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Combined inhibition of MAP kinase and KIT signaling synergistically destabilizes ETV1 and suppresses GIST tumour growth

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Abstract

Gastrointestinal stromal tumour (GIST), originating from the interstitial cells of Cajal (ICCs), is characterized by frequent activating mutations of the *KIT* receptor tyrosine kinase. Despite the clinical success of imatinib that targets KIT, most advanced GIST patients 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

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Authors' contributions

P.C. and Y.C. conceived of the project. L.R., I.S., D.M., P.C. and Y.C. performed the mouse genetics and xenograft treatment experiments with technical support from J.W.; L.R. performed all of the cellular growth assays, knockdown, drug treatment experiments with technical support from Z.C. and I.S. P.C. and Y.C. performed expression profiling and ChIP-seq with technical support from S.S. and I.S. Y.C. performed bioinformatics analysis. C.R.A. provided pathology review. P.B. provided the *Kit*^{558V/+} mice. T.T. provided the GIST-T1 cell line. M.K. and I.K.M. provided MEK1/2 constructs. C.R.A., P.B., F.R. and W.D.T. provided intellectual input. L.R., Y.C. and P.C. wrote the manuscript. All authors reviewed and revised the manuscript.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

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 tumour regression *in vivo*. The combination strategy to target ETV1 may provide an effective therapeutic strategy in GIST clinical management.

Keywords

GIST; ETV1; MAPK

Introduction

Gastrointestinal stromal tumour (GIST) represents one of the most common subtypes of human sarcomas with approximately 5,000 cases a year in the US. GIST arises from the interstitial cells of Cajal (ICCs) 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 progress 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 multi-targeted tyrosine kinase inhibitor (TKI) that targets KIT/PDGFR, is the standard first line therapy in advanced GIST with radiographic response rate of approximately 50%, and disease stabilization in another 25-30% (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 advanced GIST patients eventually die of their disease (14-18). Imatinib resistance remains the greatest challenge in the management of advanced GISTs. Due to the vast heterogeneity of resistance mechanisms both between patients and within individual patient, it is challenging to develop next generation therapies that can address the majority 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 breakthrough 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 models, we demonstrate that *Etv1* is required for GIST tumour initiation and proliferation in the physiological *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 tumour initiation and proliferation

To assess whether *Etv1* is required for GIST initiation *in vivo*, we crossed the germline *Kit*^{558V/+} knock-in mouse model that develops ICC hyperplasia throughout the gastrointestinal tract and GIST-like tumours in the cecum (22, 23) with the *Etv1*^{-/-} knockout mouse model (24) that is defective in ICC development (21). Since the *Etv1*^{-/-} mice die at postnatal day10-14 (P10-P14) (24), we examined the GI tract of *Etv1*^{-/-};*Kit*^{558V/+} and *Etv1*^{+/+};*Kit*^{558V/+} littermates at day P10. Consistent with prior observations, all three *Etv1*^{+/+};*Kit*^{558V/+} mice developed GIST-like masses in the cecum that stain positively for Kit and Etv1 (**Fig. 1A, B**) and diffuse ICC hyperplasia in the stomach and large intestines (**Fig.1C, D**). In contrast, one of the three *Etv1*^{-/-};*Kit*^{558V/+} mice developed ICC hyperplasia in the cecum and none developed cecal GIST-like tumours or ICC hyperplasia of the stomach or large intestine (**Fig. 1A-C, E**). In addition, immunohistochemistry against ICC makers, Kit and Ano1, showed that *Etv1*^{-/-};*Kit*^{558V/+} 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. 1**), phenocopying the ICC loss in *Etv1*^{-/-} mice(21). These observations suggest that *Etv1* is required for GIST tumour 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 tumour proliferation, we crossed the *Etv1*^{flox} conditional knockout mouse model where *Etv1* exon 11 that encodes the DNA binding domain has been placed between LoxP sites (25) with the *Rosa26*^{CreERT2} mouse that ubiquitously expresses the tamoxifen-activated *CreERT2* to generate a genetically engineered mouse (GEM) model where *Etv1* can be temporally ablated in adult tissues by tamoxifen treatment. Tamoxifen administration in adult *Etv1*^{flox/flox};*Rosa26*^{CreERT2/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 *Etv1*^{flox/flox};*Kit*^{558V/+};*Rosa26*^{CreERT2/CreERT2} 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 tumour samples confirmed significant but incomplete excision of *Etv1* exon 11 (**Supplementary Fig. 2A**). Vehicle treated mice exhibited an identical phenotype to

the *Kit*^{558V/+} mice, with highly proliferative GIST-like tumours of the cecum and ICC-hyperplasia of the large intestine and the stomach (**Fig. 2AC**). In contrast, tamoxifen treated mice exhibited significant reduction of cell proliferation by Ki67 IHC in cecal tumours and ICC hyperplasia (**Fig. 2A-C**). This level of Ki67 reduction is reminiscent of the imatinib treatment in *Kit*^{558V/+} mice (26). Further, *Etv1* ablation by tamoxifen treatment induced significant fibrosis indicated by Masson's trichrome stain in the cecal tumours similar to imatinib treatment (27) (**Fig. 2D**). These observations demonstrate that *Etv1* is required for GIST tumour 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*^{flox/flox};*Kit*^{V558 /+};*Rosa26*^{CreERT2/CreERT2} cecal tumours. The RNA-seq profile of *Etv1* transcript shows that tamoxifen treated tumours had a ~3.4-fold decrease in the floxed exon 11 count, implying a 3.4-fold decrease in full-length, functional *Etv1* transcript (**Supplementary Fig. 2B, C**). This decrease is due to 1) 1.7-fold decrease in *Etv1* overall transcript level and 2) ~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 tumours compared to controls (**Supplementary Fig. 2D**).

Despite the incomplete ablation of *Etv1*, tamoxifen treatment induced robust transcriptional changes as seen by hierarchical clustering (**Fig. 3A, Supplementary Table 1**). The RNA transcripts of known *Etv1* transcriptional targets including *Dusp6*, *Gpr20* and *Edn3* (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 and IHC analyses showed a consistent decrease in Kit protein levels in tamoxifen-treated cecal tumours (**Supplementary Fig. 2D and Fig. 3C-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. 3**).

To determine the biological processes perturbed by *Etv1* ablation, we performed Gene Set Enrichment Analysis (GSEA) comparing tamoxifen and corn oil treated tumours (28). Remarkably, the set of genes most down-regulated by imatinib in *Kit*^{V558 /+} mice (*Imatinib DN*) (23) is the most enriched gene set among those downregulated by tamoxifen treatment (**Fig. 3E, Supplementary Table 2 and 3**). Likewise, the set of genes most up-regulated 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 2**). These data are consistent with the decrease in Ki67 staining after tamoxifen treatment and suggest that *Etv1* is required for tumour 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 decrease in both *KIT* transcript and protein levels (**Supplementary Fig. 4A, B**). 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 was the most enriched gene set among downregulated genes by *ETV1* knockdown while genes most upregulated by imatinib was the most enriched gene set among unregulated genes by *ETV1* knockdown (**Fig. 4C**), consistent with our observation in mouse tissues (**Fig. 3**).

To determine whether *KIT* is a direct transcriptional target of ETV1, we analyzed ChIP-seq of ETV1 in human GIST cells. We found multiple binding sites of ETV1 at the *KIT* enhancer regions characterized by high H3K4me1 and low H3K4me3 marks in human GIST cells (**Fig. 4D**). The direct and specific binding of ETV1 to the enhancer regions of the *KIT* locus was confirmed by ChIP-qPCR with siRNA-mediated suppression of *ETV1* in all three GIST cell lines (**Fig. 4E-G**). These observations suggest that in addition to the regulation of ETV1 protein stability by MAP kinase signaling downstream of mutant *KIT* signaling, ETV1 directly and positively regulates *KIT* expression and therefore, it cooperates with mutant *KIT* by forming a positive feedback circuit to promote GIST tumourigenesis.

Combined inhibition of the KIT and MAP kinase signaling represents an effective strategy to target ETV1 and suppress GIST tumour 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 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. 5 A-D**). Notably, the ability of MEK162 to durably inhibit MAP kinase pathway and the ETV1 protein stability is cell line specific—GIST882 cells displayed sustained inhibition while GIST-T1 cells showed reactivation of the MAP kinase pathway and re-accumulation 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 μ M MEK162 was combined with low dose imatinib (62.5 nM in GIST882 and 40 nM in GIST-T1) (**Fig. 5C, D**). To assess whether the synergistic effect is due to on-target effect of MEK162, we expressed wild-type MEK1/2 (WT) or MEK1/2 mutants (*MEK1^{L115P}*, *MEK2^{L119P}*) that are resistant to allosteric MEK inhibitors such as MEK162 due to reduced drug binding (29). GIST-T1 cells expressing either *MEK1^{L115P}* or *MEK2^{L119P}* were more

resistant to MEK162 alone. Moreover, the combination of MEK162 to imatinib conferred less synergistic growth inhibition in the presence of *MEK1^{L115P}* or *MEK2^{L119P}* 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 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 tumour growth at the maximum tolerated doses (**Fig. 6A**). Remarkably, the combination of imatinib and MEK162 treatment resulted in a dramatic reduction (>50%) of tumour 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, B**). Combination therapy provided more potent and durable inhibition of MAP kinase signaling (**Fig. 6C, Supplementary Fig. 6A**). Importantly, the ETV1 protein levels was more potently and durably inhibited, which was associated with reduction of *ETV1* transcriptional targets (*e.g.*, *DUSP6* and *KIT*) than either imatinib or MEK162 alone (**Fig. 6C, Supplementary Fig. 6B**). When GIST882 xenografted mice were treated from the same day of cell implantation, only the combination of imatinib and MEK162 successfully prevented xenograft tumour formation, suggesting that dual lineage inhibition could also inhibit GIST tumour formation *in vivo* (**Supplementary Fig. 6C**).

In the GIST-T1 xenograft model, single agent imatinib led to tumour stabilization. However, single agent MEK162 did not significantly inhibit tumour growth (**Fig. 6D**), consistent with the inability of MEK162 to durably inhibit the MAP kinase pathway in GIST-T1 cells (**Fig. 5A, 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, E**). The treatment effects 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. 6D, E**). These observations demonstrated a clear synergistic growth inhibitory effect of imatinib and MEK162 in GIST tumour 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 *Kit^{V558} /+* 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 tumour proliferation by Ki67 and increased tumour fibrosis by trichrome staining (**Fig. 7A-D**). The combination treatment of imatinib and MEK162 lead to increased tumour fibrosis and significantly greater reduction of Ki-67 than either single agent (**Fig. 7A, B**). Moreover, the combination treatment had significantly reduced tumour weight compared to either single agent alone or to vehicle (**Fig. 7C**). These treatment effects of the combination therapy are accompanied by increased inhibition of the Kit and MAP kinase signaling pathways, decreased ETV1 protein and 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 genetically engineered mouse 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, since *ETV1* is required for the survival of GIST precursor ICCs and is required for GIST tumour initiation *in vivo*, it may also represent a therapeutic target for the Kit-low GIST progenitor/stem cell population. Importantly, targeting ETV1 will help break the positive feedback circuit and indirectly target *KIT* expression independent of the *KIT* mutational status.

While it is challenging to therapeutically target non-ligand 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 tumourigenesis activates downstream MAP kinase signaling and augmented 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 inhibit 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 genetically engineered mouse models *in vivo*, maximum tolerated doses 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 MAP kinase pathway bypassing KIT (26). The survival signals that bypass KIT may be heterogeneous dependent on the tumour contexts. Here, addition of even low doses of MEK162, leads to durable destabilization of ETV1 protein and dramatically augments tumour response, resulting in complete responses.

The response to single agent imatinib in our model systems mirrors that of patients undergoing first-lines imatinib treatment. While the majority of patients attain clinical benefits with imatinib treatment, the RESIST response rate is only ~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 upfront.

Methods

Generation of compound genetically engineered GIST models (GEM)

All mouse studies are approved by MSKCC IACUC under protocol 11-12-029. The *Kit*^{558V/+} knock-in mouse was a generous gift from Dr. Peter Besmer (30), the *Etv1*^{-/-} mice was a generous gift from Dr. Thomas Jessell (24), the *Etv1*^{flox/flox} mice was a generous gift from Dr. David Ladle (Wright State University) (25) and the *Rosa26*^{CreERT2} mice was a generous gift from Dr. Andrea Ventura (31). The *Etv1*^{-/-};*Kit*^{558V/+}, *Etv1*^{+/+};*Kit*^{558V/+}, *Etv1*^{flox/flox};*Rosa26*^{CreERT2/CreERT2} and *Etv1*^{flox/flox};*Kit*^{558V/+};*Rosa26*^{CreERT2/CreERT2} 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 (DFCI) and were maintained as previously described (21). The GIST-T1 cell line was obtained from Dr. Takahiro Taguchi (32). The GIST-T1 cell line harbors 57-nucleotide (V570-Y578) in-frame deletion in *KIT* exon 11 and was maintained in RPMI supplemented with 10% FBS, 10mM HEPES pH 7.5. All GIST cell lines have been authenticated for KIT mutations by DNA-sequencing and have been tested negative for mycoplasma infection by MycoAlert Plus MycoPlasma Detection Kit (Lonza), last in February, 2014.

Antibodies to the following were used for IHC, Immunofluorescence (IF), western blotting and ChIP: rabbit anti-ETV1 (Abcam, 1:100 for IF, 1:500 for western blotting, 2 µg for ChIP), rabbit anti-ANO1 clone SP31 (LSBio, 1:50 for IHC), rabbit anti-KIT (Cell signaling, #3074, 1:1000 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, Abcam, ab8895), rabbit anti-H3K4me3 (for ChIP, Abcam, ab8580), rabbit anti-phosphorylated KIT (Cell signaling, #3073, 1:1000 for western blotting), rabbit anti-phosphorylated ERK1/2 (Cell signaling, #4370, 1:5000 for western blotting, 1:400 for IHC), rabbit anti-ERK1/2 (Cell signaling, #4695, 1:5000 for western blotting), rabbit anti-phosphorylated AKT (Cell signaling, #4060, 1:1000 for western blotting), rabbit AKT (Cell signaling, #4685, 1:1000 for western blotting), rabbit anti-phosphorylated S6 (Cell signaling, #2317, 1:1000 for western blotting, 1:400 for IHC), rabbit anti-S6 (Cell signaling, #4856, 1:1000 for western blotting), rabbit anti-cleaved caspase 3 (Cell signaling, #9661, 1:1000 for western blotting, 1:400 for IHC), HRP-conjugated anti-GAPDH (Abcam, ab9385, 1:5000 for western blotting), HRP-conjugated anti-actin (Abcam, ab49900, 1:5000 for western blotting). The MEK162 (a MEK inhibitor) and imatinib (a KIT inhibitor) were supplied by Novartis.

Lentiviral knockdown and CRISPR/Cas9 mediated knockout

pLKO.1 constructs against *ETV1* (shETV1: TRCN0000013925, targeting CGACCCAGTGTATGAACACAA in exon 7) were purchased from Open Biosystems and pLKO.1 shScr (targeting CCTAAGGTAAAGTCGCCCTCG) was purchased from Addgene. Lentiviruses were generated by co-transfecting the shETV1 hairpin constructs with psPax2 and pVSVG (Addgene) into 293FT cells (Invitrogen) using Lipofectamine 2000 (Invitrogen). GIST882, GIST48 and GIST-T1 cells were infected shSCR or shETV1

lentivirus. RNA was collected 72 hours post infection and analyzed for *KIT* mRNA by RT-PCR.

In order to knock out *ETV1* in human GIST cell lines, we designed 3 pairs of single guide RNA (sgRNA) sequences for human *ETV1* using the design tool from Feng Zhang Lab and cloned the targeting sequences into the lentiCRISPRv2 vector obtained from Addgene. Lentiviruses for *ETV1* 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 2ug/ml puromycin for 7 days. *KIT* mRNA and protein level were analyzed 16 days post infection. The target guides sequences are:

sgETV1-1: F: CACCGTGAAGAGGTGGCCCGACGTT; R: AAACAACGTCGGGCCACCTCTTCAC; sgETV1-2: F: CACCGCAGCCCTTTAAATTCAGCTA; R: AAAGTAGCTGAATTTAAAGGGCTGC;

sgETV1-3: F: CACCGGATCCTCGCCGTTGGTATGT; R: AAACACATACCAACGGCGAGGATCC.

Stable gene expression

cDNAs for human wild-type *MEK1*, wild-type *MEK2*, *MEK1^{L115P}* mutant, *MEK2^{L119P}* 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 2ug/ml puromycin for 5 days at 48 hours post 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 MSCV-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 post infection to analyze *KIT* mRNA by qRT-PCR.

Mouse procedures

For the GI tract of mice at different postnatal age (Postnatal day 7 to 6 months old), the stomach, small intestine, large intestine, cecum or cecal GIST tumours are dissected and separated and embedded in paraffin or snap frozen as previously described(21) for subsequent analyses. For tamoxifen or corn oil treatment of *Etv1^{fl/f};Kit^{V558} /+;Rosa26^{CreERT2/CreERT2}* mice, tamoxifen (Toronto Research Chemicals) was dissolved in at 20 mg/ml in corn oil and injected intraperitoneally to 6-week old mice at a dose of 4 mg every other day for 3 doses. Mice were euthanized 2 weeks after the first tamoxifen dose.

For drug treatment studies in *Kit^{V558} /+* mice, approximately 8-10 weeks *Kit^{V558} /+* mice were treated in 4 cohorts by oral gavage, 1) Vehicle: water; 2) Imatinib: 50 mg/kg BID; 3) MEK162 30 mg/kg, BID; 4) Imatinib+MEK162: Imatinib 50 mg/kg BID + MEK162 30 mg/kg BID. Cecal tumours were isolated and weighed after 5 days of treatment and subjected for paraffin embedding and analyzed by H&E, Trichrome stain and IHC of Ki67.

For short-term treatment, the protein and RNA were isolated from cecal tumours 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 μ l of 1:1 mix of growth media and Matrigel (BD Biosciences) were subcutaneously injected into CB17-SCID mice (Taconic). Tumour sizes were measured weekly starting 6 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, xenograft were treated twice daily until the end of experiments. For treatment from the same day of implantation, GIST882 cells expressing firefly luciferase were grafted. Tumour growth was monitored by bioluminescence imaging of anesthetized mice by retro-orbitally injecting d-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).

Immunofluorescence, Immunohistochemistry and Histology

For immunofluorescence of cryostat sections of the mouse gastrointestinal tract, mouse stomach, small intestine, caecum and large intestine were dissected and fixed in 4% paraformaldehyde for 2h followed by an overnight incubation in 30% sucrose. They were then embedded in OCT, flash frozen and cut into 5- μ m sections using a cryostat. Tamoxifen treated and corn oil treated littermate controls of *Etv1^{fl/f};Kit^{V558} /+;Rosa26^{CreERT2/CreERT2}* were embedded onto the same block to ensure identical processing. Tissue sections were blocked for 1 hour using 5% goat serum, incubated with primary antibodies at 4 °C overnight and secondary antibody for 2h at room temperature. Slides were mounted using Prolong Gold (Invitrogen) and images were taken on a Nikon Eclipse TE2000-E microscope using a Photometric Coolsnap HQ camera. Images were taken with $\times 20$ (numerical aperture 0.75) or $\times 60$ (numerical aperture 1.4) objectives. Monochrome images taken with DAPI, FITC and Texas Red filter sets were pseudo-colored blue, green and red, respectively, and merged using ImageJ. The exposure, threshold and maximum were identical between Tamoxifen treated and corn oil treated littermate controls of *Etv1^{fl/f};Kit^{V558} /+;Rosa26^{CreERT2/CreERT2}* images.

Tissue paraffin embedding, sectioning and H&E staining were performed by the Histoserv, Inc. Immunohistochemistry was performed by the MSKCC HOPP automatic staining facility using a Ventana BenchMark ULTRA automated stainer.

RNA isolation and qRT-PCR

For tissue culture cells, RNA was isolated using E.Z.N.A total RNA kit (Omega). For xenograft and mouse models, explanted tissue samples were grounded in 1000 μ l Trizol (Invitrogen) using a PowerGen homogenizer (Fisher Scientific), followed by addition of 200 μ l chloroform. The samples were then centrifuged at 10,000g for 15 min. The upper

phase was mixed with an equal volume of 70% ethanol, and the RNA was further purified using E.Z.N.A total RNA kit (Omega).

For qRT-PCR, RNA was reverse transcribed using 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: CTACCCCATGGACCACAGATTT, R: CTAAAGCCTTGTGGTGGGAAG;

KIT: F: GGGATTTTCTCTGCGTTCTG, R: GATGGATGGATGGTGGAGAC;

DUSP6: F: TGCCGGGCGTTCTACCTGGA, R: GGCGAGCTGCTGCTACACGA

RPL27: F: CATGGGCAAGAAGAAGATCG, R: TCCAAGGGGATATCCACAGA;

Cell viability

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

Chromatin immunoprecipitation (ChIP) 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 h after siRNA transfection with either Scramble (siSCR, Dharmacon) or ETV1-specific siRNA (siETV1, Dharmacon). For drug treatment experiment, GIST882 chromatin was isolated 8 h after treatment and GIST-T1 chromatin was isolated 2 hours after treatment.

The human ChIP-qPCR primers pairs were:

KIT enhancer1: F: GAAGCAAACCCAGGCTGTA, R: TTTGCCAACTGTTGCTTCGG;

KIT enhancer2: F: GGGGAAGCACGAAAAACACC, R: TCGAAGACTTGTCCCTTGGC;

KIT enhancer3: F: TGGTTTCCTCGTCACAGATCC, R: GGAAGAAAGGAGCAGCGGAA;

PSA promoter: F: TGGGCGTGTCTCCTCTGC, R: CCTGGATGCACCAGGCC

H3K4me1 and H3K4me3 ChIP sequencing were 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 (hg 19) 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 RefSeq 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 and 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 tumours, we performed RNA-Seq (GSE64608). The isolated RNA was processed using the TruSeq RNA sample Prep kit (#15026495, Illumina) according to the manufacturers' protocol. Briefly, the RNA was Poly-A selected, reverse transcribed and the obtained cDNA underwent end-repair, A-tailing, ligation of the indexes & adapters, and PCR enrichment. The libraries were sequenced on an Illumina HiSeq-2500 platform with 51bp paired-end reads to obtain a minimum yield of 40 million reads per sample. The sequence data was processed and mapped to the human reference genome (hg19) using STAR v2.330(33). Gene expression was quantified using the Cuffdiff(34). Hierarchical clustering was performed using Partek Genomics Suite. Gene set enrichment analysis 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 up and downregulated by 2-fold with FDR < 0.05 in cecal tumours of *Kit*^{V558} /+ mice respectively(26).

Statistics

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations List

GIST	Gastrointestinal stromal tumour
ETV1	ets variant 1
MAPK	Mitogen-activated protein kinases

References

1. Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature*. 1995; 373:347–9. [PubMed: 7530333]
2. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science*. 1998; 279:577–80. [PubMed: 9438854]
3. Li FP, Fletcher JA, Heinrich MC, Garber JE, Sallan SE, Curiel-Lewandrowski C, et al. Familial gastrointestinal stromal tumor syndrome: phenotypic and molecular features in a kindred. *J Clin Oncol*. 2005; 23:2735–43. [PubMed: 15837988]
4. Miettinen M, Lasota J. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Arch Pathol Lab Med*. 2006; 130:1466–78. [PubMed: 17090188]
5. Nishida T, Hirota S, Taniguchi M, Hashimoto K, Isozaki K, Nakamura H, et al. Familial gastrointestinal stromal tumors with germline mutation of the KIT gene. *Nat Genet*. 1998; 19:323–4. [PubMed: 9697690]
6. Agaimy A, Wunsch PH, Sobin LH, Lasota J, Miettinen M. Occurrence of other malignancies in patients with gastrointestinal stromal tumors. *Semin Diagn Pathol*. 2006; 23:120–9. [PubMed: 17193825]
7. Agaram NP, Wong GC, Guo T, Maki RG, Singer S, Dematteo RP, et al. Novel V600E BRAF mutations in imatinib-naïve and imatinib-resistant gastrointestinal stromal tumors. *Genes Chromosomes Cancer*. 2008; 47:853–9. [PubMed: 18615679]
8. Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science*. 2003; 299:708–10. [PubMed: 12522257]
9. Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol*. 2003; 21:4342–9. [PubMed: 14645423]
10. Demetri GD. Identification and treatment of chemoresistant inoperable or metastatic GIST: experience with the selective tyrosine kinase inhibitor imatinib mesylate (STI571). *Eur J Cancer*. 2002; 38(Suppl 5):S52–9. [PubMed: 12528773]
11. Debiec-Rychter M, Dumez H, Judson I, Wasag B, Verweij J, Brown M, et al. Use of c-KIT/PDGFRα mutational analysis to predict the clinical response to imatinib in patients with advanced gastrointestinal stromal tumours entered on phase I and II studies of the EORTC Soft Tissue and Bone Sarcoma Group. *Eur J Cancer*. 2004; 40:689–95. [PubMed: 15010069]
12. Verweij J, Casali PG, Zalcberg J, LeCesne A, Reichardt P, Blay JY, et al. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. *Lancet*. 2004; 364:1127–34. [PubMed: 15451219]
13. Blanke CD, Rankin C, Demetri GD, Ryan CW, von Mehren M, Benjamin RS, et al. Phase III randomized, intergroup trial assessing imatinib mesylate at two dose levels in patients with unresectable or metastatic gastrointestinal stromal tumors expressing the kit receptor tyrosine kinase: S0033. *J Clin Oncol*. 2008; 26:626–32. [PubMed: 18235122]
14. Beadling C, Jacobson-Dunlop E, Hodi FS, Le C, Warrick A, Patterson J, et al. KIT gene mutations and copy number in melanoma subtypes. *Clin Cancer Res*. 2008; 14:6821–8. [PubMed: 18980976]

15. Blanke CD. Perforation and Stage-II Colon Cancer: Is it Always High Risk? *Gastrointest Cancer Res.* 2008; 2:103–4. [PubMed: 19259304]
16. de Raedt T, Cools J, Debiec-Rychter M, Brems H, Mentens N, Sciote R, et al. Intestinal neurofibromatosis is a subtype of familial GIST and results from a dominant activating mutation in PDGFRA. *Gastroenterology.* 2006; 131:1907–12. [PubMed: 17087943]
17. Demetri GD, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, et al. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet.* 2006; 368:1329–38. [PubMed: 17046465]
18. Demetri GD, Reichardt P, Kang YK, Blay JY, Rutkowski P, Gelderblom H, et al. Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet.* 2013; 381:295–302. [PubMed: 23177515]
19. Antonescu CR, Besmer P, Guo T, Arkun K, Hom G, Koryotowski B, et al. Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. *Clin Cancer Res.* 2005; 11:4182–90. [PubMed: 15930355]
20. Bardsley MR, Horvath VJ, Asuzu DT, Lorincz A, Redelman D, Hayashi Y, et al. Kitlow stem cells cause resistance to Kit/platelet-derived growth factor alpha inhibitors in murine gastrointestinal stromal tumors. *Gastroenterology.* 2010; 139:942–52. [PubMed: 20621681]
21. Chi P, Chen Y, Zhang L, Guo X, Wongvipat J, Shamu T, et al. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. *Nature.* 2010; 467:849–53. [PubMed: 20927104]
22. Antonescu CR, Sommer G, Sarraf L, Tschernyavsky SJ, Riedel E, Woodruff JM, et al. Association of KIT exon 9 mutations with nongastric primary site and aggressive behavior: KIT mutation analysis and clinical correlates of 120 gastrointestinal stromal tumors. *Clin Cancer Res.* 2003; 9:3329–37. [PubMed: 12960119]
23. Kwon JG, Hwang SJ, Hennig GW, Bayguinov Y, McCann C, Chen H, et al. Changes in the structure and function of ICC networks in ICC hyperplasia and gastrointestinal stromal tumors. *Gastroenterology.* 2009; 136:630–9. [PubMed: 19032955]
24. Arber S, Ladle DR, Lin JH, Frank E, Jessell TM. ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell.* 2000; 101:485–98. [PubMed: 10850491]
25. Patel TD, Kramer I, Kucera J, Niederkofer V, Jessell TM, Arber S, et al. Peripheral NT3 signaling is required for ETS protein expression and central patterning of proprioceptive sensory afferents. *Neuron.* 2003; 38:403–16. [PubMed: 12741988]
26. Rossi F, Ehlers I, Agosti V, Succi ND, Viale A, Sommer G, et al. Oncogenic Kit signaling and therapeutic intervention in a mouse model of gastrointestinal stromal tumor. *Proc Natl Acad Sci U S A.* 2006; 103:12843–8. [PubMed: 16908864]
27. Kim TS, Cavnar MJ, Cohen NA, Sorenson EC, Greer JB, Seifert AM, et al. Increased KIT inhibition enhances therapeutic efficacy in gastrointestinal stromal tumor. *Clin Cancer Res.* 2014; 20:2350–62. [PubMed: 24583793]
28. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005; 102:15545–50. [PubMed: 16199517]
29. Delaney AM, Printen JA, Chen H, Fauman EB, Dudley DT. Identification of a novel mitogen-activated protein kinase kinase activation domain recognized by the inhibitor PD 184352. *Mol Cell Biol.* 2002; 22:7593–602. [PubMed: 12370306]
30. Sommer G, Agosti V, Ehlers I, Rossi F, Corbacioglu S, Farkas J, et al. Gastrointestinal stromal tumors in a mouse model by targeted mutation of the Kit receptor tyrosine kinase. *Proc Natl Acad Sci U S A.* 2003; 100:6706–11. [PubMed: 12754375]
31. Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, et al. Restoration of p53 function leads to tumour regression in vivo. *Nature.* 2007; 445:661–5. [PubMed: 17251932]
32. Taguchi T, Sonobe H, Toyonaga S, Yamasaki I, Shuin T, Takano A, et al. Conventional and molecular cytogenetic characterization of a new human cell line, GIST-T1, established from gastrointestinal stromal tumor. *Lab Invest.* 2002; 82:663–5. [PubMed: 12004007]

33. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013; 29:15–21. [PubMed: 23104886]
34. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology*. 2010; 28:511–5.

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Statement of 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.

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Figure 1. *Etv1* is required for GIST tumour initiation *in vivo*

(A) Representative H&E staining of the cecal mass and the cecum of *Etv1*^{+/+}; *Kit*^{V558Δ/+} and *Etv1*^{-/-}; *Kit*^{V558Δ/+} mice respectively showing that *Etv1* is required for formation of GIST-like cecal tumour (yellow arrow point to malignant cells in tumour). M: mucosa; CM: circular muscle; LM: longitudinal muscle. Scale bar: 100μm.

(B) Representative immunofluorescence (IF) of KIT (red), ETV1 (green) and DAPI (blue) of the cecal tumour or cecum of *Etv1*^{+/+}; *Kit*^{V558Δ/+} and *Etv1*^{-/-}; *Kit*^{V558Δ/+} mice respectively. Yellow arrows point to the preserved submucosal ICCs (ICC-SMP) with positive KIT immunostaining. Scale bar: 50μm.

(C) Summary of the histological findings in *Etv1*^{+/+}; *Kit*^{V558Δ/+} and *Etv1*^{-/-}; *Kit*^{V558Δ/+} cecum examined at 10 days postnatal.

(D) Representative H&E and Kit immunohistochemistry (IHC) images of the large intestine and stomach in *Etv1*^{+/+}; *Kit*^{V558Δ/+} mice, demonstrating hyperplasia of the interstitial cells of Cajal (ICC) (yellow arrow pointing to Kit-positive ICC hyperplasia) in large intestine and stomach. Scale bar, 50μm.

(E) Representative H&E and Kit immunohistochemistry (IHC) images of the large intestine and stomach in *Etv1*^{-/-}; *Kit*^{V558Δ/+} mice, demonstrating the lack of Kit-positive ICC layer between the longitudinal muscle (LM) and the circular muscle (CM) layer in *Etv1*^{-/-}; *Kit*^{V558Δ/+} mice. Scale bar, 50μm.

Figure 2. *Etv1* is required for GIST tumour proliferation *in vivo*

(A) Representative images of Ki67 IHC of the cecal tumour of 8-9 weeks old, *Etv1^{flox/flox};Kit^{558V/+};Rosa26^{CreERT2/CreERT2}* mice treated with either corn oil or tamoxifen, demonstrating a significant reduction of Ki67 in the tamoxifen treated (*Etv1*-ablated) tissues. Scale bar: 50µm.

(B) Quantification of Ki67 percentage in cecal tumours of 8-9 weeks old *Etv1^{flox/flox};Kit^{558V/+};Rosa26^{CreERT2/CreERT2}* mice treated with either corn oil or tamoxifen. n=3, Mean ± SEM; two-tailed unpaired *t* test ($p=0.0005$).

(C) Representative images of Ki67 IHC of the large intestine showing a significant reduction of the Ki67 in the large intestine of the *Etv1^{fl/fl};Kit^{V558/+};Rosa26^{CreERT2/+}* treated with tamoxifen compared to corn oil control. Scale bar: 50µm.

(D) Representative images of trichrome stains of cecal mass of *Etv1^{flox/flox};Kit^{558V/+};Rosa26^{CreERT2/CreERT2}* mice treated with either corn oil or tamoxifen, demonstrating an increase in fibrosis in the tamoxifen treated cohort. Scale bar: 50µm.

Figure 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 tumours identified by RNA-seq. Clustering was based on 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 vs. corn oil-treated murine GISTs.

(C) Representative immunofluorescence (IF) images of ETV1 (green) and KIT (red) protein in cecal tumours from *Etv1^{fl/fl}; Kit^{V558Δ/+}; Rosa26^{CreERT2/CreERT2}* mice treated with tamoxifen or corn oil, demonstrating ETV1 ablation and decreased KIT protein level. Nuclei (DAPI, blue). Scale bars: 50μm.

(D) Representative KIT IHC images of the cecal tumours and ICC hyperplasia in the large intestines of mice treated as in (c). Scale bars: 50μm.

(E) GSEA plots of the ranked list of the differentially expressed genes between tamoxifen-treated vs. corn oil-treated murine GIST tumour 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 vs. corn oil-treated murine GIST tumour samples, using the ISHIDA_E2F_TARGETS gene set.

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 $\pm$ 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 $\pm$ SEM. Two-tailed unpaired t test: * $p < 0.05$; ** $p < 0.01$.

(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, read, and yellow color 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) vs. scrambled control siRNA (siSCR) in GIST882 cells. n=3, Mean $\pm$ SD.

(F) ChIP-qPCR of ETV1 at the *KIT* enhancer 2 (red mark in D) in GIST48 cells. n=3, Mean $\pm$ SD. (G) ChIP-qPCR of ETV1 at the *KIT* enhancer 2 (red mark in D) in GIST-T1 cells. n=3, Mean $\pm$ SD.

Figure 5. Combined inhibition of the 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 nM) or MEK162 (1 μM) for the indicated time points.

(B) ETV1 localization at the target gene loci (i.e. *KIT*, *DUSP6*) by ChIP-qPCR in GIST cells treated with Imatinib (1 μM) or MEK162 (500 nM) for 8 hours in GIST882, or Imatinib (80 nM) or MEK162 (500 nM) 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 $\pm$ 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 $\pm$ 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 nM); M: MEK162 (1000 nM).

Figure 6. Combined inhibition of the MAP kinase and KIT signaling synergistically suppress tumour growth in *in vivo* GIST xenograft mouse models

(A) Treatment response of GIST882 xenografts in SCID mice. The treatment cohorts are as the following: 1) Vehicle (blue): water; 2) Imatinib (green): 100 mg/kg BID; 3) MEK162 (red): 30 mg/kg BID; 4) Imatinib+MEK162 (dose 1) (magenta): Imatinib (100 mg/kg BID) + MEK162 (10 mg/kg BID); 5) Imatinib+MEK162 dose 2 (yellow): Imatinib (50 mg/kg BID) + MEK162 (30 mg/kg BID); 6) Imatinib+MEK162 dose 3 (black): Imatinib (100 mg/kg BID) + MEK162 (30 mg/kg BID) (dose 3). $n=6-8$, Mean $\pm$ 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 gavages as indicated. Vehicle: water; Imatinib: 100 mg/kg BID; MEK162: 30 mg/kg BID; Imatinib (100 mg/kg BID) + MEK162 (30 mg/kg BID).

(C) Immunoblots of three representative GIST882 xenograft tumours explanted after 2 days of drug treatment by oral gavages as indicated. Vehicle: water; Imatinib: 100 mg/kg BID; MEK162: 30 mg/kg BID; Imatinib (100 mg/kg BID) + MEK162 (30 mg/kg BID).

(D) Treatment response of GIST-T1 xenografts in SCID mice as indicated by oral gavages. The treatment cohorts are as the following: 1) Vehicle: water; 2) Imatinib: 80 mg/kg BID; 3) MEK162: 30 mg/kg BID; 4) Imatinib (80 mg/kg BID) + MEK162 (30 mg/kg BID). $n=10$, Mean $\pm$ 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 gavages as indicated. Vehicle (blue): water; Imatinib (green): 80 mg/kg BID; MEK162 (red): 30 mg/kg BID; Imatinib+MEK162 (magenta): Imatinib (80 mg/kg BID) + MEK162 (30 mg/kg BID).

(F) Immunoblots of three representative GIST-T1 xenograft tumours explanted after 2 days of drug treatment by oral gavages as indicated. Vehicle: water; Imatinib: 80 mg/kg BID; MEK162: 30 mg/kg BID; Imatinib (80 mg/kg BID) + MEK162 (30 mg/kg BID).

Figure 7. Combined inhibition of the MAP kinase and KIT signaling synergistically suppress tumour growth in genetically engineered GIST mouse model

(A) Ki67 percentage of murine cecal GISTs isolated after 5 days of drug treatment by oral gavages of the GIST GEMM (*Kit*^{V558/+}). Vehicle (black): water; Imatinib (blue): 50 mg/kg BID; MEK162 (yellow): 30 mg/kg BID; Imatinib+MEK162 (red): Imatinib (50 mg/kg BID) + MEK162 (30 mg/kg BID). n=7-9, Mean $\pm$ 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 gavages of the GIST GEMM (*Kit*^{V558/+}) under the same conditions as in (A).

(C) Tumour weight of murine cecal GISTs isolated after 5 days of drug treatment by oral gavages of the GIST GEMM (*Kit*^{V558/+}) under the same conditions as in (A). n=7-9, Mean $\pm$ SEM. Two-tailed unpaired *t* test, *p* value indicated in figure.

(D) Immunoblots of representative cecal tumours from GIST GEMM (*Kit*^{V558/+}) treated under the same drug treatment conditions as indicated in (A) for 1.5 days. Two cecal tumours from two different mice for each treatment conditions. Dusp6 is one of the transcriptional targets of Etv1.