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Glutamate Receptor GRIA3—Target of CUX1 and Mediator of Tumor Progression in Pancreatic Cancer[1,2]

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Abstract

Previously, we identified the transcription factor CUX1 as an important modulator of invasion and resistance to apoptosis. Expression profiles suggested that CUX1 regulates a complex transcriptional program mediating tumor progression. We aimed to identify functionally relevant targets of CUX1 by using RNA interference (RNAi)–based loss-of-function screens. Therefore, we generated an RNAi library containing putative transcriptional targets of CUX1 identified by microarrays and performed cell viability screens. Using this approach, several CUX1 targets with effect on tumor cell viability were identified, including the glutamate receptor GRIA3, which was validated in detail for its effects on proliferation, apoptosis, and cell migration using RNAi knock-down and overexpression strategies *in vitro*, as well as xenograft models *in vivo*. The expression of GRIA3 was evaluated in human pancreatic cancer tissues. We found that knock-down of GRIA3 significantly reduced proliferation and migration and enhanced apoptosis. In contrast, overexpression of GRIA3 significantly reduced apoptosis and enhanced both proliferation and tumor cell migration. GRIA3 could be confirmed as a downstream effector of CUX1 and was expressed in pancreatic cancer tissues. *In vivo*, GRIA3 significantly enhanced the growth of subcutaneous xenografts. Inhibitors of glutamate receptors such as GYKI52466 and SYM2206 significantly decreased survival of pancreatic cancer cells, suggesting the presence of glutamate signaling in pancreatic cancer. In conclusion, GRIA3 plays a role as a mediator of tumor progression in pancreatic cancer downstream CUX1. To our knowledge, this is the first report to identify a glutamate receptor as a modulator of tumor progression in a solid cancer outside the brain.

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Introduction

Pancreatic cancer carries the most dismal prognosis of all solid tumors, with a 5-year survival rate of less than 5% and a median survival of less than 6 months [1]. Most patients are diagnosed with pancreatic cancer at advanced stages of the disease. However, even after curative surgery at early stages, local recurrence occurs in many patients. Many reports demonstrate that perineural invasion, that is, spread of cancer cells in the perineural space, is one of the determinants of local recurrence and one of the most significant poor prognostic factors [2,3]. The molecular basis for this propensity to invade perineurally is largely unknown. Once local recurrence or metastatic stage has developed, pancreatic cancer is highly resistant to any therapeutic regimen tested so far [4].

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazol-propionate; siRNA, small interfering RNA; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand

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[2]This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W6 and are available online at www.neoplasia.com.

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The transcription factor CUX1, also known as CDP (CCAAT displacement protein) or CUTL1, belongs to a evolutionarily conserved family of homeobox transcription factors involved in the regulation of cell proliferation, embryonic development, and cell differentiation [5,6]. CUX1 is expressed as multiple isoforms and is cleaved by proteases such as cathepsin L in transcriptionally more active C-terminal fragments [7].

Transcriptional activation of CUX1 leads to increased proliferation in various cell systems [8,9]. Previously, we have shown that CUX1 stimulates tumor cell motility and invasion *in vitro* and *in vivo* by orchestrating a complex transcriptional program [10–13]. Furthermore, we recently identified CUX1 as a potent inhibitor of apoptosis and survival factor in pancreatic cancer [14]. The important role of CUX1 in promoting cell tumor progression is underlined by the fact that CUX1 expression is strongly associated with a less differentiated phenotype and decreased survival in patients with breast cancer [10,15]. Recently, these data could be corroborated by other reports that described the development of mammary tumors in a CUX1-transgenic mouse model [16] and an important role of CUX1 in the regulation of genes associated with metastasis and epithelial-mesenchymal transition [17].

To search for downstream effectors transcriptionally regulated by CUX1, we previously performed whole-genome expression profiling experiments [10]. By using this approach, we identified a list of 41 putative target genes regulated by CUX1 [10]. To functionally screen these targets for effects on survival, we generated a custom RNA interference (RNAi) library containing these 41 genes. The sequential combination of transcriptional profiles and loss-of-function screens identified several functionally relevant CUX1 targets. Interestingly, GRIA3, a subunit of ionotropic glutamate receptors, also known as α-amino-3-hydroxy-5-methyl-4-isoxazol-propionate (AMPA) receptors (AMPARs), which have been mainly described in the central nervous system (CNS), was among these hits. GRIA3 is one of four subunits of the AMPAR, which combine to form heterotetramers [18]. In the current study, we characterized GRIA3 as an important mediator of tumor progression in pancreatic cancer *in vitro* and *in vivo*.

Materials and Methods

Materials and Cell Lines

PANC1 and HT1080 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The PaTu-8988t cell line was received from the German Collection of Cell Lines (DSMZ; Braunschweig, Germany). Cells were maintained in Dulbecco modified Eagle medium (GIBCO, Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO), 100 μg/ml streptomycin, and 100 U/ml penicillin. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Recombinant human tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL/TNFSF10) was obtained from R&D Systems (Minneapolis, MN) and used at a concentration of 100 ng/ml for PANC1 and 75 ng/ml for PaTu-8988t. The AMPAR inhibitors SYM2206 and GYKI52466 were purchased from Tocris Bioscience (Bristol, UK).

RNAi Library

A total of 41 genes were selected as putative CUX1 targets, which were differentially expressed in a genome-wide microarray profile for CUX1 target genes published previously [10]. Three small interfering RNA (siRNA) duplexes from Ambion (Austin, TX) with independent silencing sequences were used for each gene. For siRNA transfection, HT1080 cells were seeded onto a 96-well plate. After 24 hours, cells were transfected with siRNA using siLentFect Lipid Reagent (BioRad, Munich, Germany) following the manufacturer's instructions. Forty-eight hours later, cell viability was measured using CellTiter-Glo luminescent assay (Promega, Madison, WI) according to the manufacturer's instructions.

Plasmids and siRNA

Myc-tagged full-length human p180 CUX1-pMX (180 kDa) and the transcriptionally active C-terminal CUX1-pXJ (75 kDa) were kind gifts from A. Nepveu (McGill University, Montreal, Quebec, Canada). GRIA3-pcDNA3.1 (flip splicing variant) was kindly provided by L. Niu (State University of New York, New York, NY). For stable expression of CUX1, C-terminal CUX1 was cloned into pcDNA3-neo. All plasmids were transfected using TransFast Transfection Reagent (Promega). Stable clones were selected with neomycin (1 mg/ml; PAA Laboratories, Pasching, Austria).

All siRNA experiments were performed using two independent silencing sequences (hCUX1_1, hCUX1_2, hGRIA3_1, and hGRIA3_2), which were purchased from Ambion, and these are available on request. All cells were transfected with siRNA using siLentFect (BioRad) according to the manufacturer's instructions. As nonsilencing control, control siRNA (Ambion) was used.

Quantitative Real-time Polymerase Chain Reaction Analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand complementary DNA was synthesized using Oligo(dt)-Primers (Ambion) and Omniscript RT Kit (Qiagen). Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed using 7500 Fast Real-time PCR and the SYBR Green PCR Master Mix Kit (Applied Biosystems, Wellesley, MA) according to the manufacturer's instructions. Primers were designed with the PrimerExpress program (Applied Biosystems), and these are available on request. Primers for the ribosomal protein RPLP0 (NM_001002) were used as internal standard.

Immunoblot Analysis

Immunoblots were performed as described before [11] and probed with primary antibodies against CUX1 [11], GRIA3 (Millipore, Billerica, MA), poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), and β-actin (Sigma-Aldrich, St Louis, MO).

Proliferation and Viability Assays

[^{3}H]thymidine incorporation was performed as described previously [11], and Bromodeoxyuridine (BrdU) incorporation was measured by using the colorimetric BrdU Cell Proliferation ELISA (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

For proliferation assays with cells transiently transfected with expression plasmids, the number of transfected cells was counted under the microscope. To selectively assess the proliferation of transiently transfected cells, cotransfections with LacZ plasmid were performed. After 24 hours, cells were fixed and stained using X-Gal stain (Sigma). Proliferation was calculated by counting blue-stained cells.

Cell viability was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) or the MTT Cell Proliferation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

FACS

FACS analysis was performed as described previously [19]. In brief, cells were stained with propidium iodide after RNase treatment and analyzed in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Cell cycle was analyzed using the FlowJo software (Tree Star, Ashland, OR).

Two-Chamber Migration Assays

Two-chamber Boyden migration assays were performed as described previously [10]. The number of migrated cells was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and normalized to the total number of proliferating cells in a neighbor well.

Apoptosis Assays

For quantification of histone-associated DNA fragments, we used the Cell Death Detection Elisa^{PLUS} (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Activities of caspase-3 and -7 were measured using a luminogenic caspase-3/7 substrate in the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. PARP cleavage was assessed using a specific antibody detecting cleaved PARP (Cell Signaling Technology).

nu/nu Mice Xenografts

Female *nu/nu* mice were injected subcutaneously with 10^6 PANC1 cells/0.1 ml of phosphate-buffered saline. Five mice per group were injected, and two clones each of cells stably transfected with GRIA3flip-pcDNA3 or empty pcDNA3 vector were used. Tumor growth was determined by regular measurements of the three diameters from day 14 until sacrifice at day 46 after tumor cell inoculation.

Immunohistochemistry

For immunohistochemical analysis, an independent set of 17 human pancreatic adenocarcinoma tissues was provided by the Institute of Pathology of the University of Marburg according to the guidelines of the local ethics committee. Immunohistochemical analysis was performed by using a rabbit polyclonal anti-GRIA2/3 (1:50; Abcam, Cambridge, UK), as described previously [10].

Statistical Analysis

For the *in vitro* experiments, statistical analyses were performed using the double-sided unpaired Student's *t* test after Bonferroni correction for multiple testing, where appropriate. Differences in tumor growth in the mouse xenografts were analyzed using paired *t* test/Wilcoxon matched pairs test.

Results

Loss-of-Function Screen of CUX1 Targets

Previously, we performed genome-wide expression profiles in NIH3T3 cells with or without stable knock-down of CUX1 by RNAi to identify transcriptional targets of CUX1 mediating its effects on tumor progression [10]. Among the lists of putative target genes resulting from these profiling experiments, we aimed to perform unbiased loss-of-function screens for functionally relevant CUX1 targets affecting cell survival. For this purpose, we designed a custom RNAi library containing 41 genes identified by microarray analysis. A detailed list of these genes is available in Table W1. Given the strong effect of CUX1 on cell survival [14], we performed cell viability assays in a 96-well plate format as readout. As the cellular system, we used HT1080 cells that we had previously used to validate our microarray results [10]. To confirm a sufficient knock-down efficacy in our experimental setting, we randomly selected five genes for all of which we could demonstrate a knock-down of more than 70% on RNA level 48 hours after transfection of siRNA oligonucleotides (Figure W1).

The loss-of-function screen led to a significant decrease in cell viability in 7 of the 41 genes (Table 1). Significance was defined as change in viability greater than 25% after 48 hours in at least two of three silencing sequences. The seven hits comprised genes involved in diverse cellular functions such as cell-cell adhesion (*GJA1* and *PVRL3*), deacetylation (*SIRT2*), glutamate signaling (*GRIA3*), and transcriptional regulation (*ARNTL*).

As a first step in our validation process, we aimed to confirm the screen data in several independent cell lines. Given our previous observations that CUX1 is highly expressed and promoting tumor progression in pancreatic cancer [10,13,14], we focused on pancreatic cancer cells to validate the screen hits. Among the seven hits, we were interested in genes that either were upregulated by CUX1 and whose knock-down reduced cell viability or in genes that were downregulated by CUX1 and whose knock-down enhanced cell viability. These genes are likely to represent targets mediating CUX1-induced tumor progression. First, we performed real-time PCR experiments with the pancreatic cancer cell lines PaTu-8988t and PANC1. Transient knock-down of CUX1 in both cells confirmed expression and up-regulation or down-regulation by CUX1 on the messenger RNA (mRNA) level in accordance with the previous microarray data for five of seven genes (*SIRT2*, *GJA1*, *GRIA3*, *PVRL3*, and *FMOD*; see Figures W2 [*PANC1*] and W3 [*PaTu-8988t*]). Four of these five genes (*SIRT2*, *GJA1*, *GRIA3*, and *PVRL3*), whose knock-down reduced cell viability, were up-regulated by CUX1, whereas one gene (*FMOD*), whose knock-down

Table 1. RNAi Target Genes.

Gene Symbol	Relative Viability (siRNA Sequence 1)	Relative Viability (siRNA Sequence 2)	Relative Viability (siRNA Sequence 3)	Mean Viability Relative to Control	Significance Criteria	Regulation by CUX1 in NIH3T3 (Microarray)
SIRT2	0.60	0.64	0.76	**0.67**	*	Up
GJA1	0.77	0.65	0.59	**0.67**	*	Up
GRIA3	0.71	0.57	0.88	**0.72**	*	Up
PVRL3	0.68	0.57	0.97	**0.74**	*	Up
CXCL12	0.45	0.95	0.42	**0.61**	*	Down
FMOD	0.88	1.46	1.25	**1.20**	*	Down
ARNTL	1.47	1.65	1.13	**1.42**	*	Up

List of the 7 of 41 genes fulfilling significance criteria in the RNAi screen. Cell viability relative to the mean viability of 11 nonsilencing control sequences, as determined by CellTiter-Glo assays, is shown as mean and separately for silencing sequences 1 to 3. The genes used for the screen were previously identified as regulated by CUX1 in a microarray approach [10]. Up-regulation or down-regulation of the genes by CUX1 in the previous microarray experiment is shown in the right column.
Significance was defined as change in viability greater than 25% after 48 hours in at least two or three silencing sequences and marked with an asterisk.

enhanced cell viability, was downregulated by CUX1 in all the examined cell lines. This indicates that these target genes identified by the combinatorial approach of transcriptional profiling and subsequent loss-of-function screening might be involved in mediating CUX1-induced tumor cell survival.

Among these screen results, GRIA3 caught our particular interest for further validation in the current study. GRIA3 is known as subunit of the ionotropic AMPAR in the CNS. To our knowledge, no data exist so far on the role of GRIA subunits in tumors outside the brain.

GRIA3 Is Regulated by CUX1

First, we verified that GRIA3 is transcriptionally regulated by CUX1. On the RNA level, we performed quantitative RT-PCR of PaTu-8988t and PANC1 cells with or without knock-down of CUX1 by siRNA. Knock-down of CUX1 significantly reduced GRIA3 mRNA in both cell lines (Figure 1*A*). Moreover, we examined the effect of transiently overexpressed CUX1 on GRIA3 mRNA expression. Because full-length CUX1 is endogenously cleaved by cathepsin L resulting in a transcriptionally active C-terminal fragment [7], we used a transcriptionally active C-terminal CUX1 p75 plasmid for transfection experiments. Transient overexpression of CUX1 resulted in significant up-regulation of GRIA3 mRNA (Figure 1*B*). To confirm the regulation of GRIA3 by CUX1 on the protein level, we performed Western blots. In analogy to the CUX1 effects seen on the RNA level, knock-down of CUX1 resulted in decreased GRIA3 protein levels (Figure 1*C*), whereas CUX1 overexpression increased GRIA3 levels in both cell lines (Figure 1*D*).

To further confirm the regulation of GRIA3 by CUX1, we analyzed stable PANC1 clones expressing the transcriptionally active C-terminal CUX1 fragment [5] at different expression levels. In these clones, GRIA3 protein (Figure W4*A*) and mRNA (Figure W4*B*) expressions were positively correlated with CUX1 levels, further corroborating the regulation of GRIA3 by CUX1.

GRIA3 Enhances Proliferation

On the basis of our screen results demonstrating the effect of GRIA3 on cell viability, we first aimed to characterize its effects on proliferation in more detail.

Proliferation was analyzed after transient knock-down and overexpression of GRIA3 in PANC1 and PaTu-8988t cells. Knock-down of GRIA3 resulted in significantly reduced [^{3}H]thymidine incorporation in both cell lines (Figure 2*A*). This was associated with a marked decrease in S phase and increase in G_1 phase, as assessed by FACS cell cycle analysis in PANC1 cells (Figure 2*B*). In contrast, transient overexpression of GRIA3 led to a significant increase in cell number, which was determined by counting X-Gal–positive cells after cotransfection of both LacZ and GRIA3 (Figure 2*C*). To investigate the long-term effects of GRIA3 on proliferation, we generated stable PANC1 clones.

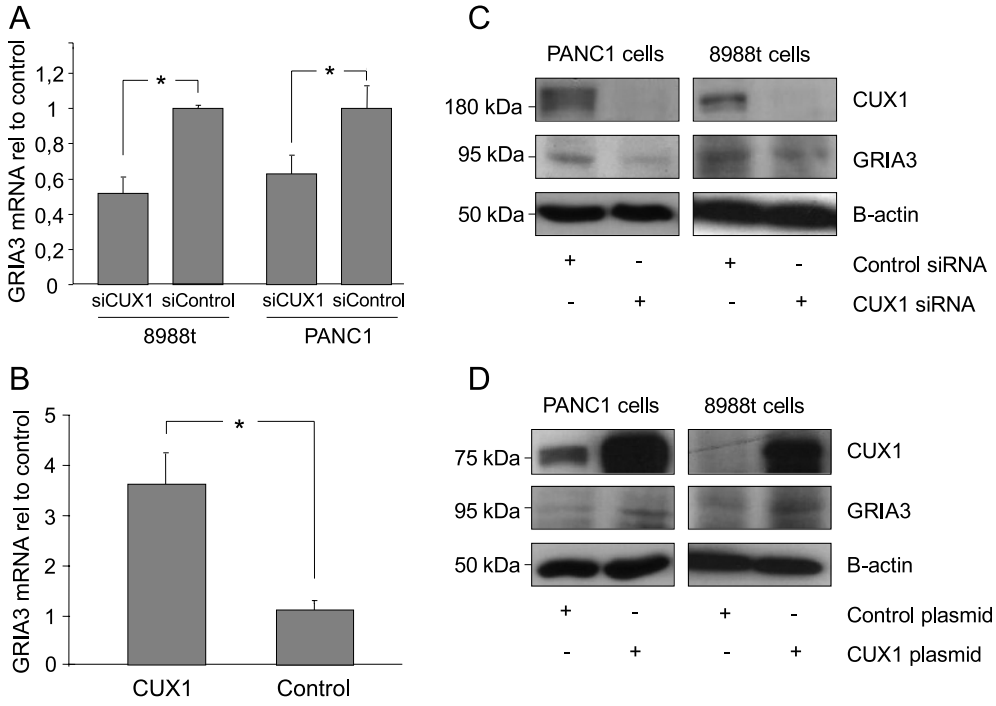

Figure 1. GRIA3 is upregulated by CUX1. (A) Knock-down of CUX1 decreases GRIA3 mRNA. PaTu-8988t and PANC1 cells were transiently transfected with CUX1-specific or nonsilencing control siRNA. GRIA3 mRNA levels quantified by quantitative RT-PCR were normalized to *RPLP0* as housekeeping gene and expressed relative to control cells. *$P < .05$. Results expressed as mean ± SEM are representative of three independent experiments. (B) Transient CUX1 overexpression increases GRIA3 mRNA. PANC1 cells were transiently transfected with the C-terminal CUX1 expression plasmid or an empty vector. GRIA3 mRNA levels were quantified by quantitative RT-PCR, normalized to *RPLP0*, and expressed relative to control cells. *$P < .05$. Results expressed as mean ± SEM are representative of three independent experiments. (C) Knock-down of CUX1 decreases GRIA3 protein. PANC1 and PaTu-8988t cells were transfected with CUX1 or control siRNA. GRIA3 protein levels were analyzed by Western blot; *β-actin* was used as a housekeeping gene. Results are representative of three independent experiments. (D) Transient overexpression of CUX1 increases GRIA3 protein. PANC1 and PaTu-8988t cells were transfected with CUX1 or an empty plasmid. GRIA3 and β-actin levels were analyzed by Western blot. Results are representative of three independent experiments.

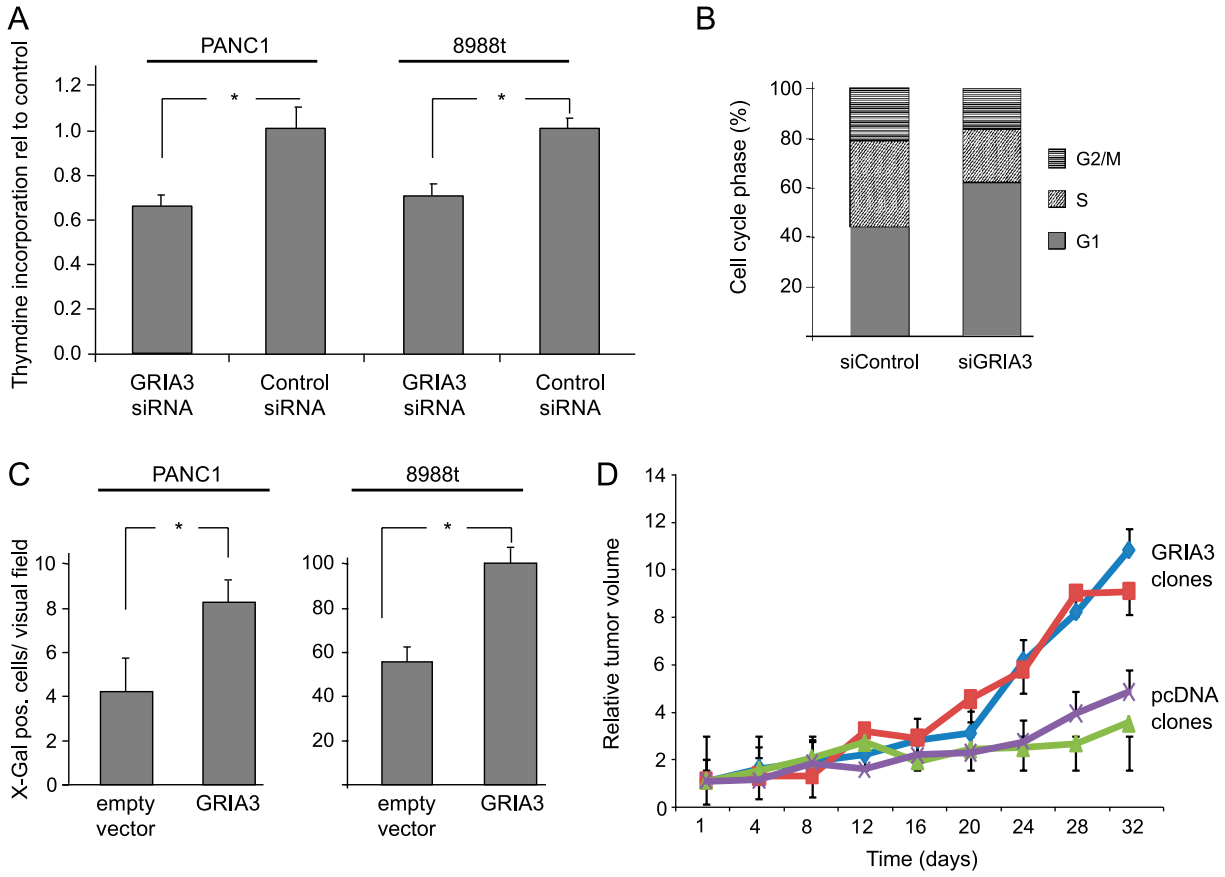

Figure 2. GRIA3 enhances tumor cell proliferation. (A) Knock-down of GRIA3 by siRNA leads to decreased proliferation compared with control-transfected cells as assessed by thymidine incorporation assays 48 hours after transfection. *$P < .05$. Results expressed as mean ± SEM are representative of three independent experiments. (B) Cell cycle analysis by FACS of PANC1 cells transfected with GRIA3 siRNA or control siRNA. Cell cycle distribution was determined after PI staining, and this was shown as a percentage of cells in the G_1, S, and G_2/M phases. Results are representative of three independent experiments. (C) PANC1 and PaTu-8988t were transiently cotransfected with GRIA3 or empty vector and LacZ plasmids. To selectively assess the number of transfected cells, fixed cells were X-Gal–stained and counted. Results are shown as X-Gal–positive cells per visual field. Results are representative of three independent experiments. *$P < .05$. (D) *In vivo* growth of PANC1 cells stably expressing GRIA3 or control plasmid (two clones each). Cells were injected as subcutaneous xenografts in *nu/nu* mice (six tumors per clone). After establishment of measurable tumors (day 1), tumor volume was measured during the next 32 days. Tumor growth is shown relative to day 1 and presented as mean ± SEM of 12 tumors per group, derived from two clones each. *$P < .05$ compared with control clones.

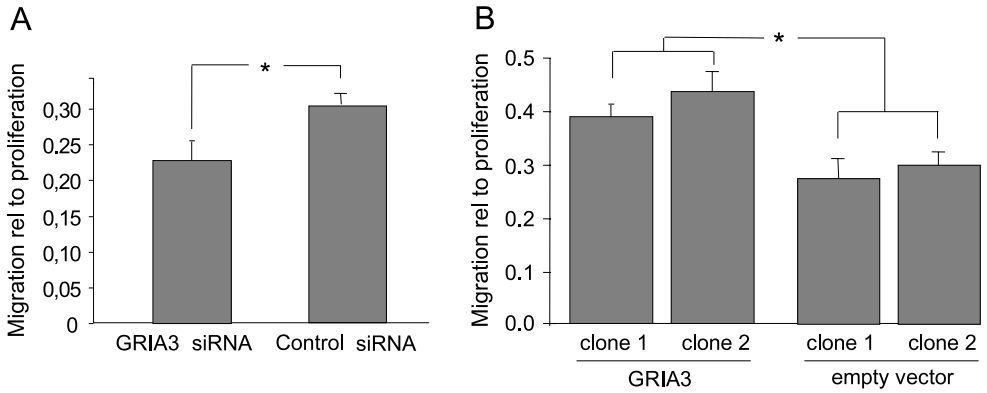

Figure 3. GRIA3 enhances tumor cell migration. (A) PANC1 cells transfected with GRIA3 or control siRNA were seeded onto Boyden chamber inserts. Cells that migrated through the 8-μm pores were quantified after 6 hours using CellTiter-Glo viability assays. (B) PANC1 cells stably overexpressing GRIA3 or empty vector (two clones each) were seeded onto Boyden chamber inserts. Cells that migrated through the pores were quantified after 6 hours using CellTiter-Glo viability assay. Results are shown as mean ± SEM after normalization to proliferating cells in neighbor wells and are representative of three independent experiments. *$P < .05$.

GRIA3 overexpression led to increased proliferation in two independent clones compared with mock-transfected control clones (Figure W5). These data indicate that GRIA3 stimulates tumor cell proliferation by enhancing S-phase progression *in vitro*.

To confirm the effect of GRIA3 on tumor proliferation *in vivo*, we performed experiments in nude mice analyzing subcutaneous xenograft tumors of GRIA3 overexpressing PANC1 cells. GRIA3 overexpression in two independent clones showed a significantly enhanced tumor growth compared with mock-transfected clones (Figure 2*D*), confirming a significant effect of GRIA3 on tumor growth *in vivo*.

GRIA3 Enhances Migration

Given the paramount role of GRIA3's upstream regulator CUX1 on motility and invasion that we had described previously [10], we investigated whether GRIA3 also affects tumor cell migration. Therefore, we performed Boyden chamber migration experiments in PANC1 cells after knock-down or overexpression of GRIA3 by examining migration through a porous membrane. We normalized for proliferation at the end of experiment to exclude a potential proliferation bias. GRIA3 knock-down significantly reduced cell migration toward serum as a chemotactic gradient (Figure 3*A*). Moreover, PANC1 clones stably overexpressing GRIA3 were also assessed for migration differences. In two independent clones, GRIA3 overexpression led to a significantly enhanced migration compared with control clones (Figure 3*B*). This indicates that, apart from its effects on proliferation, GRIA3 is also able to promote tumor cell migration.

GRIA3 Protects from TRAIL- and Drug-Induced Apoptosis

In addition to the important role of CUX1 as a mediator of proliferation and migration [10], we recently observed a pronounced effect of CUX1 on tumor cell survival and resistance to apoptosis [14]. Therefore, we investigated whether GRIA3 as CUX1 downstream effector also affects resistance to apoptosis. We induced apoptosis by TRAIL/TNFSF10. Knock-down of GRIA3 significantly enhanced both basal and TRAIL-induced apoptosis in PANC1 and PaTu-8988t cells, as determined by quantification of histone-associated DNA fragments (Figure 4*A*). This was accompanied by activation of the effector caspases-3 and -7 (Figure 4*B*) as well as enhanced PARP cleavage (Figure 4*C*) in both cell lines. In analogy, stable GRIA3 overexpression protected from TRAIL-induced apoptosis (Figure 4*D*), indicating an important role of GRIA3 in mediating resistance to apoptosis.

GRIA3 Protein Is Expressed in Pancreatic Cancer Tissues

On the basis of the high expression levels and functional relevance of CUX1 for tumor progression in pancreatic cancer [10,14], we sought to verify that the CUX1 target GRIA3 is expressed in pancreatic cancers by studying GRIA3 protein levels in a pilot series of 17 pancreatic adenocarcinomas. Unfortunately, the antibody available for immunohistochemistry detects two subunits of the glutamate receptor AMPAR, GRIA2 and GRIA3. Using this antibody, we detected strong expression levels of GRIA2/3 in 13 of 17 examined pancreatic cancer tissues (Figure 5*A*). Interestingly, a particularly strong staining was observed in some tumor cells adjacent to neural structures (Figure 5*B*).

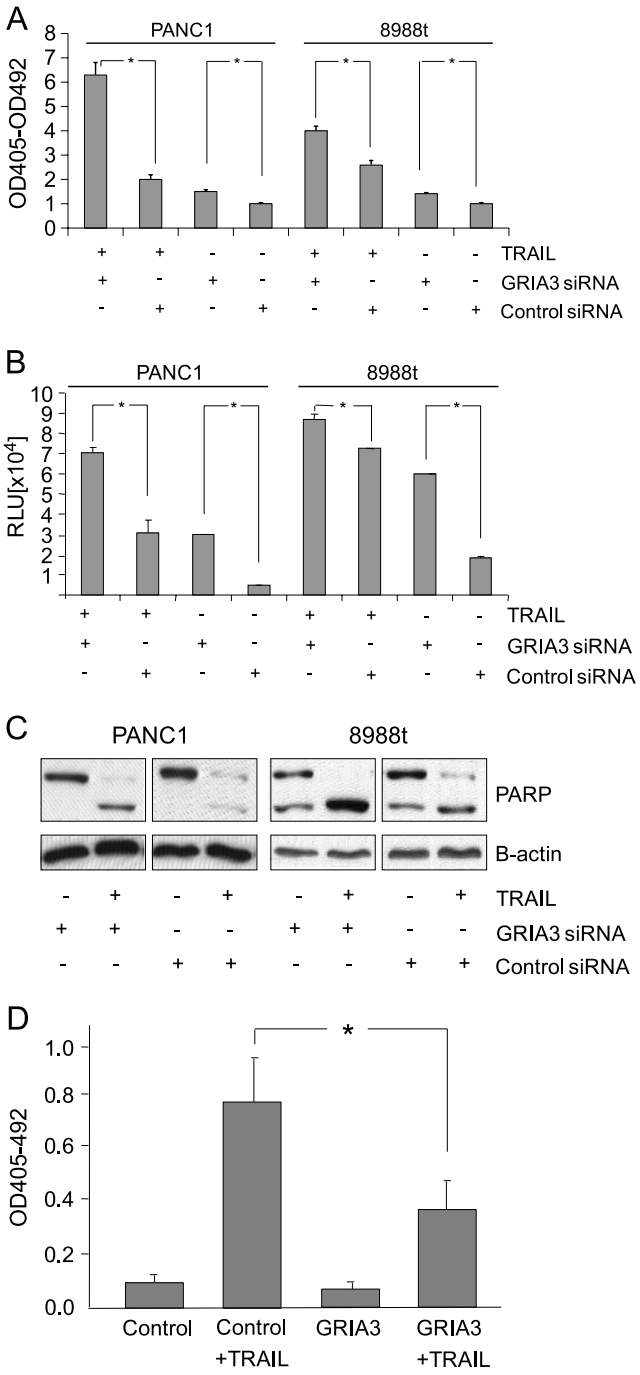

Figure 4. GRIA3 mediates resistance to TRAIL-induced apoptosis. (A–C) PANC1 and PaTu-8988t cells were transfected with GRIA3 or control siRNA. About 36 hours later, TRAIL was added for 12 hours, and apoptosis was quantified assessing histone-associated DNA fragments (A), caspase-3/7 activity assays (B), and PARP cleavage using Western blots (C). *$P < .05$. (D) PANC1 cells stably overexpressing GRIA3 or empty vector (two clones each) were incubated with TRAIL for 12 hours. Apoptosis was quantified to assess histone-associated DNA fragments and shown as mean ± SD at $OD_{405\text{-}492}$. *$P < .05$. Results are representative of three independent experiments.

Agonists and Antagonists of Glutamate Receptors Modulate Pancreatic Cancer Cell Survival

GRIA3 is a subunit of the AMPAR. AMPARs are heterotetrameric complexes composed of combinations of four subunits, termed *GRIA1-4*. Studies in the CNS revealed that AMPARs open on binding of agonists such as glutamate and become permeable for ions such as calcium, sodium, and potassium.

To assess whether GRIA3 is part of functional AMPARs in pancreatic cancer, which might be therapeutically targeted by specific antagonists,

we examined the expression levels of all four subunits in pancreatic cancer cells. Quantitative RT-PCR demonstrated the expression of GRIA1, 3, and 4 in both PANC1 and PaTu-8988t. Although expression levels were not quantitatively compared, GRIA2 seemed to be expressed less in both cell lines (Figure W6). Interestingly, CUX1 was not able to significantly regulate mRNA expression of GRIAs other than GRIA3, as assessed by quantitative RT-PCR (data not shown).

To demonstrate the functionality of glutamate receptors in pancreatic cancer cells, we incubated PANC1 and PaTu-8988t cells with increasing concentrations of glutamate in glutamate-free medium. Addition of glutamate led to a significant increase in cell viability (Figure 5*C*), suggesting the presence of functional glutamate receptors. However, because we could not rule out that the observed effect was due to glutamate depletion independently of the presence of functional glutamate receptors, we wished to corroborate our observations by using AMPAR inhibitors.

Several compounds have been described as inhibitors for AMPARs, among them are the selective noncompetitive AMPAR antagonist GYKI52466 and the noncompetitive allosteric AMPAR antagonist SYM2206. On the basis of our findings that GRIA3 strongly affects tumor cell survival, we aimed to investigate the effects of AMPAR antagonists as potential therapeutic modalities. We incubated both PANC1 and PaTu-8988t cells with increasing concentrations of GYKI52466 or SYM2206. Interestingly, both compounds dose-dependently inhibited the viability of both cell lines, suggesting that therapeutic interference with glutamate receptor signaling by application of AMPAR antagonists might represent a new treatment approach for pancreatic cancer (Figure 5*D*).

Discussion

By applying a sequential approach of transcriptional profiling and loss-of-function screening, we identified the AMPAR subunit GRIA3 as a downstream target of the transcription factor CUX1 and as an important mediator of its effects on tumor proliferation, survival, and migration in pancreatic cancer cells *in vitro* and *in vivo*. To our knowledge, this is the first report of AMPAR expression and its functional implication in tumor progression of solid cancers outside the CNS.

We report that targeting AMPARs by specific inhibitors reduced tumor cell growth, indicating that targeting AMPARs might represent a novel therapeutic approach in patients with pancreatic cancer.

Within the CNS, expression of GRIA3 and other glutamate receptors has been reported in gliomas and neuroblastomas [20,21]. In gliomas, high expression levels of GRIA1 have been implicated in tumor progression. De Groot et al. [20] described a statistically significant increase in GRIA1 expression in high-grade glioblastoma samples compared with that in low-grade tumors. In analogy to our findings on GRIA3, knock-down of GRIA1 decreased glioma cell proliferation and increased apoptosis *in vitro* and in xenografts *in vivo* [20]. Recently, Piao et al. [22] showed that overexpression of GRIA1 increased

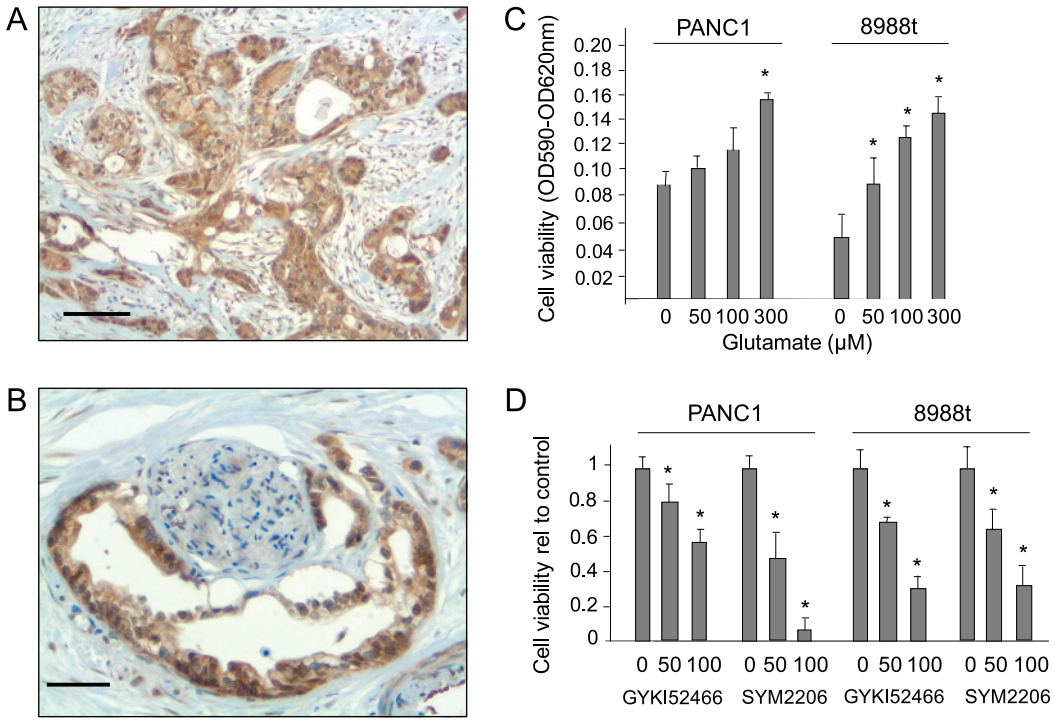

Figure 5. GRIA2/3 is expressed in pancreatic cancer, and glutamate receptor modulators affect pancreatic cancer cell viability. (A, B) Immunohistochemistry of human pancreatic cancer tissues using an antibody directed against GRIA2/3. (A) Representative picture of a GRIA2/3–positive ductal pancreatic carcinoma tissue. Bar, 100 μm. (B) GRIA2/3–positive pancreatic cancer cells adjacent to a neural structure. Bar, 40 μm. (C) Effect of glutamate on the viability of PANC1 and PaTu-8988t cells. Cells were incubated in glutamate-free medium with increasing concentrations of glutamate for 72 hours. Cell viability was determined using MTT assays. Results expressed as mean ± SEM are representative of three independent experiments. *$P < .05$ compared with cells without glutamate addition. (D) Effect of the glutamate receptor antagonists GYKI52466 (0-100 μM) and SYM2206 (0-100 μM) on cell viability of PANC1 and PaTu-8988t cells after 72 hours of incubation as assessed by MTT assays. Results are representative of three independent experiments. *$P < .05$ compared with untreated cells.

glioma cell adhesion to extracellular matrix, leading to enhanced tumor cell invasion.

The intracellular signaling events downstream GRIA3 in tumor cells remain to be elucidated. In postsynaptic neurons, stimulation of AMPARs with agonists such as glutamate or AMPA leads to the opening of an ion channel and subsequent influx of cations such as calcium, sodium, and potassium. In the cell lines used, we were unable to show a significant increase in intracellular calcium content after glutamate or AMPA stimulation, as determined by FURA- and aequorin-based calcium measurements (Griesmann et al., unpublished observations). This suggests that GRIA3 signaling occurs calcium-independently. Furthermore, the main source of glutamate in the tumor environment remains unclear to date. GRIA-positive tumor cells may be stimulated by glutamate in the serum, which is known to contain significant amounts of glutamate [23]. In addition, it can by hypothesized that cancer cells are able to metabolize glutamine into glutamate by glutaminase, a process well described in the CNS. Alternatively, glutamate may also be produced by transamination of 2-oxoglutarate, an intermediate in the citric acid cycle [24]. Moreover, a constitutive activation of GRIA3 can also not be ruled out, and a detailed functional characterization of stimulation and downstream signaling of AMPARs in cancer cells is warranted.

Likewise, the upstream regulation of GRIA3 expression by oncogenic signaling cascades requires further studies. We show that GRIA3 is regulated on mRNA and protein levels by the homeodomain transcription factor CUX1. To our knowledge, the promoter of GRIA3 has not been characterized in detail. On the basis of our functional data, we speculate that GRIA3 expression might be modulated by several tumor-promoting signaling events, among them is the CUX1-induced signaling pathway. A characterization of the GRIA3 promoter including a detailed mapping of CUX1 binding sites is warranted to prove that GRIA3 is a direct target of CUX1.

Outside the CNS, only few data exist on the expression and function of AMPARs. Ganor et al. [25] found that human T cells express GRIA3 and observed that glutamate triggers integrin-mediated adhesion as well as chemotactic migration. The same group extended their studies examining T-cell leukemias and cutaneous T-cell lymphomas [26]. They found high GRIA3 levels in both malignancies and demonstrated that glutamate enhanced growth and spreading, facilitating engraftment of T-cell leukemia cells *in vivo* and accompanied by induction of EMMPRIN/CD147, a cancer-associated matrix metalloproteinase inducer [26]. The authors hypothesized that GRIA3 may enhance spreading and engraftment of lymphoma cells in the nervous system with high glutamate levels. In pancreatic cancer, we also observed a significant effect of GRIA3 on cancer cell migration *in vitro*. By analogy, it can be speculated that GRIA3 expression in pancreatic cancer cells might stimulate the perineural invasion of the tumor, which represents a typical growth pattern in pancreatic cancer. This is in accordance to our observation of strong GRIA2/3 expression in some tumor cell clusters adjacent to neural structures. Further studies are warranted to correlate GRIA expression patterns in whole resected specimens with histopathologic criteria such as perineural invasion to verify this hypothesis.

On the basis of the high expression and the strong effects of GRIA3 on proliferation, survival, and migration, targeting GRIA3 might represent an interesting novel therapeutic approach for pancreatic cancer. To date, no specific inhibitor for GRIA3 is available. Therefore, we used inhibitors that have been shown to inhibit ionotropic AMPARs containing the subunits GRIA1, 2, 3, or 4. Apart from GRIA3, we could confirm the expression of GRIA1 and GRIA4 mRNA in pancreatic cancer cell lines. We observed a significant inhibitory effect on tumor cell viability after incubation with two different AMPAR antagonists, SYM2206 and GYKI52466. Our data are in accordance with previous reports on the cytotoxic effects of AMPA antagonists on various tumor cell lines *in vitro*. Rzeski et al. [27] demonstrated strong antiproliferative and antimigratory effects of GYKI52466 on astrocytoma, breast, colon, and lung carcinoma cells. Recently, Stepulak et al. [28] analyzed RNA expression patterns of the different members of the glutamate receptor family in various cancer cell lines and found RNA levels of AMPAR subunits including GRIA3 in several non-CNS cancer cell lines. This indicates that targeting AMPARs might represent a novel therapeutic avenue not only for pancreatic cancer but also for a variety of tumors of different origin. Although our data indicate an important role of GRIA3 in cancer progression, we cannot rule out that other GRIA subunits that are targeted by available AMPA inhibitors might also affect tumor cell survival. Besides GRIA3, we detected expressions of GRIA1 and GRIA4 in pancreatic cancer cells. Interestingly, these subunits were not significantly regulated by CUX1. Further studies are required to delineate the role of the different GRIA subunits for cancer progression and to define the most promising GRIA subunit as target of AMPA antagonists.

In summary, we identified GRIA3 as an important mediator of tumor cell survival, proliferation, and migration and as a downstream target of CUX1, which has been shown to orchestrate a complex transcriptional program enhancing tumor progression [10,11]. Targeting GRIA3 by specific inhibitors represents a promising novel target for pancreatic cancer therapy.

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Table W1. List of the 41 Genes Analyzed in the RNAi Screen.

Gene Symbol	Relative Viability (siRNA Sequence 1)	Relative Viability (siRNA Sequence 2)	Relative Viability (siRNA Sequence 3)	Mean Viability Relative to Control	Significance Criteria
SIRT2	0.60	0.64	0.76	**0.67**	*
GJA1	0.77	0.65	0.59	**0.67**	*
GRIA3	0.71	0.57	0.88	**0.72**	*
PVRL3	0.68	0.57	0.97	**0.74**	*
CXCL12	0.45	0.95	0.42	**0.61**	*
FMOD	0.88	1.46	1.25	**1.20**	*
ARNTL	1.47	1.65	1.13	**1.42**	*
S100A10	0.80	0.43	0.89	**0.71**	
LLT1	0.56	0.76	0.86	**0.73**	
TFDP1	0.84	0.45	1.07	**0.78**	
PDLIM1	0.87	0.78	0.72	**0.79**	
SEMA3A	0.61	0.81	1.06	**0.83**	
MMP3	0.82	0.90	0.82	**0.85**	
ANK1	1.16	0.78	0.65	**0.86**	
ETV4	1.01	0.66	0.93	**0.87**	
HMGN1	1.11	0.85	0.65	**0.87**	
ITGA5	0.55	0.97	1.09	**0.87**	
COL4A1	0.73	0.88	1.09	**0.90**	
SERPINA3	0.87	1.02	0.82	**0.90**	
SLIT2	0.91	0.81	1.01	**0.91**	
CDH2	1.10	0.69	1.01	**0.93**	
CHI3L2	0.82	0.94	1.08	**0.94**	
EFNA2	0.76	1.16	0.94	**0.95**	
NRAS	1.17	0.67	1.01	**0.95**	
CDH18	0.67	0.98	1.26	**0.97**	
TM4SF1	0.66	0.98	1.30	**0.98**	
ITGA6	0.82	1.26	0.86	**0.98**	
COL3A1	0.68	0.86	1.42	**0.99**	
RPS27	1.17	0.79	1.03	**1.00**	
SAA1	0.95	1.20	0.87	**1.00**	
CD34	1.12	1.22	0.75	**1.03**	
MYBL1	1.41	0.67	1.02	**1.03**	
DCK	1.13	1.13	0.84	**1.03**	
LUM	0.77	1.05	1.28	**1.03**	
GSTZ1	0.95	1.13	1.10	**1.06**	
DAB2	0.69	0.95	1.65	**1.10**	
LGALS9	0.96	1.20	1.14	**1.10**	
IL1A	1.01	1.08	1.22	**1.10**	
EPHA1	0.69	1.21	1.44	**1.11**	
ALCAM	1.31	0.99	1.07	**1.12**	
PTN	1.04	1.46	1.18	**1.23**	

Cell viability relative to the mean viability of 11 nonsilencing control sequences, as determined by CellTiter-Glo assays, is shown as mean and separately for silencing sequences 1 to 3. The genes used for the screen were previously identified as regulated by CUX1 in a microarray approach [10]. Changes in cell viability greater than 25% after knock-down of a particular gene in two of three silencing sequences were considered as significant, and these are marked with an asterisk.

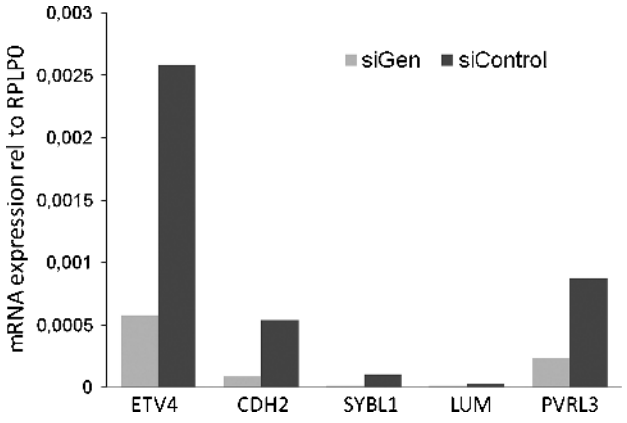

Figure W1. Knock-down efficiency of five randomly selected genes from the RNAi library as assessed by quantitative RT-PCR in HT1080 cells, normalized to *RPLP0* expression as the housekeeping gene. Results are expressed as mean ± SEM and are representative of three independent experiments.

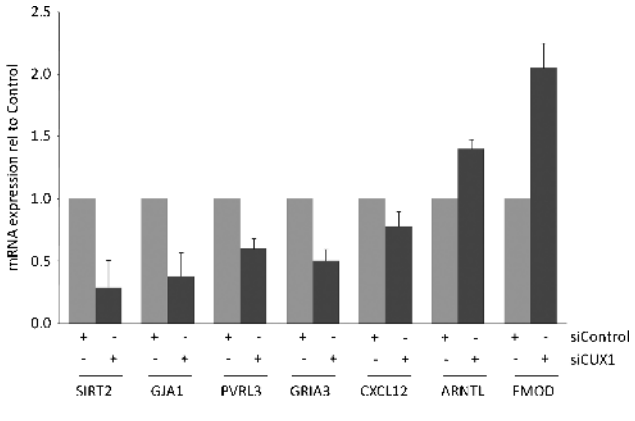

Figure W2. mRNA expression, as quantified by quantitative RT-PCR, of the seven screen hits in PANC1 cells transiently transfected with siRNA against CUX1 (siCUX) or nonsilencing control siRNA. Results are shown as mean ± SEM normalized to *RPLP0* expression as the housekeeping gene. Results are representative of three independent experiments.

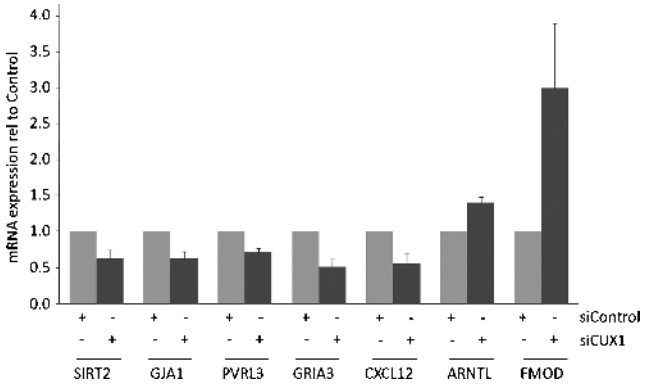

Figure W3. mRNA expression, as quantified by quantitative RT-PCR, of seven screen hits in PaTu-8988t cells transiently transfected with siRNA against CUX1 (siCUX) or nonsilencing control siRNA. Results are shown as mean ± SEM normalized to *RPLP0* expression as the housekeeping gene. Results are representative of three independent experiments.

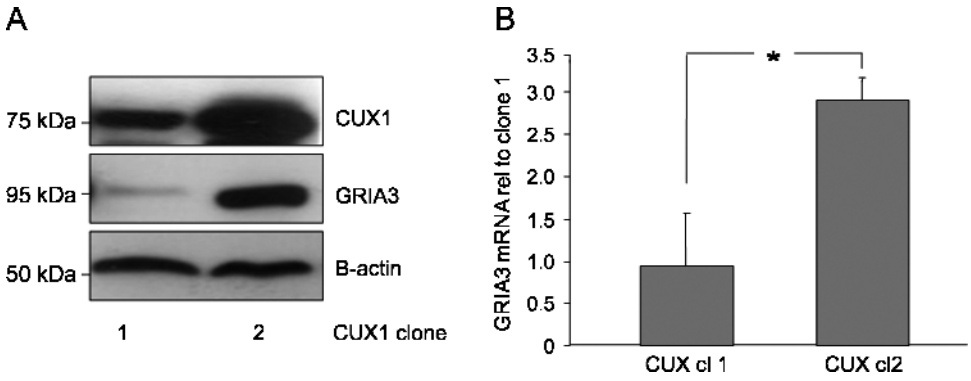

Figure W4. (A) Stable overexpression of CUX1 increases GRIA3 protein. PANC1 cells were stably transfected with the transcriptionally active CUX1 expression plasmid. GRIA3 protein levels were analyzed in two different clones with different CUX1 expression levels by Western blot using specific antibodies; *β-actin* was determined as the housekeeping gene. (B) Stable overexpression of CUX1 increases GRIA3 mRNA. PANC1 clones 1 and 2 stably expressing two different levels of the transcriptionally active CUX1 expression plasmid (shown in panel A) were analyzed for GRIA3 mRNA by quantitative RT-PCR, normalized to *RPLP0* expression as the housekeeping gene, and expressed relative to clone 1. *$P < .05$ compared with clone 1. Results are expressed as mean ± SEM and are representative of three independent experiments.

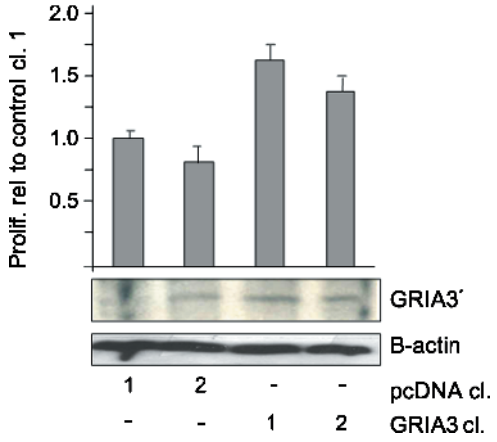

Figure W5. PANC1 cells stably expressing GRIA3 or control plasmid (two clones each) were assessed for differences in proliferation by using BrdU proliferation assays 24 hours after seeding. Results were normalized to control clone 1; these are shown as mean ± SEM and are representative of three independent experiments. GRIA3 expression level and *β-actin* as the housekeeping gene, as determined by Western blot, are shown below.

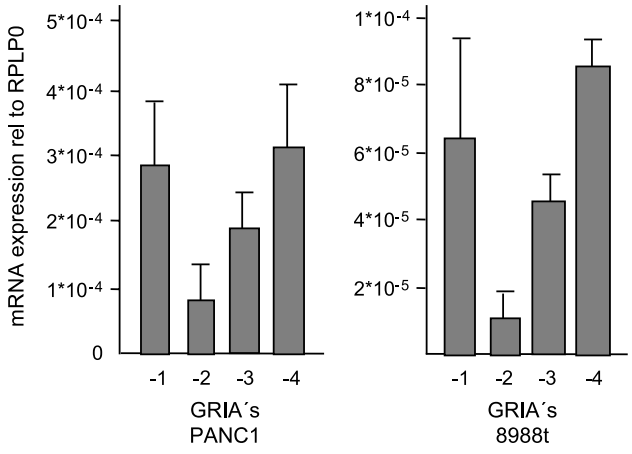

Figure W6. Expression of hGRIA1-4 in PANC1 and PaTu-8988t cells, as assessed by quantitative RT-PCR normalized to *RPLP0* expression as the housekeeping gene. Results are expressed as mean ± SEM and are representative of three independent experiments.