.

To knock out ETV1 in human GIST cell lines, we designed three pairs of single guide RNA (sgRNA) sequences for human ETV1 using the design tool from the Feng Zhang Lab and cloned the targeting sgRNAs or vector control were generated in 293FT cells by standard methods using amphotropic packaging vector. GIST48 cells were infected with lentivirus for 48 hours and selected with 2 μg/mL puromycin for 7 days. KIT mRNA and protein level were analyzed 16 days after infection. The target guides sequences are as follows:

- sgETV1-1: F: CACCGCAGCCCTTTAAATTCAGCTA; R: AAACTA GCTGAATTTAAAGGGCTGC;
- sgETV1-2: F: CACCGTGAAGAGGTGGCCCGACGTT; R: AAACAA CGTCGGGCCACCTCTTCAC;
- sgETV1-3: F: CACCGAGCTTGCCGGTGGTTAGT; R: AACACTA GCTGAATTTAAAGGGCTGC.

Stable Gene Expression

cDNAs for human wild-type MEK1, wild-type MEK2, MEK1 L115P mutant, and MEK2 L158F mutant were cloned into lentiviral-based vector plX301 (Addgene). Lentivirus was produced in 293FT cells by standard methods using amphotropic packaging vector. GIST-T1 cells were infected and selected with 2 μg/mL puromycin for 5 days at 48 hours after infection for subsequent biochemical and drug treatment studies.

To determine the effect of ETV1 overexpression on KIT transcript levels, cDNA of human ETV1 was cloned into murine stem-cell virus-based retroviral vector pMIG (Addgene). Retrovirus was produced in 293FT cells by standard methods using amphotropic packaging vector. GIST882 and GIST-T1 cells were infected with empty vector or pMIG-ETV1. RNA was isolated 48 hours after infection to analyze KIT mRNA by qRT-PCR.

Mouse Procedures

For the GI tract of mice at different postnatal ages (postnatal day 7 to 6 months old), the stomach, small intestine, large intestine, cecum, and or cecal GIST tumors were dissected, separated, and embedded in paraffin, or snap-frozen as previously described (21) for subsequent analyses. For tamoxifen or corn oil treatment of Etv1 f/f;Kit V558 Δ/+;Rosa26 +/ERT2 CreERT2 mice, tamoxifen (Toronto Research Chemicals) 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 V558 Δ/+;Rosa26 +/ERT2 CreERT2 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 lyses 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 by retro-orbitally injecting t-luciferin and imaging with the IVIS Spectrum Xenogen machine (Caliper Life Science). To generate lyses 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 cryotome. Tamoxifen-treated and corn oil–treated mouse gastrointestinal tract sections were blocked for 1 hour using 5% goat serum, and incubated with DAPI, FITC, and Texas Red filter sets were pseudocolored blue, green, and red, respectively, and merged using ImageJ. The exposure, threshold, and maximum 1.4) objectives. Monochrome images taken with DAPI, FITC, and Texas

RNA Isolation and qRT-PCR

For tissue culture cells, RNA was isolated using the E.Z.N.A total RNA Kit (Omega). For xenograft and mouse models, explanted tissue samples were ground in 1,000 μL 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 Realplex machine (Eppendorf). Expression was normalized to the ribosomal protein RPL27. The following primer pairs were used (21):

**ETV1-Exon67**: F: CTACCCCATGGAACCAGAGATT, R: CTAAAAG CCTTGGGTGGGGAAG; **KIT**: F: GGGATTTCCTCTCGGCTCTG, R: GATGGATGAGTGG GGGAGC; **DUSP6**: F: TGCCGCGGCTTCTACCTGGGA, R: GGGCAGCTGCT GCTACACGA; **RPL27**: F: CATGGGCGAAGAAGAGATCG, R: TCCAAGGGGGA TATCCACAGA.

**Cell Viability**

GIST882 and GIST-T1 cells were plated at 4 × 10⁴ and 1 × 10⁴ 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 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 (siCR; Pharmaco) or ETV1-specific siRNA (siETV1; Pharmaco). 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: GAAGCAAACCCCGCCGTGA, R: TTTGCC AACCTGGTTGCTGCG; **KIT enhancer2**: F: GGGGAAGCCAGGAAAAACACC, R: TCGAAG CTTGCTCCTGCG; **KIT enhancer3**: F: TGGTTTCTCCTGCACAGATCC, R: GGAAGA AAGGAGCCCGGAAA; **PSA promoter**: F: TGGGCCGTTGTCTCGTCC; R: CCTGGATGC ACCGGCCC.

**H3K4me1 and H3K4me3 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 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 KITΔV558Δ 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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Combined Inhibition of MAP Kinase and KIT Signaling Synergistically Destabilizes ETV1 and Suppresses GIST Tumor Growth

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