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

Leili Ran1, Inna Sirota1, Zhen Cao1, Devan Murphy1, Yuedan Chen1, Shipra Shukla1, Yuanyuan Xie1, Michael C. Kaufmann1,2, Dong Gao1, Sinan Zhu1, Ferdinando Rossi3, John Wongvipat1, Takahiro Taguchi4, William D. Tap5,6, Ingo K. Mellinghoff1,2,7, Peter Besmer3, Cristina R. Antonescu8, Yu Chen1,5,6,9, and Ping Chi1,5,6,9

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

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

Corresponding Authors: Ping Chi, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 646-888-3338; Fax: 646-888-3406; E-mail: chip@mskcc.org; and Yu Chen, Phone: 646-888-3356; Fax: 646-888-3406; E-mail: cheny1@mskcc.org

doi: 10.1158/2159-8290.CD-14-0985

©2015 American Association for Cancer Research.
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). 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 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/+ intermutes at day P10. Consistent with prior observations, all 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 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+/−;Kit558V/+ and Etv1−/−;Kit558V−/− 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+/−;Kit558V/+ and Etv1−/−;Kit558V−/− mice, respectively. Yellow arrows, preserved ICC-SMP with positive KIT immunostaining. Scale bar, 50 μm. C, summary of the histologic findings in Etv1+/−;Kit558V/+ and Etv1−/−;Kit558V−/− cecum examined at 10 days postnatal. D, representative H&E and KIT IHC images of the large intestine and stomach in Etv1+/−;Kit558V/+ 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−/−;Kit558V−/− mice, demonstrating the lack of a KIT-positive ICC layer between the longitudinal muscle and the circular muscle layers in Etv1−/−;Kit558V−/− mice. Scale bar, 50 μm.

Etv1−/−;Kit558V−/− 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−/−;Kit558V−/− 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 Etv1flm 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-activatable CreERT2 to generate a GEM model where Etv1 can be temporally ablated in adult tissues by tamoxifen treatment. Tamoxifen administration in adult Etv1flm/+;Rosa26CreERT2/+ mice caused no observable phenotype, suggesting that the degree of Etv1 ablation achieved is compatible with animal survival (data not shown). We next generated Etv1flm/+;Rosa26CreERT2/+;Etv1558V+/− 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 Kit558V/+ 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 Kit558V/+ 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 Etv1flm/+;Rosa26CreERT2/+;Kit558V−/− 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 ET1 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 ET1 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 Kit(Rosa26;Rosa26;CreERT2/CreERT2) 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 ET1 and KIT regulate a common set of core transcriptional program. This is consistent with the model that ET1 is a major downstream effector of KIT, and also that ET1 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 ET1 is required for tumor proliferation and growth in vivo.

To determine whether ET1 regulates KIT transcription in human GIST, we knocked down ET1 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 ET1 knockdown (Fig. 4A). CRISPR/Cas9–mediated knockout of ET1 in GIST48 cells also resulted in a decrease in both KIT transcript and protein levels (Supplementary Fig. S4A and S4B). We next retrovirally overexpressed ET1 in GIST882 and GIST-T1 cells and found a modest upregulation in KIT transcript level (Fig. 4B). We performed GSEA of ET1 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 those identified as downregulated by ET1 knockdown, whereas genes most upregulated by imatinib were the most enriched gene set among upregulated by ET1 knockdown (Fig. 4C), consistent with our observation in mouse tissues (Fig. 3).

To determine whether KIT is a direct transcriptional target of ET1, we analyzed chromatin immunoprecipitation and
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 GEMM (+Etv1f/f;Kitf/f;Rosa26CreERT2/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.

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). 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 MEK1/2 due to reduced drug binding (29). GIST-T1 cells expressing either MEK1L115P or MEK2L119P were more resistant to MEK162 alone. Moreover, the combination
Targeting ETV1 in GIST

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 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.

of MEK162 and imatinib conferred less synergistic growth inhibition in the presence of MEK11L115P or MEK2L115P 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 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 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 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 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, KIT, 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, 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 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).
Imatinib
Imatinib + MEK162

Targeting ETV1 in GIST

The synergy of combination is more apparent in MEK162 in GIST tumor growth. A clear synergistic growth-inhibitory effect of imatinib and the genetically engineered GIST xenograft studies than in 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: 30 mg/kg twice a day; (iv) imatinib + MEK162: 80 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; ****, 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), MEK162 (30 mg/kg twice a day). n = 5, mean ± SEM. Two-tailed unpaired t test: *, P < 0.0001; **, P < 0.0001; ***, P < 0.0001. D, 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 (100 mg/kg twice a day) + MEK162 (30 mg/kg twice a day). Scale bar, 50 μm. E, 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). n = 5, mean ± SEM. Two-tailed unpaired t test: *, P < 0.0001; **, P < 0.0001; ***, P < 0.0001. F, 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).

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 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 K253R549* 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 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 KITlow 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 synergized with lower doses of imatinib, but higher doses of MEK162 (30 mg/kg twice a day; imatinib: 50 mg/kg twice a day; MEK162: 30 mg/kg twice a day) n = 7–9, mean ± SEM. Two-tailed unpaired t test, P value indicated in figure. B, representative Trichrome and Ki67 IHC images of murine cecal GISTs isolated after 5 days of drug treatment by oral gavage of the GIST GEMM (KitΔV558/+), under the same conditions as in A. Scale bar, 50 μm. C, tumor weight of murine cecal GISTs isolated after 5 days of drug treatment by oral gavage of the GIST GEMM (KitΔV558/+), under the same conditions as in A n = 7–9, mean ± SEM. Two-tailed unpaired t test, P value indicated in figure. D, immunoblots of representative cecal tumors from GIST GEMM (KitΔV558/+), treated 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.

**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/+ 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. Thomas Jessell (Columbia University; ref. 24), the Etv1floxflox mice were a generous gift from Dr. David Ladle (Wright State University; ref. 25), and the Rosa26CreERT2/+ mice were a generous gift from Dr. Andrea Ventura (Memorial Sloan Kettering Cancer Center; ref. 31). The Etv1−/+;KitΔV558/+;Etv1−/+;KitΔV558/+;Etv1−/+;KitΔV558/+;Rosa26CreERT2/+;Rosa26CreERT2/+;Rosa26CreERT2/+;Rosa26CreERT2/+ 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 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; 1:100 for Western blotting, 1:100 for IHC), rat anti-mouse KIT (clone ACK4; Cedarlane; CL8936ap; 1:100 for IF), rabbit anti-Ki67 (Abcam; ab15580; 1:400 for IHC), rabbit anti-H3K4me1 for ChIP; anti-KIT (clone 22C10; Biolegend; 1:100 for IF).
Targeting ETV1 in GIST

Rosa26^{CreERT2}/CreERT2 mice, tamoxifen (Toronto Research Chemicals) mutant, and analyses. For tamoxifen or corn oil treatment of or cecal GIST tumors were dissected, separated, and embedded in 70% ethanol, and the RNA was further purified using the E.Z.N.A total RNA Kit (Omega).

**Stable Gene Expression**

cDNAs for human wild-type MEK1, wild-type MEK2, MEK1^{ΔΔA} mutant, and MEK2^{ΔΔA} mutant were cloned into lentiviral-based vector pLX301 (Addgene). Lentiviruses were produced 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:

sEVT1-1: 5'-CACCGGATCCTCGCCGTTGGTATGT; R: AAACACCACTCCCTTCAC
sEVT1-2: 5'-CACCGCGCCCTTTTATTCCGCTA; R: AAACACTCTGAACTTTCGTC
sEVT1-3: 5'-CACCGGAGCTGCTCGGTGTGTA; R: AAACACATCACCAAGGAGATCC

**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 colon 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^{fl/fl}, Kit^{S558A/+}, Rosa26^{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^{S558A/+} mice, approximately 8- to 10-week-old Kit^{S558A/+} 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 C57-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 luciferin and imaging with the IVIS 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. Tumor 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 luciferin and imaging with the IVIS 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).

For IHC 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. Tumor 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 luciferin and imaging with the IVIS 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).

**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 mL Tissue-Tek (Tissue) 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-Exon67F:** CTACCACCTGACACAGATTT, **R:** CTTTAAG CTTTGTTGTTGAGGAA;  
**KIT:** F: GGAGATTTTCTCTCGGTTCTG, **R:** GATGGATGGATGGTGGAGGAC;  
**DUSP6F:** TGGCGGGGGCTTACCTGGGA, **R:** GGGCGGTGCTGCTGACAGCA;  
**RPL27F:** CATGGGCAAGGAAGAGATCG, **R:** TCCAAGGGGA TATCCACAGA.

**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.

**Gene Expression Analysis**

Gene expression analysis was performed as previously described (21). For 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.

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; 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 primers were as follows:

- **KIT enhancer1F:** GAGACAAACCACCCAGGCTGTA, **R:** TTTGGCC AACTGTTGCTCCGG;  
- **KIT enhancer2F:** GGGGAAGACAGAAAAACCACCC, **R:** TCAGAGA CTTGTCCCTTGCGG;  
- **KIT enhancer3F:** TGGTTTTCCTGTCACAGATCC, **R:** GGAGA GAGGGAGCAGGGGAA;  
- **PSA promoter:** TGGGGCTGTGCTCCCTGTC, **R:** CTTGATGACTCAGGCC.

**Gene Expression Analysis**

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-Exon67F:** CTACCACCTGACACAGATTT, **R:** CTTTAAG CTTTGTTGTTGAGGAA;  
- **KIT:** F: GGAGATTTTCTCTCGGTTCTG, **R:** GATGGATGGATGGTGGAGGAC;  
- **DUSP6F:** TGGCGGGGGCTTACCTGGGA, **R:** GGGCGGTGCTGCTGACAGCA;  
- **RPL27F:** CATGGGCAAGGAAGAGATCG, **R:** TCCAAGGGGA TATCCACAGA.

**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.

**Authors’ Contributions**

Conception and design: L. Ran, Y. Xie, W.D. Tap, P. Besmer, Y. Chen, P. Chi  
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Ran, I. Sirota, D. Murphy, Y. Chen, S. Shukla, P. Rossi, J. Wongvipat, I.K. Mellinghoff, C.R. Antonescu, Y. Chen, P. Chi  
Analysis and interpretation of data (e.g., statistical analysis, biositistics, computational analysis): L. Ran, D. Murphy, D. Gao, C.R. Antonescu, Y. Chen, P. Chi  
Writing, review, and/or revision of the manuscript: L. Ran, F. Rossi, W.D. Tap, C.R. Antonescu, Y. Chen, P. Chi  
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Ran, I. Sirota, Z. Cao, D. Murphy, S. Shukla, Y. Xie, D. Gao, S. Zhu, T. Taguchi, P. Chi  
Study supervision: L. Ran, Y. Chen, P. Chi  
Other (provided constructs): M.C. Kaufmann

**Acknowledgments**

The authors thank the following MSKCC core facilities: Mouse Genetics Core (W. Mark and P. Romaniello), Genomics Core Laboratory (A. Viale) at MSKCC, and the Rockefeller University Genomics Core (S. Dewell and C. Zhao). They also thank Dr. Jonathan A. Fletcher at Brigham and Women’s Hospital for providing the GIST882 and GIST48 human GIST cell lines and Drs. Thomas Jessell (Columbia University), David Ladle (Wright State University), and Andrea Ventura (Memorial Sloan Kettering Cancer Center) for providing the Etra1 Δ/−Δ, Etra1 Δ/Δ and the Rosa26ΔCreERT2/ΔCreERT2−/− mice, respectively.

**Grant Support**

This work was supported in part by the NIH/NCI (K08CA140946, to Y. Chen; K08CA151660, to P. Chi; P50 CA140146, to P. Chi; C.R. Antonescu, and P. Besmer; R01CA102774, to P. Besmer; DP2CA174499, to P. Chi), the Starr Cancer Consortium (to Y. Chen and P. Chi), the Sidney Kimmel Foundation (Sidney Kimmel Scholar Award, to P. Chi), the Sarcoma Foundation of America Research (to P. Chi), and the GIST Cancer Awareness Foundation (to P. Chi). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 29, 2014; revised January 5, 2015; accepted January 5, 2015; published OnlineFirst January 8, 2015; DOI: 10.1158/2159-8290.CD-14-0985
REFERENCES


Growth Synergistically Destabilizes ETV1 and Suppresses GIST Tumor Growth

Leili Ran, Inna Sirota, Zhen Cao, et al.