The DREAM complex mediates GIST cell quiescence and is a novel therapeutic target to enhance imatinib-induced apoptosis

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ABSTRACT

GISTs can be successfully treated with imatinib mesylate (Gleevec), however, complete remissions are rare and patients frequently achieve disease stabilization in the presence of residual tumor masses. The clinical observation that discontinuation of treatment can lead to tumor progression suggests that residual tumor cells are in fact quiescent and hence able to re-enter the cell division cycle. In line with this notion, we have previously shown that imatinib induces GIST cell quiescence in vitro through the APC-Cdh1-Skp2-p27Kip1 signaling axis. Here, we provide evidence that imatinib induces GIST cell quiescence in vivo and that this process also involves the DREAM complex, a multi-subunit complex that has recently been identified as a additional key regulator of quiescence. Importantly, inhibition of DREAM complex formation by depletion of the DREAM regulatory kinase DYRK1A or its target LIN52 was found to enhance imatinib-induced cell death. Our results show that imatinib induces apoptosis in a fraction of GIST cells while at the same time a subset of cells undergoes quiescence involving the DREAM complex. Inhibition of this process enhances imatinib-induced apoptosis, which opens the opportunity for future therapeutic interventions to target the DREAM complex for more efficient imatinib responses.
INTRODUCTION

The majority of gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumor of the gastrointestinal tract, are characterized by oncogenic mutations in the KIT or platelet-derived growth factor receptor alpha (PDGFRA) receptor tyrosine kinase (1-3). Because these oncogenic mutations lead to constitutive, ligand-independent activation of the receptor, GISTs can be successfully treated with the small molecule kinase inhibitor imatinib mesylate (Gleevec) (4). Although approximately 85% of patients with advanced or metastatic disease benefit from imatinib treatment, complete responses are rare and the majority of patients only achieve partial response or stable disease with remaining tumor load (5).

Successful cancer therapy is often hampered by the occurrence of tumor cell quiescence, because quiescent cells remain viable and therefore are a reservoir for tumor progression (6-8). This reversible exit from the cell division cycle and entry into G0 has previously been shown to involve the anaphase promoting complex (APC)CDH1-SKP2-p27Kip1-signalling axis (9-13). In this pathway, the APC, together with its activator CDH1, promotes the polyubiquitylation and subsequent degradation of SKP2, a substrate adaptor component of the SCF (SKP1-Cullin-F-box) complex. SKP2 loss results in the accumulation of its target, the CDK inhibitor p27Kip1, and the reinforcement of a quiescent state. In a previous study we could show that this process is active in imatinib-treated GIST cells (14).

A second major group of proteins that negatively regulate the cell cycle are the retinoblastoma tumor suppressor protein (pRB) and the related “pocket protein” family members p107 (RBL1) and p130 (RBL2) (15,16). p130 has been shown to accumulate in G0, when it interacts with E2F4 to repress E2F-dependent gene transcription (17-19). p130 protein levels are also regulated by SKP2 (20), which underscores that quiescence involves multiple interconnected pathway components.
In line with this notion, it has been shown that p130/E2F4 are part of a larger, multisubunit protein complex, the mammalian DREAM complex (21). This complex consists of DP, RBL2 (p130), E2F4 and the mammalian homologs of the C. elegans synthetic multivulva class B (synMuvB) gene products LIN9, LIN37, LIN52, LIN53/RBBP4 and LIN54 (21). The DREAM complex forms in G0 and represses E2F target genes. In humans, LIN9, LIN37, LIN52, LIN53/RBBP4 and LIN54 have been shown to form a stable complex (the MuvB core) that dissociates from p130 and E2F4/DP in S phase and instead binds to B-MYB, when it is referred to as MMB (MYB-MuvB) complex (21-23). In a recent study, phosphorylation of DREAM component LIN52 at Ser28 was shown to regulate complex formation in G0 (24). This phosphorylation is mediated by members of the dual specificity tyrosine-phosphorylation-regulated kinase (DYRK) family, specifically DYRK1A and potentially DYRK1B (24).

Here, we show that imatinib induces GIST cell quiescence in vivo and in vitro and that this process involves the DREAM complex as evidenced by upregulation of p130, increased p130/E2F4/LIN37 complex formation and enhanced phosphorylation of the DREAM subunit LIN52. Importantly, abrogation of quiescence by siRNA-mediated knock-down of LIN52 or the DYRK1A kinase were both found to significantly increase imatinib-induced GIST cell apoptosis. Therefore, interference with DREAM-mediated quiescence can enhance imatinib-induced anti-GIST activity, which underscores the relevance of the DREAM complex as novel drug target warranting further preclinical and clinical investigations.

MATERIALS AND METHODS

Cell culture, inhibitor treatments and transfections

The human GIST cell line GIST882 (a generous gift from Jonathan A. Fletcher, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA) was derived from
an untreated metastatic GIST and maintained in RPMI1640 supplemented with 15% fetal bovine serum (FBS, Gemini BioProducts, West Sacramento, CA), 1% L-Glutamine, 50 U/ml penicillin (Cambrex, Walkersville, MD) and 50 µg/ml streptomycin (Cambrex) as described previously (25).

Cells were incubated in imatinib mesylate (1 µM in DMSO; LC Laboratories, Woburn, MA), harmine (10 µM in DMSO; Sigma-Aldrich, St. Louis, MO) or mock-treated with 0.1% DMSO for up to 72 h or as indicated.

For small interfering RNA (siRNA) experiments, single RNA duplexes were used to reduce protein expression of members of the DREAM complex (Qiagen, Valencia, CA) and DYRK1A (Dharmacon/Thermo Fisher Scientific, Pittsburgh PA). Briefly, GIST882 cells were trypsinized and 3 x 10^6 cells were transfected with 5 µl of 10 µM annealed RNA duplexes using nucleofection (Amaxa/Lonza, Walkersville, MD). For dual knockdown experiments, 2.5 µl of 20 µM of each annealed RNA duplexes was used. Cells were then transferred to 35 mm tissue culture dishes with 2 ml RPMI1640 free of antibiotics. Cells were either incubated for 72 h without further manipulation before further processing or treated with 1 µM imatinib of 0.1% DMSO at 24 h after transfection and incubated for another 48 h (72 h total). Knock-down efficiency was monitored by immunoblotting or qRT-PCR (for LIN54 only).

**Immunological and cell staining methods**

Protein lysates of cells growing as monolayer were prepared by scraping cells into lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate) containing protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µM phenylmethylsulfonyl fluoride). Lysates were incubated for 1 h with shaking at 4°C and then cleared by centrifugation for 30 min at 14,000 rpm at 4°C. Protein
concentrations were determined by the Bradford assay (Biorad, Hercules, CA). 30 μg of protein were loaded on a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and blotted onto a nitrocellulose membrane. Co-immunoprecipitation experiments were carried out using TrueBlot® IP/Western reagents (Rockland Immunochemicals, Gilbertsville, PA) according to manufacturer’s instructions.

For immunofluorescence analysis, cells grown on coverslips were briefly washed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then washed in PBS and permeabilized with 1% Triton-X 100 in PBS for 15 min at room temperature followed by washing in PBS and blocking with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 15 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C in a humidified chamber and incubated for another 3 hours at 37°C the next morning. After a brief wash in PBS, cells were incubated with FITC-anti-mouse secondary antibodies (Jackson ImmunoResearch) for at least 2 hours at 37°C, washed with PBS and counterstained with DAPI (Vector Laboratories, Burlingame, CA). Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

Immunofluorescence microscopic staining of paraffin-embedded sections was performed as described previously (26). In brief, slides were deparaffinized in xylene and rehydrated in a graded ethanol series. After microwaving in 0.01 M citrate buffer (pH 6.0) for 10 min, slides were washed in dH2O and PBS before continuing with staining as described above.

Primary antibodies used for immunoblotting and immunofluorescence were actin (Sigma, St. Louis, MO), BrdU (Roche Applied Science, Indianapolis, IN), cleaved caspase 3 (Cell Signaling, Beverly, MA), cyclin A (Novocastra, New Castle upon Tyne, United Kingdom), DYRK1A, E2F4 (both Santa Cruz, Santa Cruz, CA), pKIT Y719 (Cell Signaling, Beverly, MA), KIT (DakoCytomation, Carpinteria, CA), LIN9, LIN37, LIN52,
pLIN52 S28 and LIN54 (all from Litovchick et al, 2007 or Litovchick et al, 2011), p27^{Kip1} (BD Biosciences Pharmingen, San Diego, CA), p27^{Kip1} (Invitrogen/Zymed Laboratories, South San Francisco, CA), p130 (RBL2, Santa Cruz), PARP and SKP2 (both Invitrogen/Zymed Laboratories).

**BrdU assay**

To assess the proliferating cell fraction, GIST882 cells were incubated in BrdU labeling medium (Roche Applied Science) for 2 hours. Cells were then washed in PBS and fixed in ice-cold methanol for 10 min. After another wash in PBS, DNA was denatured by incubation in 2 N HCl for 1 hour at 37°C. The HCl was neutralized by immersion in 0.1 M borate buffer, pH 8.5, before incubation with a mouse monoclonal anti-BrdU antibody (Roche) overnight at 4°C in a humidified chamber. Detection and analysis was performed as described previously (14).

**Senescence-associated beta-galactosidase activity**

Senescence-associated beta-galactosidase activity was assessed using the Senescence β-Galactosidase Staining Kit (Cell Signaling) according to manufacturer’s instructions.

**Quantitative real time reverse transcriptase (RT)-PCR**

For quantitative real time RT-PCR (qRT-PCR), cell were transfected with control siRNA or siRNA duplexes targeting LIN54 and RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. RNA was treated with DNase I enzyme (Fermentas, St. Leon Rot, Germany) to remove any contaminating traces of genomic DNA and cDNA was transcribed by RT-PCR using
random primers and the Maxima First Strand cDNA Synthesis Kit (Fermentas). LIN54 and \(\beta\)-actin cDNA was amplified using exon-overlapping, mRNA/cDNA-specific primers to LIN54 (forward: 5'-ATTGCTAAGAAGCCTCGAACG-3', reverse: 5'-TGGTGAAACTTGAGTTGTCTGTC-3'; IDT, Leuven, Belgium) and \(\beta\)-actin (forward: 5'-CCAAGGCCAACCGGAGGAGATGAC-3', reverse: 5'-AGGGTACATGGTGGTGCCGCCAGAC-3') and measured using the SsoFast EvaGreen Kit (Bio-Rad). Cycling conditions were 95\(^\circ\)C (30 sec, activation), 95\(^\circ\)C (5 sec, denaturation), 55\(^\circ\)C (10 sec, annealing/extension) for 40 cycles on a Bio-Rad CFX96 Real-Time System run on a C1000 Thermal Cycler platform (Bio-Rad, Hercules, CA, USA). \(\beta\)-Actin served as reference gene for relative quantification.

**Cell cycle analysis**

Cell cycle analysis was performed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed cells. In brief, cells were harvested by trypsinization, washed twice with pre-chilled PBS (1% FBS), and fixed with ice-cold 70% ethanol. After the fixation step, the cells were washed with PBS/1% FBS, resuspended in propidium iodide (PI, Sigma-Aldrich)/RNase staining solution (50 µg/ml PI, 10 mM Tris pH 7.5, 5 mM MgCl₂, 10 µg/ml RNase) and incubated at 37\(^\circ\)C in the dark for 30 min. The analysis was performed in the University of Pittsburgh Cancer Institute Flow Cytometry Core Facility using a Gallios Flow Cytometer (Beckman Coulter) and the Kaluza 5 acquisition software.

**GIST xenograft models**

For mouse xenograft models, GIST882 cells (carrying a \(KIT\) p.K642E [exon 13] mutation) or tumors originating from the biopsies of two patients (bearing \(KIT\) p.V650D
[exon 11] or KIT p.A502_Y503dup [exon 9] mutations, respectively) were implanted in both flanks of two mice as described previously (27). Second passage xenografts were generated by explanting established xenografts and implanting them into the flanks of a second set of mice. When tumors were 1 cm in diameter, mice were treated with placebo or imatinib (100 mg/kg, p.o. daily) for twelve days. Tumor volume, weight and general health of the mice were recorded as previously described (27). After the mice were sacrificed, tumors were excised and processed for histopathological examination. The animal experiment was approved by the Ethics Committee of KU Leuven (Leuven, Belgium).

RESULTS

Imatinib induces GIST cell quiescence in vivo and in vitro

To analyze imatinib-induced quiescence in vivo, several cell line- and patient-derived GIST xenograft models were analyzed (Fig. 1) (27). Mice were treated with placebo or imatinib and tumor tissues were stained for the quiescence marker p27Kip1. Imatinib treatment led to a significant increase of tumor cells exhibiting nuclear p27Kip1 staining in GIST882 xenografts (Fig. 1A) and patient-derived xenografts carrying a mutation in KIT exon 11 (Fig. 1B), thereby confirming the ability of imatinib to induce quiescence in GIST cells in vivo. A xenograft derived from a tumor with a KIT exon 9 mutation showed only a minimal increase in nuclear p27Kip1 staining. This was not due to differences in the expression of its upstream regulator SKP2 (Suppl. Fig. 1), indicating that baseline SKP2 levels do not determine levels of p27Kip1 after imatinib treatment. Remarkably, KIT exon 9 mutations are known to be associated with a reduced sensitivity to imatinib in GIST patients (28) but there are currently no GIST cell line models available to dissect the precise underlying molecular mechanisms.
A main characteristic of quiescence is the fact that it is reversible, in contrast to cellular senescence, which is largely irreversible (29). To further corroborate the fact that imatinib treatment leads to quiescence and not senescence in GIST cells, GIST882 cells were treated with imatinib for 72 h (three days), after which the drug-containing media was removed and cells were cultured without drug for up to eleven days. The percentage of cells in S-phase was measured by BrdU incorporation and was found to decrease significantly during imatinib treatment (from 26.9% to 1.3%; Fig. 1C) with a subset of cells showing morphological signs of apoptosis (Suppl. Fig. 2). However, five days after drug wash-out, a significant increase of cells in S phase was observed, and cellular proliferation was restored to pre-treatment levels at seven to nine days after imatinib removal (Fig. 1C).

These findings were corroborated by immunoblot analyses (Fig. 2A) showing that inhibition of KIT phosphorylation by imatinib is completely reversible after imatinib wash-out. KIT reactivation was associated with a cell cycle reentry (as measured by expression levels of SKP2, p27Kip1 and cyclin A) at day 5 after drug removal. These results confirm clinical findings showing that metastatic or inoperable GISTs resume growth when imatinib treatment is discontinued (30).

The same study (30) reported that GISTs, which progress after discontinuation of imatinib, usually do retain their responsiveness to imatinib during further treatment. To recapitulate these findings in vitro and to test whether there would be any detectable differences between imatinib-naïve and imatinib-pretreated cells, we re-exposed GIST cells that had completed one imatinib wash-out cycle (i.e., 11 days after removal of drug) with imatinib in comparison to imatinib-naïve GIST cells. As shown in Fig. 2B, prior imatinib-induced quiescence had no effect on inhibition of KIT phosphorylation, induction of apoptosis or induction of cell cycle arrest when cells were re-exposed to the drug. There was in fact a more pronounced PARP cleavage band that most likely reflects cell
synchronization. These results provide *in vitro* evidence that pretreated GIST cells retain their responsiveness to antineoplastic activities of imatinib as suggested by previous clinical reports.

The results shown above imply that GIST cells either undergo apoptosis or enter quiescence after imatinib treatment and, furthermore, that imatinib does not lead to a senescent phenotype i.e., permanent cell cycle exit. To corroborate the latter notion, we stained imatinib-treated GIST882 cells for senescence-associated (SA) beta-galactosidase (beta-gal) activity (Suppl. Fig. 3A). There was a low (<5%) baseline level of SA beta-gal-positive multinucleated cells, but no change in the percentage of SA beta-gal positive cells was detected when imatinib-treated cells were compared to control-treated cells. Furthermore, immunofluorescence microscopic staining of the senescence-associated marker p16^{INK4A} (CDKN2A) of imatinib-treated GIST882 cell populations did not reveal any changes in total protein levels (Suppl. Fig. 3B). Taken together, these results indicate that indeed most, if not all, GIST cells undergo either apoptosis or quiescence when treated with imatinib, and that imatinib does not induce a senescence phenotype in these cells.

*The DREAM complex is involved in imatinib-induced quiescence*

The multiprotein DREAM complex was recently identified as one of the key regulators of cellular quiescence in addition to the APC^{CDH1}-SKP1- p27^Kip1 signaling axis (21). We therefore tested whether imatinib-induced quiescence in GIST cells involves DREAM complex-mediated mechanisms.

To test this hypothesis, we initially focused on the core DREAM complex members p130 (RBL2) and the mammalian MuvB homolog LIN37. As expected, imatinib induced a time-dependent increase of p130 protein expression (Fig. 3A) indicating cellular quiescence. Conversely, p130 expression decreased when imatinib was
removed indicating exit from quiescence and cell cycle re-entry (Fig. 3B). At the same time, LIN37 levels remained largely unchanged, which is line with a previous report (22).

These results were corroborated by immunofluorescence microscopic analyses showing a significantly enhanced nuclear staining of p130 after imatinib treatment from 13.2% to 49.2% (p<0.0015, Student’s t-test for independent samples, Fig. 3C), in line with what previously has been reported for quiescent cells (31).

We next sought to directly test imatinib-induced DREAM complex assembly. Co-immunoprecipitation experiments confirmed an enhanced formation of a complex between p130, E2F4 and LIN37 after imatinib treatment of GIST cells (Fig. 3D).

Taken together, these results provide compelling evidence for a critical role of the DREAM complex in the induction of quiescence in imatinib-treated GIST cells.

**The DREAM complex is a modulator of the cellular response to imatinib and is a potential therapeutic target**

Having shown that the DREAM complex is involved in the induction of imatinib-induced quiescence, we next wanted to test whether its disruption could be exploited therapeutically.

Knock-down of single DREAM complex subunits (p130, E2F4, LIN9, LIN37 or LIN54) did not result in a significant increase of imatinib-induced GIST cell apoptosis (Suppl. Fig. 4A) indicating a certain redundancy of DREAM complex components as previously suggested (21).

A combined knock-down of E2F4 and LIN54, however, was able to enhance GIST cell apoptosis from 36.6% in control siRNA-transfected and imatinib-treated cells to 53.1% in E2F4/LIN54 siRNA-transfected and imatinib-treated cells (p<0.001; Suppl. Fig. 4B). E2F4/LIN54 knock-down lead to an increased DNA synthesis in comparison to control siRNA-transfected cells as measured by BrdU incorporation (30.0% vs. 21.0%
BrdU-positive cells, respectively) suggesting that inhibition of efficient DREAM complex formation resulted in increased baseline proliferation in GIST cells (p<0.001; Suppl. Fig. 4C).

Phosphorylation of DREAM complex component LIN52 at serine 28 by the DYRK1A protein kinase has previously been reported to play an important role in DREAM complex assembly and activity (24). We therefore asked whether this process also plays a role in imatinib-induced GIST cell quiescence. LIN52-S28 levels in fact increased in imatinib-treated GIST cells as shown by immunoblotting (Fig. 4A) while basal expression levels of DYRK1A and LIN52 remained unchanged, which is line with a previous report (24). LIN52 phosphorylation and hence DREAM complex formation were reversible since LIN52-S28 expression was undetectable after removal of imatinib (Fig. 4B).

We next analyzed GIST882 cells in which LIN52 or DYRK1A have been depleted by siRNA (Fig. 4C). Flow cytometry showed a cell cycle arrest in G2/M in LIN52-depleted GIST cells (Fig 4D). However, when LIN52-depleted cells were treated with imatinib, we found a statistically significant, 19.5-fold increase of apoptosis from 2.64% in DMSO-treated, LIN52-depleted cells to 37.51% in imatinib-treated, LIN52-depleted GIST cells (Fig. 4D,E; p<0.0001).

We then tested if interference with DYRK1A can also modulate GIST cell apoptosis in response to imatinib. Since DYRK1A is a protein kinase, it is possible to inhibit its activity with pharmacological agents such as harmine. Harmine is a beta-carboline alkaloid with ATP-competitive activity towards DYRK1A (32). We treated GIST882 cells with harmine and found an enhanced cell death in combination with imatinib when compared to imatinib as single agent (Fig. 5A, B). The abrogation of imatinib-induced quiescence by harmine is shown by the reduction of p130 protein expression levels (Fig. 5A). We next used siRNA to knock-down DYRK1A expression
and found an induction of apoptosis in DYRK1A-depleted GIST cells (Fig. 5C). When DYRK1A-depleted cells were treated with imatinib, a statistically significant, 24-fold increase of apoptosis was detected (Fig. 5C,D; *, p<0.0001).

Collectively, these results show that abrogation of DREAM complex components can significantly increase the apoptotic response of GIST cells to imatinib.

DISCUSSION

GISTs can successfully be treated with imatinib, but complete remissions are rare and most patients with advanced or metastatic tumors only experience disease stabilization or a partial remission. It has been shown that the remaining tumor mass contains viable GIST cells even while still responding to imatinib and that these cells resume proliferation when imatinib treatment is discontinued (14,30,33).

In the present report, we corroborate that imatinib induces GIST cell quiescence not only in vitro but also in vivo using cell line- and patient-derived mouse xenografts. In line with clinical observations, we find that drug removal leads to reactivation of cellular proliferation, yet does not affect the sensitivity of GIST cells when re-challenged with imatinib. We have previously shown that imatinib-induced quiescence involves the APC<sup>CDH1</sup>-SKP2-p27<sup>Kip1</sup> axis (14). Here, we provide evidence for a second pathway that contributes critically to imatinib-induced quiescence in GIST cells that involves the DREAM complex. This multisubunit complex consists of the pRB-related protein p130, E2F4, LIN9, LIN37, LIN52, LIN53/RBBP4 and LIN54 (21,22). DREAM complex formation occurs in G0 to repress E2F target genes in a process that involves phosphorylation of DREAM component LIN52 at Ser28 by the DYRK1A protein kinase. Our results are in full agreement with this model since we show increased p130 expression and enhanced p130 nuclear translocation, p130/E2F4/LIN37 complex formation and increased LIN52-S28 phosphorylation in imatinib-treated GIST cells. It is noteworthy that SKP2 provides a
link between the two quiescence-associated signaling pathways since it also regulates p130 levels (20).

One of the key findings of the present study is that abrogation of quiescence by either siRNA or a pharmacological agent that inhibits DYRK1A kinase activity can significantly enhance imatinib-induced GIST cell apoptosis. While the precise mechanism warrants further investigation, in the most simplistic model, GIST cells that are prevented from entering a state of quiescence now undergo apoptosis when exposed to imatinib. We have previously shown that imatinib-induced cell death involves upregulation of core histone variant H2AX, followed by chromatin condensation and a transcriptional block (25). Although H2AX mRNA is synthesized in all phases of the cell division cycle (34), more than 90% of total histone synthesis takes place in S phase (35). It is conceivable that cells that are unable to enter quiescence and continue to proliferate are more vulnerable to imatinib-induced H2AX upregulation and subsequent cell death than cells that have exited the cell cycle and have a reduced or no H2AX expression.

The precise mechanism of imatinib-induced quiescence including p130 upregulation and enhanced phosphorylation of LIN52 leading to DREAM complex activation remains to be determined. Whereas the former event could be related to reduced SKP2 expression leading to p130 accumulation, it is unlikely that DYRK1A activity is acutely regulated on the level of gene expression or protein abundance, because its levels did not change after imatinib treatment or during the cell cycle (24). In contrast, stimulation of DYRK1A likely involves activity through modulation of feedback loops, such as LATS2 activity (36) or interaction with SPRED1 (37) or 14-3-3 (38). These possibilities warrant further experimentation.

The fact that formation of the DREAM complex is regulated by phosphorylation of its subunit LIN52 offers a window of opportunity for therapeutic interventions. The plant alkaloid harmine, which was used in the current study, displays specificity for DYRK1A
but its strong inhibitory effect on monoamine oxidase A and hallucinogenic properties unfortunately limit its use in patients. A recently described synthetic inhibitor of DYRK1A, INDY, was found to be active in several \textit{in vitro} and \textit{in vivo} models and may show an improved toxicity profile. Further support for the concept of quiescence inhibition as a strategy for cancer therapy stems from a previous study in ovarian cancer. This report focused on DYRK1A family member DYRK1B, which is overexpressed in ovarian carcinoma cells and has also been shown to be capable of phosphorylating LIN52. Inhibition of DYRK1B by RO5454948, a compound that also inhibits DYRK1A, led to escape from quiescence and an increased apoptotic response. Importantly, treatment with RO5454948 did not affect the viability of normal ovarian epithelial cells.

Results shown here lend further support to the notion that induction of tumor cell quiescence can negatively affect antineoplastic therapies. It is remarkable that imatinib itself is able to stimulate quiescence in a subset of GIST cells, thereby intrinsically limiting its own effectiveness. Our findings hence underscore the need to exploit synthetic lethal approaches such as KIT and DYRK1A inhibition as shown here to increase antitumor efficacy.

One of the key remaining questions is what determines whether GIST cells undergo apoptosis or quiescence when treated with imatinib. It is possible that the cell cycle stage at the time of treatment plays a role, but other mechanisms that can modulate the switch between cell death and survival, merit further exploration. It is possible that whole genome or transcriptome analyses may allow a stratification of GISTs as being more prone to apoptosis or quiescence. In addition, it will be important to determine whether E2F4 target genes, loss of the molecular components of the DREAM complex or members of the APC\textsuperscript{CDH1}/SKP2/p27\textsuperscript{Kip1} signaling axis may play a
role. Thus far, mining of available databases did not yield any insights into these open questions that warrant more detailed investigations.

Taken together, we show here that the DREAM complex is a critical mediator of imatinib-induced GIST cell quiescence and that abrogation of quiescence enhances the pro-apoptotic activity of imatinib. The DREAM complex is hence a promising therapeutic target to make imatinib treatment more effective with an aim towards more complete patient responses.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

None.

AUTHOR CONTRIBUTIONS

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FIGURE LEGENDS

Figure 1. Imatinib induces GIST cell quiescence in vivo and in vitro.

(A) Immunofluorescence microscopic analysis of p27^Kip1 expression in human GIST cell line xenografts (GIST882) treated with placebo or imatinib (left panel). Note the significant increase of p27^Kip1-positive cells after imatinib treatment (bottom). Quantification of the percentage of p27^Kip1-positive GIST882 cells after treatment with placebo or imatinib (right panel). Nuclei were stained with DAPI. Scale bar, 50 µm.

(B) Quantification of the percentage of p27^Kip1-positive cells as assessed by immunofluorescence microscopic analysis in patient-derived GIST xenografts carrying mutations in KIT exons 11 or 9 after treatment with placebo or imatinib.

(C) Immunofluorescence microscopic analysis of BrdU incorporation in GIST882 cells. After treatment with imatinib for 72 h, imatinib-containing media was washed out and cells were cultured in regular medium for up to 11 days. Left panels, examples of BrdU incorporation after DMSO treatment or treatment with imatinib for 72 h or 9 days after washout of the drug. Quantification of the percentage of GIST882 cells showing BrdU incorporation detected during imatinib treatment for 3 days and after the drug was washed out for the following 11 days. Nuclei were stained with DAPI. Scale bar, 50 µm.

Figure 2. Imatinib-induced GIST cell quiescence does not prevent apoptosis upon imatinib re-challenge.

(A) Immunoblot analysis of GIST882 cells for KIT activation and markers of cell cycle regulation as indicated. Note the robust cell cycle re-entry after removal of imatinib.

(B) Immunoblot analysis for KIT activation, markers of apoptosis and cell cycle regulation after treatment with imatinib for 72 h. Imatinib-naïve GIST882 cells or cells pretreated with imatinib for 72 h following wash-out for 11 days are shown (pretreated).
Note PARP cleavage as sign of apoptosis in both naïve GIST cells and GIST cells that have been manipulated to enter and exit from quiescence.

**Figure 3. The DREAM complex mediates imatinib-induced quiescence in GIST cells.**

(A) Immunoblot analysis of GIST882 cells treated with imatinib for the indicated times and probed for DREAM complex members p130 and LIN37. Immunoblot for actin is shown to demonstrate equal loading. Note the upregulation of p130 following imatinib treatment.

(B) Immunoblot analysis of GIST882 cells for p130 and LIN37 expression in GIST882 cells treated with imatinib for 72 h and cultured for up to 11 days after removal of the drug. Note the changes in p130 expression indicating entry into and exit from quiescence.

(C) Immunofluorescence microscopic analysis of p130 expression and subcellular localization in GIST882 cells treated with DMSO or imatinib for 48 h (left panels). Quantification of the percentage of GIST882 cells showing nuclear p130 after imatinib treatment (right panels). Nuclei were stained with DAPI. Scale bar, 50 µm. *, p<0.0015, Student’s t-test for independent samples.

(D) Interactions between p130, E2F4 and LIN37 proteins in GIST cells as shown by co-immunoprecipitation (IP)-immunoblot of GIST882 cells treated with DMSO or imatinib for 72 h. IP was performed with either anti-p130 (left panel) or anti-E2F4 antibodies (right panel) followed by Western blotting for the proteins indicated. Whole cell lysates were used as a positive control to demonstrate an equal input.
**Figure 4. LIN52 is activated by imatinib and attenuates its pro-apoptotic activities.**  
(A) Immunoblot analysis for DYRK1A, phosphorylated LIN52-S28 and LIN52 in GIST882 cells after treatment with imatinib for the indicated time intervals.  
(B) Immunoblot analysis of DYRK1A, phosphorylated LIN52-S28 and total LIN52 expression levels after treatment with imatinib for 72 h, wash-out of the drug and culture in regular medium for up to 11 days. Note the increase of pLIN52 S28 (arrow) during treatment and loss of phosphorylation after drug removal and exit from quiescence. **, unspecific band.  
(C) Immunoblot analysis of DYRK1A and LIN52 expression (arrow) levels after transient transfection control siRNA or siRNAs targeting DYRK1A or LIN52 for 72h. **, unspecific band.  
(D) Representative flow cytometry histograms showing the cell cycle distribution of GIST882 cells transfected with control siRNA or siRNAs targeting LIN52 (24 h) and treated with DMSO (upper panels) or imatinib (lower panels) for another 48 h. Experiments were performed in quadruplicate and at least 100,000 cells were counted per experiment. Note the increase of apoptotic cells as evidenced by a sub-G1 peak in cells depleted of LIN52 and treated with imatinib (arrow).  
(E) Quantification of cell cycle distribution as measured by flow cytometry described in (D).

**Figure 5. Inhibition of DYRK1A enhances imatinib-induced GIST cell apoptosis.**  
(A) Immunoblot analysis of GIST882 cells for markers of apoptosis (PARP cleavage, cleaved caspase 3) and quiescence (p130) after treatment with DMSO, imatinib or the DYRK1A inhibitor harmine alone or in combination for 48 h. Actin demonstrates equal protein loading. Note the significant increase of cleaved caspase 3 in cells treated with harmine in combination with imatinib.
(B) DAPI stain to morphologically detect apoptotic cells after treatment with DMSO, imatinib (IM) or the DYRK1A inhibitor harmine (HA) alone or in combination for 48 h (left panels). Quantification of the percentage of apoptotic cells (right panel).

(C) Immunoblot analysis of GIST882 cells transiently transfected with control siRNA or siRNA targeting DYRK1A and further treated with DMSO or imatinib (IM) for 48 h. Actin demonstrates equal protein loading. Note the enhanced cleavage of caspase 3 in DYRK1A-depleted and imatinib-treated GIST cells.

(D) Representative flow cytometry histograms illustrating the cell cycle distribution of GIST882 cells transfected with control siRNA or siRNAs targeting DYRK1A. 24 h post-transfection, the cells were treated with DMSO (upper panels) or imatinib (Lower panels) for another 48 h. Experiments were performed in quadruplicate and at least 100,000 cells were counted per experiment. Note the enhanced apoptosis in DYRK1A-depleted and imatinib-treated cells (arrow). Lower panel, quantification of cell cycle distribution.
Figure 1, Boichuk et al.
Figure 2, Boichuk et al.

**A**

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**B**

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A. Western blot analysis of DYRK1A, pLIN52 S28, LIN52, and actin treated with imatinib over time (0-72 hours).

B. Time course of imatinib and post-imatinib washout treatment, showing changes in DYRK1A, pLIN52 S28, LIN52, and actin expression.

C. Western blot analysis of DYRK1A, LIN52, and actin in siCtrl, siDYRK1A, and siLIN52 treated cells.

D. Flow cytometric analysis of DNA content and cell cycle phases for siCtrl and siLIN52 treatments.

E. Summary of flow cytometric analysis showing DNA content and cell cycle phases for different treatments. 

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* p<0.0001 versus DMSO, Student’s t-test for independent samples
**Figure 5, Boichuk et al.**

(A) Western blot analysis showing the effects of IM and harmine on PARP, cl. caspase 3, p130, and actin expression.

(B) Fluorescence microscopy images comparing DMSO, IM, HA, and IM + HA treatments, and a bar graph showing the percentage of apoptotic cells.

(C) Western blot analysis comparing siCtrl and siDYRK1A treatments for IM, DYRK1A, cl. caspase 3, PARP, and actin expression.

(D) Flow cytometry analysis showing DNA content distributions for siDYRK1A and siDYRK1A + IM.

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<th>G1</th>
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* p<0.0001, compared to DMSO, Student's t-test for independent samples
The DREAM complex mediates GIST cell quiescence and is a novel therapeutic target to enhance imatinib-induced apoptosis

Sergei Boichuk, Joshua A Parry, Kathleen R Makielski, et al.

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