Oncogene
https://doi.org/10.1038/s41388-018-0508-5

ARTICLE

LMTK3 is essential for oncogenic KIT expression in *KIT*-mutant GIST and melanoma

Lillian R. Klug[1,2,3] · Amber E. Bannon[1,2,3] · Nathalie Javidi-Sharifi[2,3] · Ajia Town[1,2,3] · William H. Fleming[2,3,4] · Judy K. VanSlyke[5] · Linda S. Musil[5] · Jonathan A. Fletcher[6,7] · Jeffrey W. Tyner[2,3] · Michael C. Heinrich[1,2,3]

Received: 13 March 2018 / Revised: 1 August 2018 / Accepted: 29 August 2018

Abstract

Certain cancers, including gastrointestinal stromal tumor (GIST) and subsets of melanoma, are caused by somatic *KIT* mutations that result in KIT receptor tyrosine kinase constitutive activity, which drives proliferation. The treatment of *KIT*-mutant GIST has been revolutionized with the advent of KIT-directed cancer therapies. KIT tyrosine kinase inhibitors (TKI) are superior to conventional chemotherapy in their ability to control advanced *KIT*-mutant disease. However, these therapies have a limited duration of activity due to drug-resistant secondary *KIT* mutations that arise (or that are selected for) during KIT TKI treatment. To overcome the problem of KIT TKI resistance, we sought to identify novel therapeutic targets in *KIT*-mutant GIST and melanoma cells using a human tyrosine kinome siRNA screen. From this screen, we identified lemur tyrosine kinase 3 (*LMTK3*) and herein describe its role as a novel KIT regulator in *KIT*-mutant GIST and melanoma cells. We find that LMTK3 regulated the translation rate of KIT, such that loss of LMTK3 reduced total KIT, and thus KIT downstream signaling in cancer cells. Silencing of *LMTK3* decreased cell viability and increased cell death in KIT-dependent, but not KIT-independent GIST and melanoma cell lines. Notably, *LMTK3* silencing reduced viability of all *KIT*-mutant cell lines tested, even those with drug-resistant KIT secondary mutations. Furthermore, targeting of *LMTK3* with siRNA delayed KIT-dependent GIST growth in a xenograft model. Our data suggest the potential of LMTK3 as a target for treatment of patients with *KIT*-mutant cancer, particularly after failure of KIT TKIs.

Introduction

The class III receptor tyrosine kinase KIT and its cognate ligand, stem cell factor (SCF), play important roles during development, as well as in adult stem cell maintenance [1]. Normal KIT activity is induced upon SCF binding and signals to numerous downstream pathways, including PI3K/AKT and MEK/ERK, to drive proliferation and survival of cells [2, 3]. Gain-of-function mutations in KIT causing ligand-independent kinase activation are known to drive neoplastic growth in multiple tissues [4–6]. *KIT*-mutant tumors include the majority of gastrointestinal stromal tumors (GIST) and mastocytosis, as well as subsets of melanoma, acute myeloid leukemia, and seminoma [7–11]. Somatic activating *KIT* mutations are rare overall, but within certain tumor types or subtypes, the frequency can be quite high. Of the 5000 new cases of GIST that are diagnosed each year in the U.S., over 70% of cases are caused by *KIT* mutations [12]. In melanoma, *KIT* mutations make up the most common oncogenic driver mutations in acral and mucosal subtypes, as well as melanomas arising from

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41388-018-0508-5) contains supplementary material, which is available to authorized users.

✉ Lillian R. Klug
klugl@ohsu.edu

1 Portland VA Health Care System, Portland, OR, USA

2 Knight Cancer Institute, Oregon Health and Science University, Portland, OR, USA

3 Division of Hematology and Medical Oncology, Oregon Health and Science University, Portland, OR, USA

4 Department of Pediatrics, Oregon Stem Cell Center, Oregon Health and Science University, Portland, OR, USA

5 Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, OR, USA

6 Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

7 Department of Pediatrics, Brigham and Women's Hospital, Boston, MA, USA

Published online: 21 September 2018

chronically sun-damaged skin [7, 13]. Both GIST and these melanoma subtypes have poor response to conventional cytotoxic therapies and radiation [14, 15]. However, KIT TKIs, such as imatinib, have improved outcomes for these patients. The median overall survival of patients with advanced GIST is estimated to be 7–8 years, and a subset of patients live more than 10 years [16–18]; this is in contrast to an overall survival of 12–18 months with conventional chemotherapies [19]. Although no KIT-targeted treatments are yet approved for *KIT*-mutant melanoma, early clinical trials have shown responses in some patients [13, 20–22].

While KIT TKI treatments, can provide significant clinical benefit to patients, they are rarely curative. The majority of GIST patients will develop drug resistance over the course of KIT TKI treatment; the median time to tumor progression on first-line imatinib therapy is 20–24 months [23]. GIST second- and third-line GIST therapies, sunitinib and regorafenib, respectively, are used to treat patients after failure on imatinib, but are also limited by resistance of their own [24, 25]. Resistance to KIT TKIs in GIST is almost exclusively caused by secondary *KIT* mutations, most commonly affecting the ATP binding pocket (V654A, T670I) or the activation loop (codons 816, 820, 822, 823, or 829 with multiple amino acid substitutions reported for most of these codons) [26–29]. Primary mutations that affect these domains can also confer drug resistance. Nonetheless, KIT TKI-resistant GIST remain dependent on KIT, and therefore KIT is still a relevant target. Disease management is complicated in the advanced setting with the existence of inter- and intra-lesional heterogeneity of *KIT* mutations. Patients can have various secondary mutations between and within lesions, and each mutation can have different sensitivity profiles to individual KIT TKIs [25, 27].

In the face of heterogeneous *KIT* mutations in these tumors, KIT TKIs have limited ability to control *KIT*-mutant disease once resistance develops, leaving patients with few treatment options. Because of this, we sought to identify novel targets in *KIT*-mutant cancer cells using a previously described siRNA screen methodology called RNAi Assisted Protein Identification (RAPID), which knocks down potential targets, including all predicted members of the human tyrosine kinome [30, 31]. Using this approach, we identified the protein kinase lemur tyrosine kinase 3 (LMTK3) as an essential gene in *KIT*-mutant GIST and melanoma cells. Despite its name, LMTK3 has been found to be a serine/threonine kinase with only a few identified kinase substrates or functions [32, 33]. LMTK3 has been implicated in promoting cancer growth, and some roles of LMTK3 have been described in breast cancer, including driver protein and transcriptional regulation [34–37]. However, the roles for LMTK3 are not fully understood in other tumor types.

This study describes our findings of the role for LMTK3 in promoting the viability of all *KIT*-mutant GIST and melanoma cells studied to date, including those with mutations conferring KIT TKI resistance. Our data show that LMTK3 is both necessary and sufficient for KIT protein expression. Specifically, we show that loss of LMTK3 reduces KIT translation in GIST cells, and thereby reduces KIT expression and downstream signaling. These data support that LMTK3 is a novel regulator of KIT expression, and because of its essential role in *KIT*-mutant cells regardless of KIT mutation, LMTK3 represents a potential therapeutic target in TKI-resistant *KIT*-mutant cancers.

Results

LMTK3 identified as essential for viability of mutant KIT-dependent cells

To identify novel targets in *KIT*-mutant cancers, we performed a siRNA screen using cell viability as a read-out. The siRNA library encompassed all known and predicted human tyrosine kinases, as well as *NRAS* and *KRAS* (93 genes total) [30, 31, 38]. We measured viability 96 h after transfecting cells with siRNA pools against each target in three *KIT*-mutant cell lines: two GIST and one melanoma (GIST430 [exon 11], GIST-T1 [39], and MaMel) (Supplementary Table 1).

Candidate genes that negatively affected viability were determined for each cell line by calculating the median and standard deviation across each entire screen experiment, as has been described previously [31, 40]; candidates for further comparison were those that reduced cell viability greater than one standard deviation from the median. All candidates within this cut off were found to have a statistically significant effect on cell viability (Supplementary Fig. 1). These genes were then compared across the three cell lines to identify common candidates. There were two candidates besides *KIT* (a positive control) that were shared by all three cell lines: *PTK2* and *LMTK3* (Fig. 1a). Protein tyrosine kinase 2 (PTK2), or focal adhesion kinase (FAK) has been described to have a role in GIST viability and imatinib resistance [41–43]. LMTK3, however, is a novel candidate in KIT-mutant cancers.

LMTK3 was validated in independent siRNA experiments using silencing of the essential cell cycle gene *PLK1* served as a positive control as indication of efficiency of transfection. *KIT* siRNA served as an additional positive control in mutant KIT-dependent cell lines and showed significant negative effect on cell viability in GIST-T1, GIST430 (ex11), and MaMel, in most cases comparable to *PLK1* silencing; the silencing of *LMTK3* decreased viability to similar levels in all three cell lines (Fig. 1b). Moreover, to

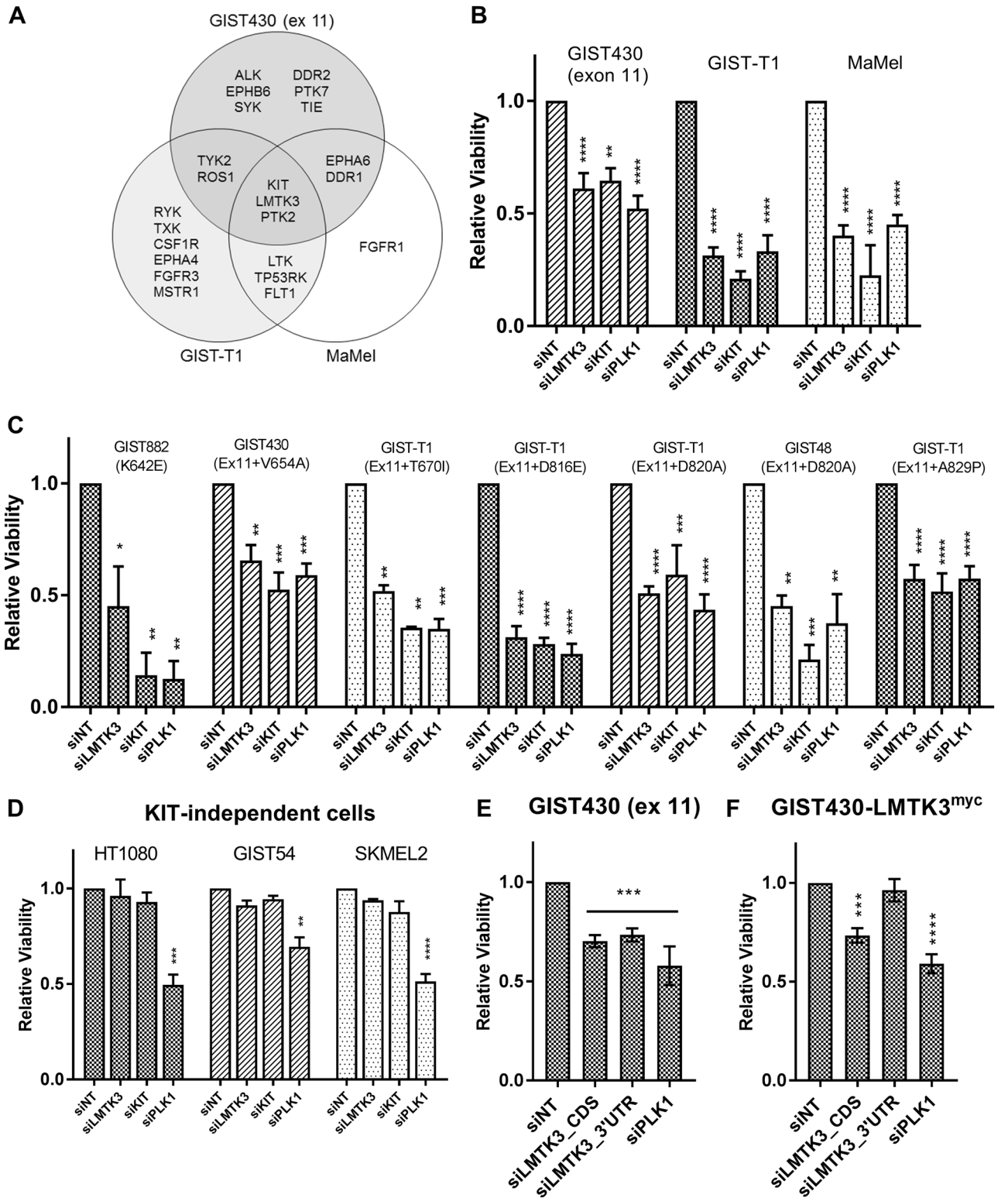

Fig. 1 Silencing of the protein kinase LMTK3 specifically reduces viability of mutant KIT-dependent GIST and melanoma cells. **a** Venn diagram of hits from RAPID tyrosine kinase siRNA screens performed in *KIT*-mutant GIST430 (ex11), GIST-T1, and MaMel cell lines. **b** Viability 96 h post-transfection with non-targeting (NT), *LMTK3*, and *KIT* siRNA. **c** Viability of *KIT*-mutant GIST cell lines was measured 96 h post-transfection with indicated siRNAs. **d** Viability of KIT-independent GIST and melanoma cells measured 96 h post-transfection with indicated siRNA. **e**, **f** Viability of GIST430 (ex11) and GIST430-LMTK3^{myc} cells 96 h post-transfection with shown siRNA. The p values of one-way ANOVA for each cell line to NT siRNA are indicated by asterisks: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$; ****, $p < 0.0001$. ($N > 3$)

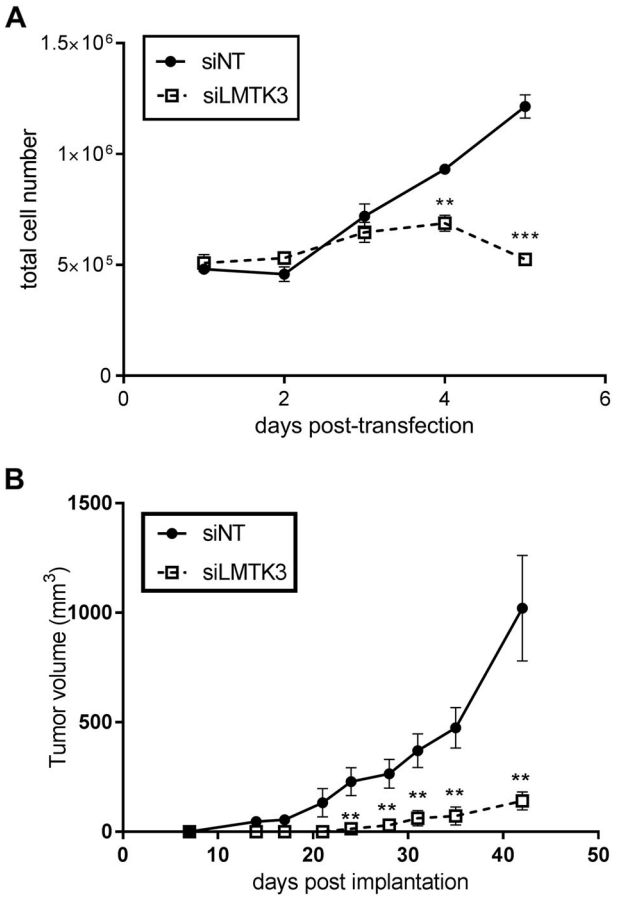

Fig. 2 *LMTK3* silencing inhibits proliferation of KIT-dependent cells in vitro and in vivo. **a** Total cell number over time of GIST430 (exon 11) after *LMTK3* silencing, ($N = 3$). **b** Subcutaneous tumor volume after implantation of 1×10^6 GIST430 (ex11) cells treated with the indicated siRNAs into each flank of an NRG mouse, ($N = 8$). The p values for t tests between NT and *LMTK3* siRNA on each day are indicated by asterisks: **, $p < 0.005$

corroborate these data, we found that multiple individual siRNAs against *LMTK3* decreased viability in *KIT*-mutant GIST and melanoma cell lines, as well as knocked down LMTK3 at the protein level (Supplementary Fig. 2).

LMTK3 is specifically essential for cell viability of KIT-dependent GIST and melanoma

To determine the breadth of the effect of *LMTK3* silencing in *KIT*-mutant cells, we expanded experiments to include a library of GIST and melanoma cell lines. These included GIST cell lines derived from those used in our initial screens (GIST430 [ex 11] and GIST-T1) and others that have secondary *KIT* mutations conferring resistance to KIT TKIs (Supplementary Table 2). Similar to *KIT* or *PLK1* silencing, *LMTK3* silencing in all mutant KIT-dependent cell lines, including those with KIT TKI-resistance mutations, decreased cell viability relative to non-targeting (NT) control siRNA (Fig. 1c). In contrast, KIT-independent fibrosarcoma (HT1080), GIST (GIST54), and melanoma (SKMEL2) cell lines showed no significant change in cell viability after *LMTK3* silencing when compared to the NT siRNA (Fig. 1d).

To further determine the specificity of the effects of *LMTK3* silencing on *KIT*-mutant cells, we created a stable GIST430 (ex 11) cell line expressing a c-myc epitope-tagged LMTK3 by lentiviral transduction (GIST430-LMTK3^{myc}). This construct contained the coding DNA sequence (CDS) of *LMTK3*, but lacked 5′ and 3′ untranslated regions (UTRs). Experiments were then performed in these, as well as control GIST430 (ex 11) cells using siRNAs targeting the *LMTK3* CDS (siLMTK3_CDS), which knocks down both endogenous and exogenous versions, or the *LMTK3* 3′UTR (siLMTK3_3′UTR), which only knocks down the endogenous version. LMTK3 knockdown with either the CDS-targeting or 3′UTR-targeting siRNAs significantly decreased cell viability in GIST430 (ex 11) cells, which only express endogenous LMTK3 (Fig. 1e). However, only siRNA targeting the *LMTK3* CDS, but not the 3′ UTR, decreased cell viability in the GIST430-LMTK3^{myc} cells (Fig. 1f), suggesting LMTK3^{myc} is sufficient to maintain cell viability and the impact of *LMTK3* silencing is due to on-target effects on endogenous LMTK3.

Silencing *LMTK3* reduces proliferation in vitro and in vivo in KIT-dependent cells

To understand the role of LMTK3 on the proliferation of KIT-dependent cells, we measured total cell number over time after *LMTK3* silencing. The proliferation of GIST430 (exon 11, Fig. 2a), GIST-T1, and MaMel cells in vitro (Supplementary Fig. 3) was significantly impaired by 96 h post-transfection with *LMTK3* siRNA. To understand the role of LMTK3 on the in vivo growth of *KIT*-mutant GIST cells, we injected siRNA-treated GIST430 (ex 11) cells subcutaneously into NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*$^{tm1Wjl/SzJ}$ (NRG) mice. GIST430 (ex 11) cells were transfected with NT or *LMTK3* siRNA 24 h prior to injection. *LMTK3* or NT siRNA-treated cells were implanted separately into the right or left flank, respectively. Non-targeted tumors were palpable within 3 weeks and animals were euthanized 6 weeks post-implantation. Non-targeting tumors grew at a rapid rate after becoming palpable, reaching an average volume of 1 cm [26] at the time of euthanasia. In contrast, tumors in which LMTK3 had been knocked down were not palpable until at least 4 weeks. These tumors grew much slower and were significantly smaller at the time of sacrifice (0.1 cm [26] average volume, $p < 0.005$, Fig. 2b). Taken together these data suggest that GIST cells in which *LMTK3* has been transiently silenced have reduced cell viability and proliferation in both in vitro and in vivo experiments.

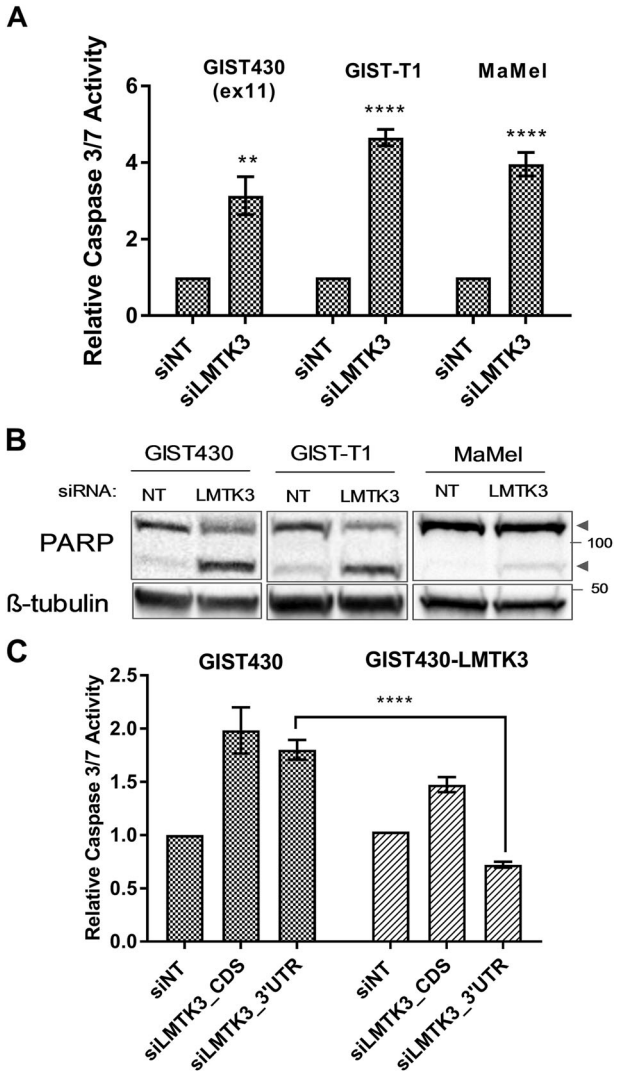

Fig. 3 *LMTK3* silencing induces apoptosis. **a** Activity of caspases 3 and 7, 96 h post-transfection with NT or *LMTK3* siRNA in *KIT*-mutant cells. ($N = 5$) **b** Immunoblot showing cleavage (lower arrowhead, 90 kDa) of full-length PARP (upper arrowhead, 110 kDa), 72 h post-siRNA transfection. **c** Activity of caspases 3 and 7, 96 h post-transfection with NT, *LMTK3* CDS or 3'UTR siRNA in GIST430 (ex11) or GIST430-LMTK3^{myc} cells. ($N = 3$) The p values of t test for each cell line are indicated by asterisks: **, $p < 0.005$; ***, $p < 0.001$; ****, $p < 0.0001$

LMTK3 silencing induces cell death in KIT-dependent cells

To clarify the mechanism by which *LMTK3* silencing decreased viability in KIT-dependent cells, we investigated the role of apoptosis. We measured the activity of caspases 3 and 7 in KIT-dependent cell lines 96 h post-siRNA transfection. We observed a significant increase in caspase 3/7 activity after *LMTK3* silencing (Fig. 3a), as well as a concordant increase in the cleavage of PARP, a downstream target of caspases, by immunoblotting (Fig. 3b). Further, a greater percentage KIT-dependent cells were permeable to propidium iodide (PI) after exposure to *LMTK3* siRNA (Supplementary Fig. 4), indicating they were undergoing cell death.

Apoptotic markers were also measured in GIST430 (ex11) vs. GIST430-LMTK3^{myc} cells treated with *LMTK3* CDS siRNA or *LMTK3* 3′UTR siRNA to determine the specificity of LMTK3 knockdown for this phenotype. We observed that LMTK3^{myc} expression was sufficient to prevent the induction of caspase activity seen when endogenous LMTK3 is knocked down, further indicating that *LMTK3* silencing specifically induces apoptosis to reduce viability in mutant KIT-dependent cells (Fig. 3c).

LMTK3 positively regulates KIT protein expression

Since *KIT*-mutant cells specifically depend on KIT expression and activity for proliferation and survival, as evidenced by sensitivity to KIT knockdown, we hypothesized that *LMTK3* silencing was affecting KIT. To test this possibility, we examined KIT protein after *LMTK3* siRNA transfection by immunoblotting. LMTK3 silencing significantly reduced the total amount of KIT protein in the imatinib-sensitive GIST and melanoma cell lines used in our screen (Fig. 4a–c) and in imatinib-resistant *KIT*-mutant GIST cells (Fig. 4b, c). The reduction in total KIT protein upon *LMTK3* silencing was reflected in a proportional reduction in KIT phosphorylation (Y721, Y703, and pan-phospho-tyrosine, Fig. 4a–d, Supplementary Fig. 5). Loss of KIT phosphorylation translated downstream to a reduction in the activity of pro-survival and proliferative signaling effectors, AKT (phospho-S473) and ERK1/2 (phospho-T202/pY204) in KIT-dependent cells (Fig. 4a, b).

Because loss of KIT can result in cell death in mutant KIT-dependent cells, we tested the ability of exogenous LMTK3^{myc} to affect this phenotype by targeting GIST430-LMTK3^{myc} cells with *LMTK3* CDS siRNA or *LMTK3* 3′ UTR siRNA. Maintenance of LMTK3 expression partially restored the loss of KIT protein in GIST430-LMTK3^{myc} cells (Fig. 4e). These results are in agreement with our above data showing the restoration of cell viability under the same conditions.

Upon observing the effect of LMTK3 knockdown on KIT abundance, we investigated how KIT expression was affected by LMTK3 overexpression. We measured KIT in GIST430-LMTK3^{myc} clones with variable levels LMTK3^{myc} expression. We found that LMTK3^{myc} protein abundance was highly correlated with KIT protein abundance (Supplementary Figures 6, $R2 = 0.9459$). Collectively, these data suggest that LMTK3 positively regulates KIT protein expression to support proliferative signaling and viability in mutant KIT-dependent cells, regardless of driver KIT mutation.

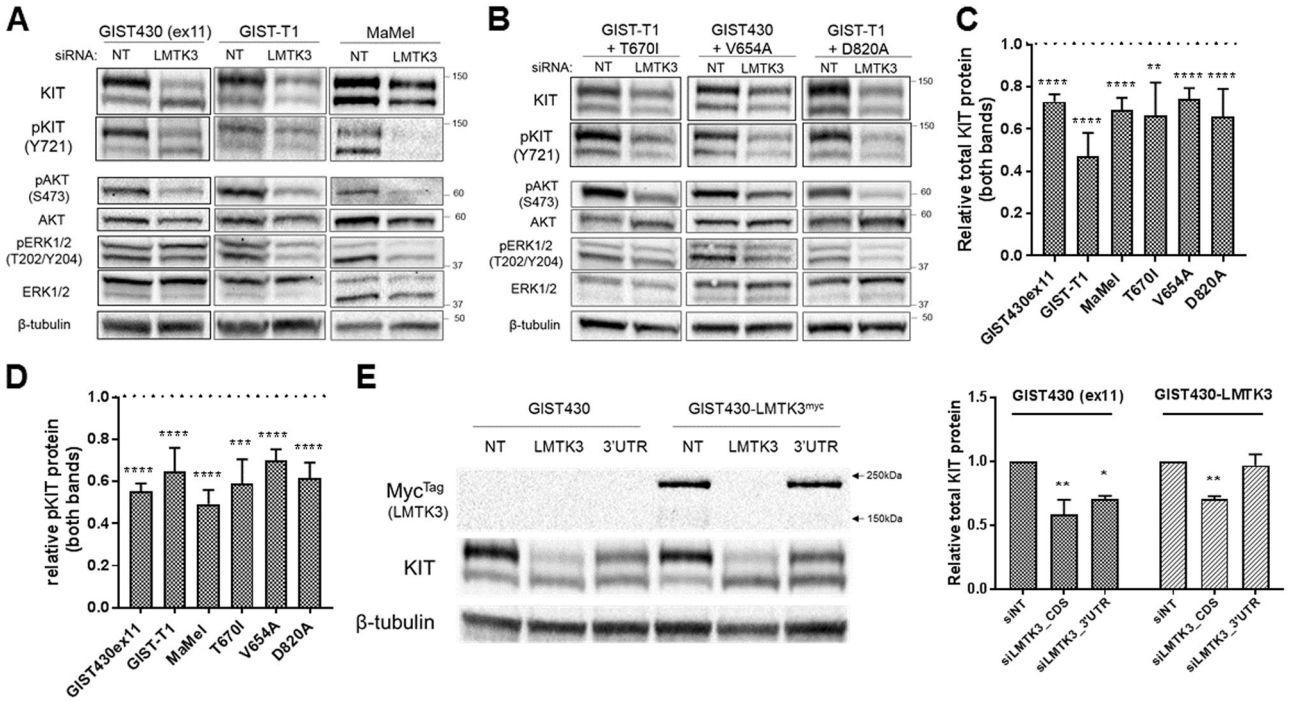

Fig. 4 Silencing of *LMTK3* in *KIT*-mutant GIST and melanoma cells reduces KIT protein expression. Immunoblotting of imatinib-sensitive GIST and melanoma cell lines (**a**) or imatinib-resistant GIST cell lines (**b**) 72 h post-transfection with NT or *LMTK3* siRNA. **c**, **d** Quantification of phospho-KIT (Y721) and total KIT protein from immunoblots, normalized to β-tubulin ($N = 3$). **e** Immunoblot and quantification of GIST430-LMTK3^{myc} stable cells 72 h post-transfection with NT, *LMTK3* CDS, or *LMTK3* 3′UTR siRNA, ($N = 4$). Bars show average protein relative to NT siRNA. The p values of t tests for each cell line compared to NT indicated by asterisks: **, $p < 0.005$; ***, $p < 0.001$; ****, $p < 0.0001$

LMTK3 regulates KIT translation in KIT-dependent GIST cells

There are multiple points of KIT regulation at which LMTK3 may play a role to control total KIT protein abundance, including transcription, translation, or protein stability. Because LMTK3 has been previously implicated to have roles in controlling transcription of oncogenes and cancer promoting genes [34, 36, 37, 44], we began by investigating whether LMTK3 is involved in regulating *KIT* transcription. We measured *KIT* mRNA abundance by qRT-PCR 72 h post-transfection with NT or *LMTK3* siRNA in GIST and melanoma cells. We found that *LMTK3* siRNA did not significantly change *KIT* transcript abundance, despite a significant decrease in *LMTK3* mRNA (Fig. 5a, Supplementary Figure 7A, C).

LMTK3 has also been implicated in regulating protein stability of ERα in breast cancer models [34]. To determine if LMTK3 may be playing a similar role in *KIT*-mutant GIST or melanoma cells, we calculated the half-life of KIT protein. We transfected cells with NT or *LMTK3* siRNA then treated with the translation inhibitor cycloheximide (CHX) at least 48 h post-transfection. We measured total KIT protein in whole cell lysates over a time course. Knockdown of LMTK3 did not shorten KIT protein half-life in GIST430 (ex 11) cell lines (Fig. 5b, Supplementary Figure 7E), nor was KIT protein half-life significantly different in MaMel and GIST-T1 cell lines transfected with NT vs. *LMTK3* siRNA (Supplementary Figure 7B, D, F-G). These results suggest that LMTK3 has a role that is independent of controlling KIT transcription or KIT protein stability in *KIT*-mutant cells.

We next investigated a role for LMTK3 in KIT translational control. At 48 h post- siRNA transfection, we labeled nascent proteins in GIST430 (ex 11) cells with radioactive (S^{35}) methionine and isolated KIT protein by immunoprecipitation and SDS-PAGE to quantify S^{35}-KIT. We observed a significant reduction in the abundance of S^{35}-labeled KIT with two individual *LMTK3* targeting siRNAs (CDS and 3′UTR) compared to NT control (Fig. 5c). These data provide evidence that LMTK3 plays an important role in regulating translation of KIT in KIT-dependent GIST cells to promote proliferative signaling that maintains these cancer cells.

Discussion

Despite the therapeutic success of KIT TKIs for treating *KIT*-mutant cancers, drug resistance presents a significant

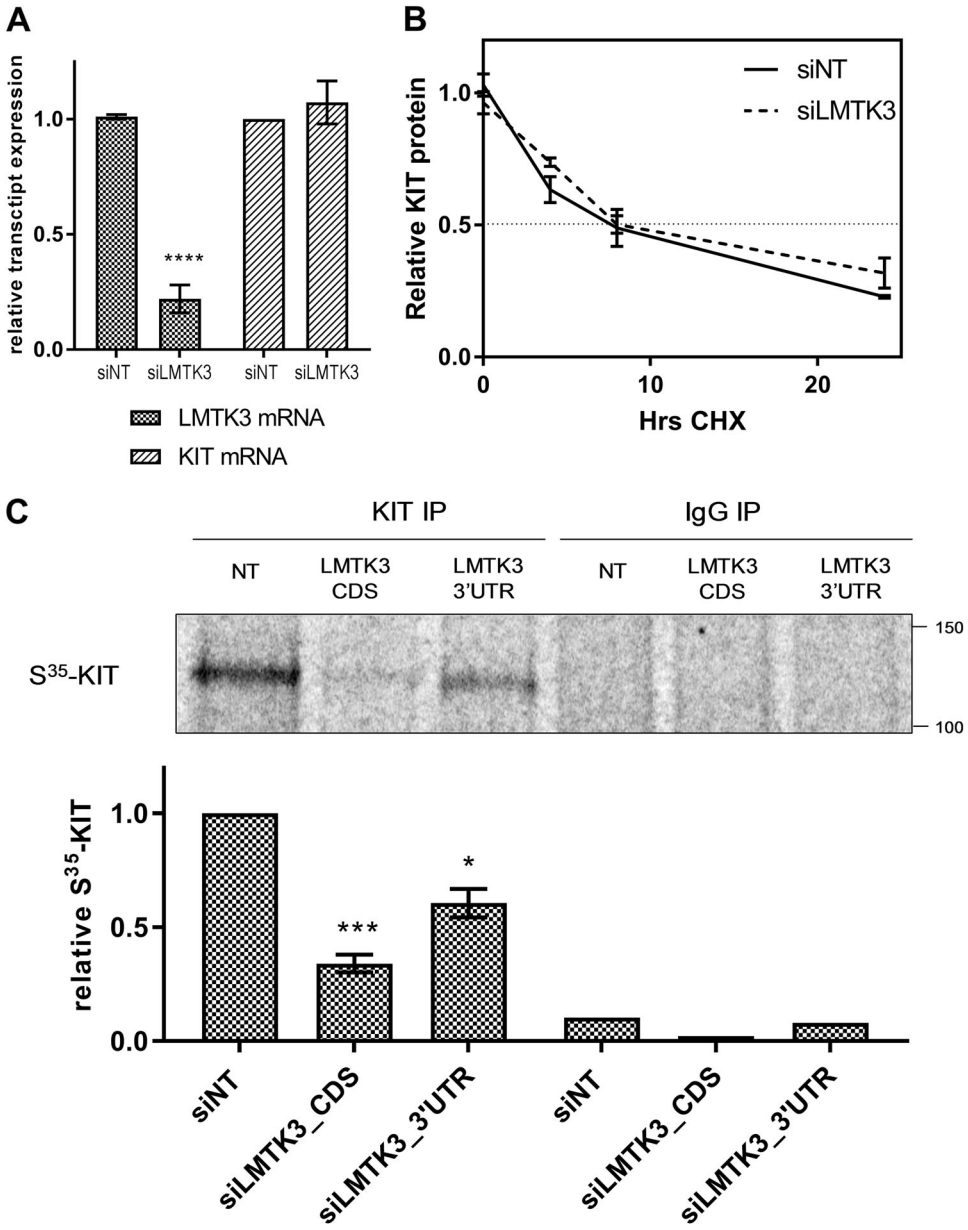

Fig. 5 LMTK3 regulates KIT translation in KIT-dependent GIST. **a** *LMTK3* and *KIT* transcript abundance relative to NT siRNA at 72 h post-transfection with *LMTK3* 3′UTR siRNA in GIST430 (ex 11). **b** KIT protein abundance after inhibition of translation with cycloheximide in GIST430 (ex 11) 48 h post-siRNA transfection. Protein half-life calculated by one-phase decay. **c** Gel showing immunoprecipitated S^{35}-KIT and quantification relative to NT ($N = 4$). GIST430 (ex 11) cells labeled 48 h post- siRNA transfection. The p values of one-way ANOVA indicated by asterisks: *, $p < 0.05$; ***, $p < 0.001$

clinical barrier. With this in mind, we used a siRNA screen to identify novel therapeutic candidates in *KIT*-mutant solid tumor cells. In this report, we describe a novel target, *LMTK3*, which was identified using three individual mutant KIT-dependent cell lines, two GIST and one melanoma. We found that silencing *LMTK3* specifically killed KIT-dependent cells bearing various *KIT* mutations, including those that confer imatinib resistance, and severely slowed GIST growth in vivo. We show that LMTK3 supports the viability of KIT-dependent GIST and melanoma cells by maintaining KIT expression.

The positive gene-specific regulation of KIT translation in KIT-dependent cells represents a novel function of LMTK3. LMTK3 has been shown to promote the expression of other oncogenes, including ERα in breast cancer, but via regulation of transcription and protein stability [34]. LMTK3 has not yet been directly shown to play roles in translation regulation in breast cancer models, but *LMTK3* silencing was shown to alter the abundance of many proteins in breast cancer [36], thus, LMTK3 may have translational regulation in this cancer as well, but this remains to be investigated.

Our data suggest that LMTK3 plays a role as a *KIT* gene-specific translational regulator in *KIT*-mutant GIST and melanoma cells, not as a regulator of global translation. Upon LMTK3 knockdown in mutant KIT-dependent cells we do not see global reduction in protein levels; for example, ERK1 and ERK2 protein remain unchanged.

Moreover, *LMTK3* silencing is not toxic to all cells, as would be the case if LMTK3 controlled total protein synthesis. KIT-dependent cells are uniquely sensitive to loss of LMTK3, supporting that LMTK3 can affect translation of KIT specifically. Prior to this study, it was not known that mutant KIT was regulated by gene-specific translation, although, WT *KIT*-specific translation has been reported in hematopoietic cells [45]. Gene-specific translational regulation is not uncommon, especially in cancer cells. Oncogenes can be specifically translated in a cap-dependent manner, often bypassing conventional translation regulation [46]. It is not yet clear if LMTK3 controls the translation of mutant KIT in this way, or if LMTK3 controls WT KIT in other cell types. This will be important to understand for assessing the therapeutic utility of LMTK3 as a target because some normal cells, such as hematopoietic stem cells depend on WT KIT. Interestingly, LMTK3 seems to have a specific role in *KIT*-mutant GIST and melanoma that is distinct from other *KIT*-mutant disease; this RAPID screen has been run on many cell lines and primary leukemia samples, including those with activating KIT mutations. *LMTK3* silencing does not affect the cell viability of the human *KIT*-mutant mastocytosis cell line, HMC-1.1 [30], nor do most *KIT*-mutant primary leukemia, suggesting it regulates KIT specifically in solid *KIT*-mutant tumor cells.

Most significantly, both KIT TKI-sensitive and -resistant cell lines showed decreased viability and decreased KIT expression after *LMTK3* silencing. Secondary KIT mutations, T670I, V654A, or D820A, confer resistance to imatinib and have variable sensitivity to second and third line KIT TKI (sunitinib and regorafenib) in GIST [25, 47]. Moreover, in advanced GIST, these mutations often co-occur to create complex intra- and inter-lesional heterogeneity, which cannot be controlled by any single clinically available KIT TKI. Resistant *KIT*-mutant GIST cells still depend on KIT for proliferation and survival, therefore KIT remains an important target. Silencing *LMTK3* can affect KIT T670I, V654A, or D820A, because LMTK3 regulates KIT at an early step of KIT expression, synthesis, suggesting it could be useful as a treatment strategy in advanced patients with various resistance mutations.

Collectively, these data presented here support the potential of LMTK3 as a therapeutic target for mutant KIT-driven GIST and melanoma. However, strategies for targeting LMTK3 have not been developed. Moreover, it is not yet clear what specific function of LMTK3 is responsible for regulating KIT translation in KIT-mutant cells. LMTK3 has indeed been shown to have serine/threonine kinase activity [32], but, it has also been shown to play roles in normal cells that are independent from its kinase activity, instead acting as a protein scaffold to bring together multi-protein regulatory complexes [48]. Either kinase activity or protein complex scaffolding could play important roles in translation, but targeting enzymatic activity of kinases has had the most success in the clinic historically. Future studies will be necessary to determine the crucial function of LMTK3 in order to design therapeutic strategies targeting LMTK3 in *KIT*-mutant solid tumors.

Materials and methods

Cell lines

A summary of the *KIT*-mutant cell lines used in this study are shown in Supplementary Table 2. *KIT*-mutant cell lines were authenticated by *KIT* sequencing and KIT TKI sensitivity experiments. Upon receipt, GIST and MaMel cell lines were tested for mycoplasma contamination by Venor™ GeM Mycoplasma Detection Kit (Sigma, St. Louis, MO) and were found to be negative. HEK293 (ATCC® CRL-1573™), HT1080 (ATCC® CCL-121™) and SKMEL2 (ATCC® HTB-68™) were purchased from ATCC (Manassas, VA) and experiments were performed within 6 months of purchase. The stable $LMTK3^{myc}$ expressing GIST430 (ex11) cell line was created by viral transduction and selection in G418 (800 ng/mL, Sigma). Cell lines were maintained in culture at 37 °C with 5% CO_2 for the minimum necessary time to perform experiments, generally not exceeding one month from thaw.

RAPID siRNA screen and siRNA transfection

Human tyrosine kinase siRNA screen (RAPID) was performed as previously described [30, 31, 38] for each cell line ($N = 3$). Cells were plated in 96-well plates and transfected with siRNA using oligofectamine (Invitrogen, Carlsbad, CA) following manufacturer's instructions. After 96-hour incubation at 37 °C, cell viability was assayed using MTS reagent. Data were corrected for row or column plating bias and triplicate experiments were averaged. The median effect and standard deviation of the data from each screen experiment were calculated as described previously [40]. Genes exceeding one standard deviation from the median of each screen were considered for comparison between the three *KIT*-mutant cell lines to identify common hits. For other siRNA experiments ($N > 3$), siGENOME siRNA pools were purchased from Dharmacon: *LMTK3*: M-005338-03-0005, *KIT*: M-003150-02-0005, NT: D-001206-13-05, *PLK1*: M-003290-01-0005, custom *LMTK3* 3′UTR: 5′CAGAAGAGGGGUUGAGAAUUU-3′.

Cell viability and caspase activity assays

Cells were plated, transfected, and incubated in opaque 96-well plates (Corning, Corning, NY). Cell viability was

measured using the Cell Titer Glo reagent (Promega, Madison, WI) according to manufacturer's instructions. Activity of caspases 3 and 7 was measured using Caspase 3/7 Glo (Promega). Both assays were measured on the Glo-Max luminometer (Promega). (N > 3)

Flow cytometry

Total cell number and percent of cells with inclusion of PI were determined by flow cytometry on the GUAVA EasyCyte5 (Millipore, Burlington, MA) by staining with ViaCount reagent (Millipore) ($N = 3$).

GIST xenograft

One million viable GIST430 (ex 11) cells suspended in 50% matrigel (Corning) were implanted subcutaneously in the flank of four week old male NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*$^{tm1Wjl/SzJ}$ (NRG) mice (Jackson Laboratories, Bar Harbor, ME) 24 h post-transfection with non-targeting or *LMTK3*-targeting siRNA using oligofectamine reagent in vitro, as described above. Each mouse bore one tumor with each siRNA on opposing flanks ($N = 8$). Once a palpable mass was detected, tumors were measured using calipers every three days until sacrifice. Animals were humanely euthanized before total tumor burden reached 2 cm [26], as dictated by our IACUC protocol. Neither randomization nor blinding were necessary for these experiments. Sample size was determined by performing power calculations based on preliminary experiments.

Quantitative RT-PCR

Total RNA was extracted (RNeasy, Qiagen, Hilden, Germany), cDNA was synthesized using 1 ug of total RNA (MultiScribe RT, Applied Biosystems, Foster City, CA), and quantitative RT-PCR (qRT-PCR) was performed on a LightCycler 480 (Roche, Basel, Switzerland) using Probes Master Mix (Roche). FAM Taqman primers for *LMTK3* (Hs01090726_g1) and *KIT* (Hs00174029_m1) were purchased from Life Technologies (Carlsbad, CA). Custom primers and hydrolysis probe (IDT, San Jose, CA) were used to detect a 66-bp *GAPDH* amplicon: GAPDH forward CACTAGGCGCTCACTGTTCT, GAPDH reverse GCGAACTCCCCGTTG, GAPDH probe 5′TexRd-XN/TGGGGAAGGTGAAGGTCGGA/3′IAbRQSp.

Protein harvest and immunoblotting

Cells were scraped from flasks for lysis with lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 1% Triton X-100, 10% Glycerol) with 1X protease and phosphatase inhibitor cocktail (Cell signaling, Danvers, MA). Immunoblotting was performed by standard SDS-PAGE protocol using the Criterion electrophoresis system (Biorad, Hercules, CA), Transblot Turbo transfer system (Biorad), and imaged and quantitated using the Chemidoc imaging system (Biorad).

Antibodies

Antibodies used for immunoblotting are shown in Supplementary Table 3. Total KIT antibody (0.5 ug, Abcam) bound to Protein A/G PLUS beads (sc-2003, Santa Cruz, Dallas, TX) was used for KIT immunoprecipitations.

Reagents

Imatinib mesylate (STI571, Novartis, Basel, Switzerland), cycloheximide (Sigma), and G418 (Sigma) were dissolved in sterile water or PBS. The expression plasmid pCMV6-LMTK3myc-DDK (cat # RC223140) was obtained from Origene (Rockville, MD) and used for construction of pLENTI-LMTK3myc-DDK (using pLENTI-C-myc-DDK-IRES-Neo, PS100081).

Cycloheximide time course

Cells were treated with cycloheximide (20 ug/mL, Sigma), for the indicated times 48–72 h post-siRNA transfection. KIT protein was quantified from whole cell lysates using PathScan Total c-KIT ELISA kit (Cell Signaling, #7197C), following manufacturer's instructions. Experiments were performed in triplicate and KIT half-life was estimated by one-phase decay.

(S^{35}) Methionine labeling

At 48 h post-siRNA transfection, GIST430 (ex 11) cells were starved in DMEM lacking methionine (and cysteine, Invitrogen Cat# 21013), supplemented with 5% dialyzed FBS and 2 mM glutamine ($N = 4$). Cells were then labeled with 100 µCi/mL S^{35}-methionine (EXPRE^{35}S^{35}S Protein Labeling Mix, PerkinElmer, Waltham, MA) for 30 min, washed twice with PBS, and lysed in RIPA lysis buffer. The supernatant was collected and immunoprecipitation was performed with KIT antibody (Abcam), or control rabbit IgG (Santa Cruz). Immunoprecipitated proteins were resolved by SDS-PAGE. Newly synthesized S^{35}-KIT was visualized and quantitated on a Bio-Rad FX Molecular Imager using Quantity One Software (Bio-Rad).

Statistics

All quantitative experimental data represent mean ± SEM (unless otherwise indicated), performed with a minimum of

three independent biological replicates. Statistical tests performed as appropriate, including one-way ANOVA (with multiple comparisons), unpaired, two-tailed *t* tests, or one phase-decay (to calculate protein half-life), using PRISM (Graph Pad, La Jolla, CA) software. *p* values indicated by asterisks: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$; ****, $p < 0.0001$.

Acknowledgements Thank you to Dr. Sebastian Bauer (West German Cancer Center, Essen, Germany) for generously providing *KIT*-mutant GIST cell lines. Thank you to Dr. Dirk Schadendorf (West German Cancer Center, Essen, Germany) for generously providing the MaMel (144aI) cell line. Deepest thanks to Arin McKinley, Diana Griffith, and Ashley Young for their technical and administrative support during the course of this study.

Financial support L.R. Klug, A. Town, A.E. Bannon, and M.C. Heinrich were supported by VA Merit Review Grants 1I01BX000338-01 and 2I01BX000338-05. M.C. Heinrich was also supported by the GIST Cancer Research Fund and the Life Raft Group. L.R. Klug, W. H. Fleming, J.W. Tyner, and M.C. Heinrich were supported by the V Foundation for Cancer Research. J.W. Tyner was supported by the Leukemia & Lymphoma Society, Gabrielle's Angel Foundation for Cancer Research, and the National Cancer Institute (5R00CA151457-04; 1R01CA183947-01). N. Javidi-Sharifi was supported by the Oregon Clinical and Translational Research Institute (OCTRI), grant number TL1 RR024159 from the National Center for Advancing Translational Sciences (NCATS), a component of the NIH, and NIH Roadmap for Medical Research.

Compliance with ethical standards

Conflict of interest MCH is a consultant for Novartis, Deciphera Pharmaceuticals, Blueprint Medicines, Ariad Pharmaceuticals, Bayer Pharmaceuticals, and Molecular MD. MCH has provided expert testimony and has a patent licensed to Novartis. MCH receives research support from Ariad, Deciphera, Blueprint Medicines and has equity interest in Molecular MD. JWT receives research support from Agios Pharmaceuticals, Array Biopharma, Aptose Biosciences, AstraZeneca, Constellation Pharmaceuticals, Genentech, Gilead, Incyte Corporation, Janssen Pharmaceutica, Seattle Genetics, Syros, Takeda Pharmaceutical Company and is a consultant for Leap Oncology.

References

1. Witte ON. Steel locus defines new multipotent growth factor. Cell. 1990;63:5–6.
2. Lev S, Givol D, Yarden Y. A specific combination of substrates is involved in signal transduction by the kit-encoded receptor. EMBO J. 1991;10:647–54.
3. Lev S, Yarden Y, Givol D. Dimerization and activation of the kit receptor by monovalent and bivalent binding of the stem cell factor. J Biol Chem. 1992;267:15970–7.
4. Kajimoto N, Nakai N, Ohkouchi M, Hashikura Y, Liu-Kimura NN, Isozaki K, et al. Characterization of various types of mast cells derived from model mice of familial gastrointestinal stromal tumors with KIT-Asp818Tyr mutation. Int J Clin Exp Pathol. 2015;8:11970–82.
5. Nakai N, Ishikawa T, Nishitani A, Liu NN, Shincho M, Hao H, et al. A mouse model of a human multiple GIST family with KIT-Asp820Tyr mutation generated by a knock-in strategy. J Pathol. 2008;214:302–11.
6. Rubin BP, Antonescu CR, Scott-Browne JP, Comstock ML, Gu Y, Tanas MR, et al. A knock-in mouse model of gastrointestinal stromal tumor harboring kit K641E. Cancer Res. 2005;65:6631–9.
7. 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.
8. Kemmer K, Corless CL, Fletcher JA, McGreevey L, Haley A, Griffith D, et al. KIT mutations are common in testicular seminomas. Am J Pathol. 2004;164:305–13.
9. Larizza L, Magnani I, Beghini A. The Kasumi-1 cell line: a t (8;21)-kit mutant model for acute myeloid leukemia. Leuk & Lymphoma. 2005;46:247–55.
10. Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. Proc Natl Acad Sci USA. 1995;92:10560–4.
11. Tian Q, Frierson HF Jr., Krystal GW, Moskaluk CA. Activating c-kit gene mutations in human germ cell tumors. Am J Pathol. 1999;154:1643–7.
12. Corless CL, Barnett CM, Heinrich MC. Gastrointestinal stromal tumours: origin and molecular oncology. Nat Rev Cancer. 2011;11:865–78.
13. Hodi FS, Corless CL, Giobbie-Hurder A, Fletcher JA, Zhu M, Marino-Enriquez A, et al. Imatinib for melanomas harboring mutationally activated or amplified KIT arising on mucosal, acral, and chronically sun-damaged skin. J Clin Oncol. 2013;31:3182–90.
14. Dematteo RP, Heinrich MC, El-Rifai WM, Demetri G. Clinical management of gastrointestinal stromal tumors: before and after STI-571. Hum Pathol. 2002;33:466–77.
15. Spencer KR, Mehnert JM. Mucosal Melanoma: Epidemiology, Biology and Treatment. Cancer Treat Res. 2016;167:295–320.
16. Blanke CD, Demetri GD, von Mehren M, Heinrich MC, Eisenberg B, Fletcher JA, et al. Long-term results from a randomized phase II trial of standard- versus higher-dose imatinib mesylate for patients with unresectable or metastatic gastrointestinal stromal tumors expressing KIT. J Clin Oncol. 2008;26:620–5.
17. 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.
18. 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.
19. Edmonson JH, Marks RS, Buckner JC, Mahoney MR. Contrast of response to dacarbazine, mitomycin, doxorubicin, and cisplatin (DMAP) plus GM-CSF between patients with advanced malignant gastrointestinal stromal tumors and patients with other advanced leiomyosarcomas. Cancer Invest. 2002;20:605–12.
20. Carvajal RD, Lawrence DP, Weber JS, Gajewski TF, Gonzalez R, Lutzky J, et al. Phase II study of nilotinib in melanoma harboring KIT alterations following progression to prior KIT inhibition. Clin Cancer Res. 2015;21:2289–96.
21. Guo J, Si L, Kong Y, Flaherty KT, Xu X, Zhu Y, et al. Phase II, open-label, single-arm trial of imatinib mesylate in patients with metastatic melanoma harboring c-Kit mutation or amplification. J Clin Oncol. 2011;29:2904–9.
22. Hodi FS, Friedlander P, Corless CL, Heinrich MC, Mac Rae S, Kruse A, et al. Major response to imatinib mesylate in KIT-mutated melanoma. J Clin Oncol. 2008;26:2046–51.
23. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, et al. Efficacy and safety of imatinib

mesylate in advanced gastrointestinal stromal tumors. N Engl J Med. 2002;347:472–80.
24. Bannon AE, Klug LR, Corless CL, Heinrich MC. Using molecular diagnostic testing to personalize the treatment of patients with gastrointestinal stromal tumors. Expert Rev Mol Diagn. 2017;17:445–57.
25. Gramza AW, Corless CL, Heinrich MC. Resistance to tyrosine kinase inhibitors in gastrointestinal stromal tumors. Clin Cancer Res. 2009;15:7510–8.
26. 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.
27. Liegl B, Kepten I, Le C, Zhu M, Demetri GD, Heinrich MC, et al. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. J Pathol. 2008;216:64–74.
28. Roberts KG, Odell AF, Byrnes EM, Baleato RM, Griffith R, Lyons AB, et al. Resistance to c-KIT kinase inhibitors conferred by V654A mutation. Mol Cancer Ther. 2007;6:1159–66.
29. Weisberg E, Griffin JD. Resistance to imatinib (Glivec): update on clinical mechanisms. Drug Resist Update: Rev Comment Antimicrob Anticancer Chemother. 2003;6:231–8.
30. Tyner JW, Walters DK, Willis SG, Luttropp M, Oost J, Loriaux M, et al. RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia. Blood. 2008;111:2238–45.
31. Tyner JW, Deininger MW, Loriaux MM, Chang BH, Gotlib JR, Willis SG, et al. RNAi screen for rapid therapeutic target identification in leukemia patients. Proc Natl Acad Sci USA. 2009;106:8695–8700.
32. Tomomura M, Morita N, Yoshikawa F, Konishi A, Akiyama H, Furuichi T, et al. Structural and functional analysis of the apoptosis-associated tyrosine kinase (AATYK) family. Neuroscience. 2007;148:510–21.
33. Wang H, Brautigan DL. A novel transmembrane Ser/Thr kinase complexes with protein phosphatase-1 and inhibitor-2. J Biol Chem. 2002;277:49605–12.
34. Giamas G, Filipovic A, Jacob J, Messier W, Zhang H, Yang D, et al. Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer. Nat Med. 2011;17:715–9.
35. Stebbing J, Filipovic A, Lit LC, Blighe K, Grothey A, Xu Y, et al. LMTK3 is implicated in endocrine resistance via multiple signaling pathways. Oncogene. 2013;32:3371–80.
36. Xu Y, Zhang H, Lit LC, Grothey A, Athanasiadou M, Kiritsi M, et al. The kinase LMTK3 promotes invasion in breast cancer through GRB2-mediated induction of integrin beta(1). Sci Signal. 2014;7:ra58.
37. Xu Y, Zhang H, Nguyen VT, Angelopoulos N, Nunes J, Reid A, et al. LMTK3 represses tumor suppressor-like genes through chromatin remodeling in breast cancer. Cell Rep. 2015;12:837–49.
38. Javidi-Sharifi N, Traer E, Martinez J, Gupta A, Taguchi T, Dunlap J, et al. Crosstalk between KIT and FGFR3 promotes gastrointestinal stromal tumor cell growth and drug resistance. Cancer Res. 2015;75:880–91.
39. 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.
40. Agarwal A, Tyner JW. RNAi screening of leukemia cells using electroporation. Methods Mol Biol. 2016;1470:85–94.
41. Rossi F, Yozgat Y, de Stanchina E, Veach D, Clarkson B, Manova K, et al. Imatinib upregulates compensatory integrin signaling in a mouse model of gastrointestinal stromal tumor and is more effective when combined with dasatinib. Mol Cancer Res. 2010;8:1271–83.
42. Sakurama K, Noma K, Takaoka M, Tomono Y, Watanabe N, Hatakeyama S, et al. Inhibition of focal adhesion kinase as a potential therapeutic strategy for imatinib-resistant gastrointestinal stromal tumor. Mol Cancer Ther. 2009;8:127–34.
43. Takahashi T, Serada S, Ako M, Fujimoto M, Miyazaki Y, Nakatsuka R, et al. New findings of kinase switching in gastrointestinal stromal tumor under imatinib using phosphoproteomic analysis. Int J Cancer. 2013;133:2737–43.
44. Jacob J, Favicchio R, Karimian N, Mehrabi M, Harding V, Castellano L, et al. LMTK3 escapes tumour suppressor miRNAs via sequestration of DDX5. Cancer Lett. 2016;372:137–46.
45. An N, Cen B, Cai H, Song JH, Kraft A, Kang Y. Pim1 kinase regulates c-Kit gene translation. Exp Hematol Oncol. 2016;5:31.
46. Grzmil M, Hemmings BA. Translation regulation as a therapeutic target in cancer. Cancer Res. 2012;72:3891–3900.
47. Garner AP, Gozgit JM, Anjum R, Vodala S, Schrock A, Zhou T, et al. Ponatinib inhibits polyclonal drug-resistant KIT oncoproteins and shows therapeutic potential in heavily pretreated gastrointestinal stromal tumor (GIST) patients. Clin Cancer Res. 2014;20:5745–55.
48. Gagnon KB, England R, Diehl L, Delpire E. Apoptosis-associated tyrosine kinase scaffolding of protein phosphatase 1 and SPAK reveals a novel pathway for Na-K-2C1 cotransporter regulation. Am J Physiol Cell Physiol. 2007;292:C1809–1815.