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DOG1 regulates growth and IGFBP-5 in gastrointestinal stromal tumors

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Abstract

Gastrointestinal stromal tumors (GIST) are characterized by activating mutations of KIT or platelet-derived growth factor receptor alpha (PDGFRA) which can be therapeutically targeted by tyrosine kinase inhibitors (TKI) such as imatinib. Despite long lasting responses most patients eventually progress after TKI therapy. The calcium-dependent chloride channel DOG1 (ANO1/TMEM16A), which is strongly and specifically expressed in GIST, is used as a diagnostic marker to differentiate GIST from other sarcomas. Here we report that loss of DOG1 expression occurs together with loss of KIT expression in a subset of GIST resistant to KIT inhibitors, and we illustrate the functional role of DOG1 in tumor growth, KIT expression and imatinib response. While DOG1 is a crucial regulator of chloride balance in GIST cells, we found that RNAi-mediated silencing or pharmacological inhibition of DOG1 did not alter cell growth or KIT signaling in vitro. In contrast, DOG1 silencing delayed the growth of GIST xenografts in vivo. Expression profiling of explanted tumors after DOG1 blockade revealed a strong upregulation in the expression of IGFBP5, a potent antiangiogenic factor implicated in tumor suppression. Similar results were obtained after selection of imatinib-resistant DOG1- and KIT-negative cells derived from parental DOG1 and KIT-positive GIST cells, where a 5000-fold increase in IGFBP5 mRNA

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transcripts were documented. In summary, our findings establish the oncogenic activity of DOG1 in GIST involving modulation of IGF/IGFR signaling in the tumor microenvironment through the antiangiogenic factor IGFBP5.

Keywords

GIST; DOG1; KIT; ANO1; TMEM16a

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract and are characterized by activating mutations in the *KIT* or *platelet-derived growth factor receptor alpha (PDGFRA)* genes (1–3).

Imatinib (IM) is a small molecule inhibitor of several oncogenic tyrosine kinases, including KIT and PDGFRA. About 85% of patients with metastatic GIST derive substantial clinical benefit from IM treatment, however, imatinib does not cure metastatic GIST and the majority of patients eventually progress. Secondary, imatinib-resistant KIT mutations within the ATP-binding and activation loop domain are commonly found in IM-resistant GIST and are believed to be the major mechanism of resistance (4–7).

The protein DOG1 (Discovered on GIST-1) encoded by *ANO1* (also known as *TMEM16A*) is a calcium-dependent chloride channel (CaCC) (8–10). Calcium-dependent chloride channels are involved in diverse physiological processes including gastrointestinal rhythmic contractions [11, 12]. Notably, DOG1 was found to be highly expressed both in GIST (13) and in ICC (interstitial cells of Cajal), the putative cell-of-origin of GIST [14, 15].

In clinical practice, DOG1 is a sensitive immunohistochemical marker for GIST and is preserved in 36% of GIST that lack KIT expression or activating mutations of *KIT* or *PDGFRA* (16–18). However, DOG1 biologic functions have not been characterized in GIST. In order to shed light on the relevance of DOG1 for GIST tumorigenesis, we evaluated the impact of DOG1 expression and activity in various GIST models, both *in vitro* and *in vivo*.

Material and Methods

Cell lines

GIST-T1 and GIST882 were established from human, untreated, metastatic GISTs. GIST-T1 contains a 57bp deletion in *c-KIT* exon 11 (19). GIST882 harbors a homozygous exon 13 missense mutation, resulting in a single amino acid substitution, K642E (20). GIST48 and GIST430 were established from GIST that had progressed, after initial clinical response, during IM therapy. GIST48 has a primary, homozygous exon 11 missense mutation (V560D) and a heterozygous secondary exon 17 (kinase activation loop) mutation (D820A). GIST430 has a primary heterozygous exon 11 in-frame deletion and a heterozygous secondary exon 13 missense mutation. GIST882B, GIST48B and GIST430B are sublines which, despite retaining the activating KIT mutation in all cells, expresses KIT transcript and protein at essentially undetectable levels. GIST62 was derived from an untreated KIT-positive GIST with KIT exon 11 in-frame mutation, but the cell line, despite retaining the activating KIT mutation in all cells, expresses KIT transcript and protein at essentially undetectable levels (21). GIST5 and GIST474 were established from imatinib-treated GISTs, and lacked KIT expression in the primary and subsequent cultures, although they retain the KIT exon 11 mutations of the parental GIST population.

Stable shRNA transfection

shRNA lentivirus for human DOG1 (NM_018043) was obtained from Sigma-Aldrich (MISSION® shRNA Lentiviral Transduction Particles TRCN0000040263). GIST cells were grown to 80% confluence and then infected with 1 MOI (multiplicity of infection) of either non-targeting scrambled shRNA (SHC002V) control particles or DOG1 shRNA lentiviral particles in medium containing 8µg/ml polybrene. Fresh medium containing 4µg/ml puromycin was added after 48h to select for puromycin-resistant cells.

Reagents and Antibodies

Imatinib mesylate (IM) was purchased from Selleck Chemicals (Houston, TX USA). 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Calbiochem (Merck, Darmstadt Germany). A rabbit polyclonal antibody against KIT was from DAKO (Carpinteria, CA USA) and a monoclonal rabbit antibody against DOG1 was from Diagnostic BioSystems (Pleasanton, CA USA). Polyclonal rabbit antibodies for phospho-KIT Y703 were from Cell Signaling (Beverly, MA USA). B-Actin Antibody was purchased from Sigma-Aldrich (St. Louis, MO USA).

In vitro assays

BrdU incorporation assay—Cells were incubated with 1 mM bromodeoxyuridine (BrdU) for 2,5h (GIST-T1) or 24h (GIST882) at 37°C and processed using the fluorescein isothiocyanate (FITC) BrdU Flow Kit (BD Biosciences, San Diego, CA USA) following the manufacturer's instructions. Briefly, 1.5×10^6 trypsinized cells were fixed, permeabilized, and digested with DNase. Cells were then stained with FITC-conjugated anti-BrdU and 7-amino-actinomycin (7-AAD) followed immediately by flow cytometric analysis. Ten thousand events of each sample were acquired on a Beckman Coulter FC500 Flow Cytometer.

SRB

The sulforhodamine B (SRB) assay was used according to the method of Skehan (22). Cells were plated in 96-well flat-bottomed plates. After 24h culture medium was replaced with fresh medium (with or without respective drugs) in triplicate or quadruplicate cultures. At the end of drug exposure (72h), cells were fixed for 1h and stained with 0.4% SRB (Sigma Aldrich, St. Louis, MO USA) and the optical density was detected at 560nm. Each experiment was repeated three times and Figures depict a representative result. The specific DOG1 inhibitor A01 was kindly provided by Prof. Alan Verkman (University of California, San Francisco).

Whole cell patch-clamp

Whole-cell membrane currents were recorded in GIST-T1 and GIST882 cell lines. The extracellular (bath) solution had the following composition: 150mM NaCl, 1mM CaCl₂, 1mM MgCl₂, 10mM glucose, 10mM mannitol, 10mM Na-HEPES (pH = 7.4). The pipette (intracellular) solution contained 130mM CsCl, 10mM EGTA, 1mM MgCl₂, 10mM HEPES, 1mM ATP (pH 7.4) plus CaCl₂ to obtain the desired free Ca²⁺ concentration: 8mM for 305nM (calculated with Patcher's Power Tool developed by Dr. Francisco Mendes and Franz Wurriehausen, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany).

During experiments, the membrane capacitance and series resistance were analogically compensated using the circuitry provided by the EPC7 patch-clamp amplifier. The usual protocol for stimulation consisted of 600ms-long voltage steps from −100 to 100mV in 20mV increments starting from a holding potential of −60mV. The waiting time between steps was 4s. Membrane currents were filtered at 1kHz and digitized at 5kHz with an

ITC-16 (Instrutech) AD/DA converter. Data were analyzed using the Igor software (Wavemetrics) supplemented by custom software kindly provided by Dr. Oscar Moran.

Western blotting

Whole cell protein lysates were prepared from cell line monolayers according to standard protocols (23). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA USA). Proteins were separated by SDS/PAGE as described by Laemmli (24) and transferred to Hybond P membranes (Amersham Pharmacia Biotech, Uppsala Sweden). Changes in protein expression and phosphorylation as visualized by chemiluminescence (ECL chemiluminescent reagent, Amersham Pharmacia Biotech, Uppsala Sweden) were captured and quantified using a FUJI LAS3000 system with Science Lab 2001 ImageGauge 4.0 software (Fujifilm Medial Systems, Stamford CT, USA).

In vivo studies

Tumor growth *in vivo* was evaluated by subcutaneously injecting the rear flanks of 6- to 8-wk old female adult athymic nude mice (NMRI nu/nu) with 10 million cells/per flank transfected with scrambled or DOG1 shRNA. Tumor growth was monitored biweekly with a caliper and tumor volumes were calculated by $[(\text{length} \times \text{width}^2) / 2]$. The experiment was stopped, mice were sacrificed and tumors were harvested when controls reached app. 1 cm³. Statistical analysis of the mean tumor volumes was done by pairwise comparison using one-tailed homoscedastic t-test analysis.

Immunohistochemical staining

Four micrometer sections of paraffin-embedded tissues of xenograft samples were cut and mounted onto SuperFrost[®] Plus coated slides (Langenbrinck, Emmerdingen, Germany) for immunohistochemical staining. Heat-induced antigen retrieval (waterbath) was carried out with Target Retrieval Solution Citrate buffer (Dako, Glostrup, Denmark) at pH 6.0 or HIER T-EDTA buffer (Zytomed Systems, Berlin, Germany) at pH 9.0. Specimens were stained with a monoclonal rabbit anti-DOG1 (IgG; clone, SP31; dilution, 1:100; 20 min, pH 6.0, Zytomed Systems, No. 504-3315), a monoclonal anti-Ki-67 (IgG1; clone, K-2; dilution, 1:2000; 30 min, pH 6.0, Zytomed Systems, No. MSK018) and a polyclonal anti-KIT antibody (dilution 1:200; 20 min, pH 9.0, Zytomed Systems, No. RP063) together with a highly sensitive and specific polymer detection system utilizing horseradish peroxidase (ZytoChem-Plus HRP Polymer Kit, Zytomed Systems). The process for development was performed using a permanent brown chromogenic substrate system (Permanent AEC Kit, Zytomed Systems). Finally, nuclei were counterstained with hematoxylin for 5min.

RNA isolation and Microarray Gene Expression Profiling

Total RNA of cell line monolayers and tumor samples were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Residual traces of genomic DNA were removed with DNase I (Qiagen, Hilden, Germany). RNA concentration and purity were determined photometrically (NanoDrop, Thermo scientific, Waltham, MA USA). Preparation of cRNA targets (5 µg total RNA), fragmentation, hybridization of HG-U133 plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA), washing, staining, and scanning were performed according to manufacturer's protocols (Affymetrix, Santa Clara, CA, USA) by the BioChip-Labor (Dr. Klein-Hitpass, Institute for Cell Biology, University of Duisburg-Essen, Essen, Germany). Signal intensities and detection calls were determined using Affymetrix microarray suite, version 5.0. Comparison files were further filtered to detect differentially expressed genes.

Quantitative real time polymerase chain reaction (qRT-PCR)

RNA extraction was performed as described above and cDNA synthesis was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit from Thermo Scientific (Waltham, MA USA). qRT-PCR was carried out using TaqMan® chemistry on the Roche LightCycler® 480 (Roche, Grenzach-Wyhlen, Germany) using the standard curve method in triplicate, as previously described (25). Probes for DOG1 (Hs00216121_m1), LTN1 (listerin E3 ubiquitin protein ligase 1, ID: Hs00391630_m1), MNK1 (MAP kinase interacting serine/threonine kinase 1, ID: Hs00374376_m1), CDC14A (CDC14 cell division cycle 14 homolog A, ID: Hs00186432_m1), DDX17 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, ID: Hs00428757_m1), IGFBP5 (insulin-like growth factor binding protein 5, ID: Hs00181213_m1), EPHA4 (EPH receptor A4, ID: Hs00177874) and CDKN1C (cyclin-dependent kinase inhibitor 1C, ID: Hs00175938_m1) were purchased from Applied Biosystems (all FAM-labelled). Expression levels of the housekeeping gene *beta-Actin* (hs99999903_m1, FAM labelled) were assessed for normalization.

Whole transcriptome sequencing

rRNA was depleted from 5µg of total RNA using biotinylated oligonucleotides (Ribominus, Invitrogen), and libraries were constructed from the rRNA-depleted RNA according to the SOLiD Total RNA-seq Kit Protocol (Applied Biosystems). Briefly, library construction involved fragmentation of the RNA by RNAse III to an average size of 150 bases, ligation of the fragmented RNA to adaptors in a directed orientation, then cDNA synthesis and PCR amplification of the resulting library. Approximately 50 bases were sequenced from one end of each fragment using either the SOLiD 3+ or SOLiD 4 instrument and reagents (Applied Biosystems). The resulting sequence data was mapped to the human reference genome, hg18, using Bioscope v1.2 (Applied Biosystems). Sequences that mapped to unique locations were quantified per transcriptional unit, defined as "reads per kilobase of transcript per megabase of total sequence (RPKM)."

A weighted score, described by the following expression:

$$S_k = \frac{Y_k - X_k}{\sqrt{\max(X_k Y_k, 1000)}} \times \log_{10}[\max(X_k Y_k, 1000)]$$

was used to rank the difference in reads for each transcript between samples.

Results

KIT and DOG1 are coexpressed in KIT-positive versus KIT-negative GIST cell lines

Whole transcriptome sequencing analyses of KIT-positive parental KIT cell lines and KIT-negative sublines showed a 47 to 157-fold reduction of KIT and a 7 to 77-fold reduction of DOG1 sequencing counts, suggestive of a coregulation (Figure 1A). Immunoblot studies confirm this observation with an 83 to 99-fold reduction of DOG1 protein levels in KIT-negative GISTs (Figure 1B). Direct (IM) or indirect (17-AAG) pharmacologic inhibition of KIT did not abolish DOG1 expression (Figure 1C).

DOG1 knockdown does not affect KIT expression, cell proliferation or IM sensitivity of GIST cells in vitro

To investigate the biological role of DOG1 in GIST, GIST-T1 and GIST882 cells were transduced with lentiviral particles carrying shRNAs against DOG1 (Sequence: CCGGCGTCGAGTTCAACGACAGAACTCGAGTTTCTGTCGTTGAACTCGACGTT TTTG). This resulted in a 91% reduction of DOG1 protein levels while nonsense shRNA

(scrambled) treatment did not alter DOG1 expression. Expression and activation of KIT and KIT-dependent signaling pathways was not altered by DOG1 knockdown (Figure 2A).

Whole-cell patch-clamp experiments were carried out in order to confirm the functional effect of DOG1 reduction on chloride currents. Notably, DOG1 knockdown resulted in a 96% inhibition of chloride efflux in GIST-T1 and 90% in GIST882 compared to the controls, underscoring that DOG1 is a crucial regulator of GIST cell chlorine balance (Figure 2B).

To assess the impact of DOG1 knockdown on cell proliferation we performed Bromodeoxyuridine (BrdU) incorporation assays. Suppression of DOG1 did not significantly alter the proliferation of GIST-T1 and GIST882 cells *in vitro* (Figure 2C). Moreover, IM sensitivity was maintained in both cell lines despite DOG1 knockdown, with IC50 values of 20nM and 50nM for GIST-T1 and GIST882 (Figure 2D).

Biochemical inhibitors of CaCCs do not affect GIST cell growth in vitro

To further explore the functional impact of the DOG1 CaCC on GIST survival, GIST-T1 and GIST882 cells were treated for three days with three biochemical inhibitors of CaCCs, niflumic acid (NAC), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and A01 (being most specific for DOG1). At active concentrations (26) none of these CaCC inhibitors significantly reduced cell viability. The inhibition of GIST-T1 by A01 at 10 μ M (34%) was seen in both DOG1 knockdown and control cells and is therefore not a DOG1-specific effect (Figure 3). Taken together, these findings argue against a cell-autonomous activity of DOG1 in GIST biology.

DOG1 knockdown inhibits growth of GIST xenografts in nude mice

As DOG1 might be involved tumor-host interactions in GIST, we next investigated the effect of DOG1 knockdown on tumor growth of GIST-T1, GIST882 and GIST430 *in vivo*. As shown in Figure 4, DOG1 knockdown effectively suppressed DOG1 in all xenograft models (exemplified for GIST-T1 and GIST882) while xenografts from parental cells (no lentiviral transduction) and control cells (expressing scrambled shRNA) retained DOG1 expression as measured by immunoblot (Figure 4A) and immunohistochemistry (Figure 4B). Interestingly, GIST-T1 tumors with DOG1 knockdown had lower proliferative activity (Ki-67: 60% positive cells in knockdown tumors versus 90% in control tumors) resulting in a significant reduction (mean 43%) of tumor size after 19 days (n=8; p=0.003) compared to controls (Figure 4C). A substantial growth delay (mean 31%) was also observed in GIST430, which exhibits an intrinsically slower tumor growth compared to GIST-T1. Notably, DOG1 knockdown did not alter the growth of GIST882 xenografts.

Analysis of gene expression data suggests IGFBP5 regulation by DOG1

In order to better understand the molecular consequences of DOG1 knockdown we performed expression arrays using Affymetrix HG-U133 plus 2.0 arrays containing 54,675 probe sets representing more than 24,568 human genes. Changes in expression were found in more than 1,500 genes after DOG1 knockdown in cell lines and in more than >3,500 genes after DOG1 knockdown in xenografts when compared with controls. As expected, *DOG1 (ANO1)* was among the top ranking differentially expressed genes. Other genes with strong differential expression are shown in Figure 5 and suppl. table 1 and 2. Comprehensive pathway analyses were performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA). For those, a fold-change cutoff was set at 1 to identify genes whose expression was significantly differentially up- or downregulated.

Comparison of microarray data and qRT-PCR data

We performed a literature search on the 40 top ranking, differentially expressed genes. Genes that might have an impact on proliferation or apoptosis were selected for qRT-PCR validation. Among these candidates a substantial (> 3-fold) difference in expression levels was found for *DOG1* and *IGFBP5* (Insulin-like growth factor-binding protein 5), while levels of other candidates were only marginally changed (Figure 6A). IGFBP5 levels decreased in GIST882 upon DOG1 knockdown (Figure 6B). In DOG1 negative GIST882B *IGFBP5* transcript counts were unchanged compared to the parental DOG1-positive GIST882 cell line. Notably, DOG1-negative GIST430B cell line showed *IGFBP5* transcriptome sequencing counts that were 5,000-fold higher than in the parental DOG1-positive GIST430 cell line (Figure 6C). The Ingenuity pathway analysis suggested the IGF-pathway together with paxillin-signaling as the top-ranking pathways affected by the DOG1 knockdown.

Discussion

With the introduction of imatinib, a potent KIT-inhibitor, the treatment of GIST as a mainly KIT/PDGFR-driven sarcoma has been revolutionized. Most patients whose tumors harbor activating KIT-mutations benefit from IM-treatment with a median progression-free survival of 12 to 24 months (27). While a subset of patients remains free of progression the majority eventually progress, at which point their therapeutic options are limited. Novel treatment strategies to prevent or overcome resistance are therefore urgently needed.

DOG1 (Ano1 / TMEM16A), a calcium-dependent chloride channel, was identified in microarray studies among a number of genes whose expression was significantly higher in GIST than in other soft tissue sarcomas [13, 26]. It has since then proven to be a reliable immunohistochemical marker in pathological practice (17). Mutations of *DOG1* have not been found in GIST (29) but expression levels are also high in ICC (interstitial cells of Cajal), the cells thought to share a common progenitor with GIST [14, 15]. Whether DOG1 is a lineage-specific marker that plays a role in differentiation or also might play a transforming role in GIST is yet unclear.

To our knowledge we are the first to investigate DOG1 biologic roles and to assess DOG1 relevance as therapeutic target in GIST.

Pathological and genetic analyses from resected metastases progressing on imatinib have revealed secondary KIT-mutations as a common mechanism of resistance. In a subset of patients, GIST metastases have lost KIT expression, indicating that KIT oncogenic programs have been supplanted by yet unidentified alternative oncogenic drivers (30). We observed a similar phenomenon in sublines of several GIST cell lines, which lost KIT expression during cultivation *ex vivo*. Interestingly, these cell lines also lost DOG1 expression suggesting interdependence of expression. Notably, the majority of KIT-negative GIST tumors (64%) in clinical practice also do not express DOG1 (17).

In the studies reported herein, DOG1 knockdown resulted in strong functional inhibition of chloride currents but did not affect expression or activation of KIT and KIT-dependent signaling pathways (Figure 2A, data not shown). In our models, DOG1 therefore does not act as key regulator of KIT, and our studies suggest that DOG1 inhibition will not synergize with KIT kinase-inhibitor drugs in inactivating KIT. Whether DOG1 expression is directly coregulated with KIT is yet unknown, however, biochemical inhibition of KIT does not affect DOG1 expression (Figure 1C) *in vitro*. We further showed that neither DOG1 knockdown nor biochemical inhibitors of DOG1 alter the growth modulating effect of imatinib *in vitro*. However, we observed a substantial growth delay when GIST-T1 and

GIST430 were grown as xenografts compared to scrambled controls in nude mice. This effect was not seen in GIST882.

DOG1 is expressed in many organs (e.g. salivary glands) and DOG1 knock-out mice die soon after birth [10, 25, 26]. Little is known about DOG1 expression in human tissues but the UniGene database suggests an expression pattern similar to that in mice (33). Nonetheless, DOG1 expression is still remarkably high in both GIST and ICCs compared to non-GIST sarcomas (13) (16). Notably, Stanich et al. showed growth inhibitory effects after DOG1 knockdown and DOG1 biochemical inhibition in ICC short term cultures (34). They concluded that regulation of proliferation by DOG1 is related to its function as a Cl^- entry pathway by reducing the Cl^- concentration in the culture media. In contrast to Stanich and colleagues we did not observe a reduction of phosphorylated retinoblastoma tumor suppressor protein (Rb) (data not shown), as a possible explanation for a cell cycle arrest (35).

DOG1 has also been linked to other types of cancers. Amplification of the chromosomal band 11q13, the genomic region containing DOG1, is frequently seen in breast, bladder, head and neck and esophageal cancer (36). Patients with squamous cell carcinomas of the head and neck (SCCHN) harbouring 11q13 amplifications were associated with a poor prognosis. These findings were recently confirmed by functional studies in SCCHN cell lines that showed that DOG1 amplification was associated with increased spreading, detachment and invasion of tumor cells (37). Similar to our findings Duvvuri et al. observed inhibition of tumor growth in SCCHN xenografts after knockdown of DOG1 (44). Notably, this effect was also seen in vitro.

We did not find published evidence that provides simple answers as to why a DOG1 knockdown would affect cell growth in vivo but not in vitro, as seen in our models. We speculate that the more complex cell-cell interaction in 3-dimensional tumors compared to mono-layers as well as a tumor/host-interaction (e.g. vasculature) may be responsible for this effect.

Using gene expression analyses we investigated differentially expressed genes that are directly associated with proliferation or survival. Pathway analyses using Ingenuity software suggested IGF- and paxillin signaling as the most relevant pathways affected by DOG1 knockdown. While the change of expression levels of genes involved in the paxillin-pathway were subtle, IGFBP5 was the strongest differentially expressed gene besides DOG1 as confirmed by qRT-PCR. In line with these findings the GIST cell line GIST430B, a KIT-negative and DOG1-negative subline of imatinib-resistant GIST430, showed a 5000-fold increase of IGFBP5 transcripts compared to its parental DOG1-positive GIST430 cell line. IGFBP5 was not upregulated in GIST882 following DOG1 knockdown and no growth delay was observed in vivo suggesting that IGFBP5 may not be relevant to all GIST.

IGFBP5 is one of the six IGFBP family members and is dysregulated in diverse types of cancer including breast cancer (38), ovarian cancer (39) and retinoblastoma (40). IGFBP5 overexpression results in “trapping” of IGF1 and IGF2 with subsequent inhibition of the IGF axis pathway (41). Of note, IGFBP5 has recently been shown to suppress tumor growth and metastasis of human osteosarcomas (42). In another model, IGFBP5 overexpression prevented tumor growth by inhibition of tumor vascularity which might explain the different biologic outcomes we found between our *in vivo* and *in vitro* experiments. Interestingly, IGF2 expression has recently been shown to predict a high mitotic index correlating with outcome in GIST underlining the relevance of the IGF-axis in GIST (43). DOG1 expression was also reported to promote tumor growth through activation of the MAPK pathway (44),

which we did not observe in our models (data not shown). Of note, MAPK activation in imatinib-sensitive GIST models is usually strongly dependent on KIT activation (45).

Given its high levels of expression, DOG1 could represent a tumor specific target in GIST. Tumor growth in our models was not dependent on DOG1 *in vitro* and only partially dependent *in vivo*. These findings suggest a potential therapeutic role only in combination with other drugs. In addition, further efforts are needed to identify biochemical inhibitors with improved selectivity for DOG1 which recapitulate the effects seen upon DOG1 knockdown *in vivo*. At present, clinical evaluation of DOG1 inhibitors may be constrained by their concomitant inhibition of other chloride channels.

Our results highlight the functional relevance of DOG1 in a subset of GIST and suggest that further studies are warranted to better understand DOG1 and IGF1-axis growth-regulation roles in GIST.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference list

1. Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen C-J, Joseph N, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science*. 2003; 299:708–710. [PubMed: 12522257]
2. Hirota S. Gain-of-Function Mutations of c-kit in Human Gastrointestinal Stromal Tumors. *Science*. 1998; 279:577–580. [PubMed: 9438854]
3. Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, et al. KIT Activation Is a Ubiquitous Feature of Gastrointestinal Stromal Tumors. *Cancer Res*. 2001; 61:8118–8121. [PubMed: 11719439]
4. 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; 26:620–625. [PubMed: 18235121]
5. Demetri G, von Mehren M, Blanke CD, Abbeele van den, Eisenberg BL, Roberts PJ, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med*. 2002; 347:472–480. [PubMed: 12181401]
6. Wardelmann E, Thomas N, Merkelbach-bruse S, Pauls K, Speidel N, Büttner R, et al. Case Report Acquired resistance to imatinib in gastrointestinal stromal tumours caused by multiple KIT mutations. *Lancet Oncol*. 2005; 6:249–251. [PubMed: 15811621]
7. Heinrich MC, Corless CL, Blanke CD, Demetri GD, Joensuu H, Roberts PJ, et al. Molecular correlates of imatinib resistance in gastrointestinal stromal tumors. *J Clin Oncol*. 2006; 24:4764–4774. [PubMed: 16954519]
8. Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, et al. TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science*. 2008; 322:590–594. [PubMed: 18772398]
9. Schroeder BC, Cheng T, Jan YN, Jan LY. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell*. 2008; 134:1019–1029. [PubMed: 18805094]

10. Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim W-S, et al. TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature*. 2008; 455:1210–1215. [PubMed: 18724360]
11. Huang F, Rock JR, Harfe BD, Cheng T, Huang X, Jan YN, et al. Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proc Natl Acad Sci U S A*. 2009; 106:21413–21418. [PubMed: 19965375]
12. Hwang SJ, Blair PJA, Britton FC, Hennig G, Bayguinov YR, Rock JR, et al. Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. *J Physiol*. 2009; 587:4887–4904. [PubMed: 19687122]
13. West RB, Corless CL, Chen X, Rubin BP, Subramanian S, Montgomery K, et al. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. *Am J Pathol*. 2004; 165:107–113. [PubMed: 15215166]
14. Chen H, Ordog T, Chen J, Young DL, Bardsley MR, Redelman D, et al. Differential gene expression in functional classes of interstitial cells of Cajal in murine small intestine. *Physiol Genomics*. 2007; 31:492–509. [PubMed: 17895395]
15. Gomez-Pinilla PJ, Gibbons SJ, Bardsley MR, Lorincz A, Pozo MJ, Pasricha PJ, et al. Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. *American journal of physiology*. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:G1370–G1381. [PubMed: 19372102]
16. Espinosa I, Lee C-H, Kim MK, Rouse B-T, Subramanian S, Montgomery K, et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. *Am J Surg Pathol*. 2008; 32:210–218. [PubMed: 18223323]
17. Liegl B, Hornick JL, Corless CL, Fletcher CDM. Monoclonal antibody DOG1.1 shows higher sensitivity than KIT in the diagnosis of gastrointestinal stromal tumors, including unusual subtypes. *Am J Surg Pathol*. 2009; 33:437–446. [PubMed: 19011564]
18. Miettinen M, Wang Z-F, Lasota J. DOG1 antibody in the differential diagnosis of gastrointestinal stromal tumors: a study of 1840 cases. *Am J Surg Pathol*. 2009; 33:1401–1408. [PubMed: 19606013]
19. Taguchi T, Sonobe H, Toyonaga S-ichi, 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. [PubMed: 12004007]
20. Tuveson, Da; Willis, Na; Jacks, T.; Griffin, JD.; Singer, S.; Fletcher, CDM., et al. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene*. 2001; 20:5054–5058. [PubMed: 11526490]
21. Bauer S, Yu LK, Demetri GD, Fletcher Ja. Heat shock protein 90 inhibition in imatinib-resistant gastrointestinal stromal tumor. *Cancer Res*. 2006; 66:9153–9161. [PubMed: 16982758]
22. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst*. 1990; 82:1107–1112. [PubMed: 2359136]
23. Duensing A, Medeiros F, McConarty B, Joseph NE, Panigrahy D, Singer S, et al. Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). *Oncogene*. 2004; 23:3999–4006. [PubMed: 15007386]
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
25. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol*. 2000; 25:169–193. [PubMed: 11013345]
26. Namkung W, Phuan P-W, Verkman AS. TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells. *J Biol Chem*. 2011; 286:2365–2374. [PubMed: 21084298]
27. Heinrich MC, Owzar K, Corless CL, Hollis D, Borden EC, Fletcher CDM, et al. Correlation of kinase genotype and clinical outcome in the North American Intergroup Phase III Trial of imatinib mesylate for treatment of advanced gastrointestinal stromal tumor: CALGB 150105 Study by Cancer and Leukemia Group B and Southwest Oncology Group. *J Clin Oncol*. 2008; 26:5360–5367. [PubMed: 18955451]

28. Allander SV, Nupponen NN, Ringnér M, Ringne M, Hostetter G, Maher GW, et al. Gastrointestinal Stromal Tumors with KIT Mutations Exhibit a Remarkably Homogeneous Gene Expression Profile Advances in Brief Gastrointestinal Stromal Tumors with KIT Mutations Exhibit a Remarkably Homogeneous Gene Expression Profile 1. *Cancer Res.* 2001; 61:8624–8628. [PubMed: 11751374]
29. Miwa S, Nakajima T, Murai Y, Takano Y, Sugiyama T. Mutation assay of the novel gene DOG1 in gastrointestinal stromal tumors (GISTs). *J Gastroenterol.* 2008; 43:531–537. [PubMed: 18648740]
30. Bauer S, Hartmann JT, de Wit M, Lang H, Grabellus F, Antoch G, et al. Resection of residual disease in patients with metastatic gastrointestinal stromal tumors responding to treatment with imatinib. *Int J Cancer.* 2005; 117:316–325. [PubMed: 15900603]
31. Rock JR, Futtner CR, Harfe BD. The transmembrane protein TMEM16A is required for normal development of the murine trachea. *Dev Biol.* 2008; 321:141–149. [PubMed: 18585372]
32. Schreiber R, Uliyakina I, Kongsuphol P, Warth R, Mirza M, Martins JR, et al. Expression and function of epithelial anoctamins. *J Biol Chem.* 2010; 285:7838–7845. [PubMed: 20056604]
33. Kunzelmann K, Kongsuphol P, Aldehni F, Tian Y, Ousingsawat J, Warth R, et al. Bestrophin and TMEM16-Ca(2+) activated Cl(–) channels with different functions. *Cell calcium.* 2009; 46:233–241. [PubMed: 19783045]
34. Stanich JE, Gibbons SJ, Eisenman ST, Bardsley MR, Rock JR, Harfe BD, et al. Ano1 As a Regulator of Proliferation. *Am J Physiol Gastrointest Liver Physiol.* 2011; 301:1044–1051.
35. Giacinti C, Giordano A. RB and cell cycle progression. *Oncogene.* 2006; 25:5220–5227. [PubMed: 16936740]
36. Akervall JA, Jin Y, Wennerberg JP, Zätterström UK, Kjellén E, Mertens F, et al. Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck. *Cancer.* 1995; 76:853–859. [PubMed: 8625189]
37. Ayoub C, Wasylyk C, Li Y, Thomas E, Marisa L, Robé A, et al. ANO1 amplification and expression in HNSCC with a high propensity for future distant metastasis and its functions in HNSCC cell lines. *Br J Cancer.* 2010; 103:715–726. [PubMed: 20664600]
38. Ahn BY, Elwi AN, Lee B, Trinh DLN, Klimowicz AC, Yau A, et al. Genetic screen identifies insulin-like growth factor binding protein 5 as a modulator of tamoxifen resistance in breast cancer. *Cancer Res.* 2010; 70:3013–3039. [PubMed: 20354179]
39. Walker G, MacLeod K, Williams ARW, Cameron Da, Smyth JF, Langdon SP. Insulin-like growth factor binding proteins IGFBP3, IGFBP4, and IGFBP5 predict endocrine responsiveness in patients with ovarian cancer. *Clin Cancer Res.* 2007; 13:1438–1444. [PubMed: 17332286]
40. Xu XL, Lee TC, Offor N, Cheng C, Liu A, Fang Y, et al. Tumor-associated retinal astrocytes promote retinoblastoma cell proliferation through production of IGFBP-5. *Am J Pathol.* 2010; 177:424–435. [PubMed: 20508032]
41. Beattie J, Allan GJ, Lochrie JD, Flint DJ. Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. *Biochem J.* 2006; 395:1–19. [PubMed: 16526944]
42. Su Y, Wagner ER, Luo Q, Huang J, Chen L, He B-C, et al. Insulin-like growth factor binding protein 5 suppresses tumor growth and metastasis of human osteosarcoma. *Oncogene.* 2011; 30:3907–3917. [PubMed: 21460855]
43. Braconi C, Bracci R, Bearzi I, Bianchi F, Sabato S, Mandolesi A, et al. Insulin-like growth factor (IGF) 1 and 2 help to predict disease outcome in GIST patients. *Ann Oncol.* 2008; 19:1293–1298. [PubMed: 18372285]
44. Duvvuri U, Shiarski DJ, Xiao D, Bertrand C, Huang X, Edinger RS, et al. TMEM16A, induces MAPK and contributes directly to tumorigenesis and cancer progression. *Cancer Res.* 2012; 72:3270–3281. [PubMed: 22564524]
45. Bauer S, Duensing A, Demetri GD, Fletcher Ja. KIT oncogenic signaling mechanisms in imatinib-resistant gastrointestinal stromal tumor: PI3-kinase/AKT is a crucial survival pathway. *Oncogene.* 2007; 26:7560–7568. [PubMed: 17546049]

Figure 1.

Coexpression of KIT and DOG1 in different GIST cell lines. A, Whole transcriptome sequencing data for KIT and DOG1 in different GIST cell lines. B, Western blot analyses of KIT, pKIT and DOG1 expression in KIT-positive (GIST882, GIST430, GIST48) and KIT-negative (GIST882B, GIST430B, GIST48B, GIST5, GIST474, GIST62) GIST cell lines. C, Western blot analyses of GIST-T1 and GIST882 after 24h of incubation with imatinib (1 μ M) and 17-AAG (500nM).

Figure 2. Effects of DOG1 knockdown. A, Western blot analyses of DOG1 expression in GIST-T1 and GIST882 cells. B, Whole-cell patch-clamp measurements of scrambled and DOG1 knockdown cells. C, BrdU cell proliferation assay. D, Cells were treated for 72h with increasing doses of IM (1nm – 10 μ M) and cytotoxicity was measured using SRB assays. Results represent the mean $\pm$ S.D. of quadruplicate values of a representative experiment.

Figure 3.

Cytotoxicity studies with the CaCC inhibitors A01, NAC and NPPB. GIST-T1 and GIST882 cells with or without DOG1 knockdown were treated with increasing doses of inhibitors for 72h and the relative amount of remaining cells was measured using the SRB assay. Results represent the mean $\pm$ S.D. of quadruplicate values of a representative experiment.

Figure 4.

In vivo growth of DOG1 knockdown xenografts. A, Western blot analyses of DOG1 and KIT expression in GIST-T1 and GIST882 xenografts. B, Immunohistochemical analysis of GIST-T1 and GIST882 xenografts. Samples were stained with H&E and with antibodies against DOG1, KIT and Ki-67. C, Tumor volume over time in nude mice implanted with GIST-T1, GIST882 and GIST430 cells after shRNA-mediated DOG1 suppression compared to scrambled shRNA controls.

Figure 5.

Heat map representing color-coded expression levels of differentially expressed genes (up-regulated (red) or down-regulated (green)) in GIST-T1 xenograft and cell line.

Figure 6.

Analysis of gene expression data. A, Quantitative Real Time RT-PCR evaluation of KIT, DOG1, MNK1 (MAP kinase interacting serine/threonine kinase 1), LTN1 (listerin E3 ubiquitin protein ligase 1), CDC14A (CDC14 cell division cycle 14 homolog A), DDX17 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 17), EPHA4 (EPH receptor A4), CDKN1C (cyclin-dependent kinase inhibitor 1C) and IGFBP5 mRNA in GIST-T1 xenografts. B; Quantitative Real Time RT-PCR evaluation of IGFBP5 in GIST-T1 and GIST882. Values were normalized to the scrambled control. C, Whole transcriptome sequencing data for IGFBP5 in GIST882 (KIT- and DOG1-positive), GIST882B (KIT- and DOG1-negative), GIST430 (KIT- and DOG1-positive) and GIST430B (KIT- and DOG1-negative) cells.