# Endoplasmic reticulum protein GliPR1 regulates G protein signaling and the cell cycle and is overexpressed in AML

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**Abstract.** Glioma pathogenesis-related protein 1 (GliPR1) is a pleiotropic protein involved in cell proliferation, tumor growth and apoptosis. The aim of the present study was to further characterize GliPR1 in regard to its subcellular localization and its overall effect on cellular gene expression. Knockdown of GliPR1 and Affymetrix microarray mRNA expression analysis revealed 262 GliPR1-dependent differentially expressed genes, of which 40 were induced and 222 were suppressed. Differentially expressed genes were overrepresented in five Gene Ontology categories: G protein signaling pathways, regulation of cyclin-dependent protein kinase activity, ER to Golgi vesicle-mediated transport, axon guidance and dephosphorylation. GliPR1-EGFP fusion protein co-localized with the endoplasmic reticulum (ER) or with cytoplasmic vesicles as demonstrated by confocal microscopy. GliPR1 expression was found to be significantly increased in acute myeloid leukemia (AML) bone marrow samples, while markedly reduced in acute lymphoblastic leukemia, unchanged in myelodysplastic syndrome and slightly decreased in chronic lymphocytic leukemia as well as in chronic myelocytic leukemia (CML) when compared to normal samples. GliPR1 was localized and involved in the ER secretory protein pathway. GliPR1 affects G protein signaling and cell cycle regulation. Based on the observed overexpression in AML samples, GliPR1 should be further explored as a potential target for AML.

## Introduction

Glioma pathogenesis-related protein 1 (GliPR1) was originally identified in human glioblastomas (1) and is also named related to testes-specific, vespid and pathogenesis protein 1 (RTVP-1) (2). Increased expression of GliPR1 was associated with myelomonocytic differentiation in macrophages (3). While GliPR1 has been reported to act as a tumor-suppressor gene inducing apoptosis in prostate cancer (4-7), it appears to be an oncogene in glioblastomas, that enhances proliferation (8) and is overexpressed in Wilms' tumors (9). Previously, we described GliPR1 as an HIV-1 dependency factor (10).

RTVP-1 (GliPR1) protein was reported to contain an N-terminal signal peptide sequence, a putative enzymatic center, a CRISP domain of unknown function and a transmembrane domain (11).

In the present study, the effect of GliPR1 knockdown on cellular gene expression was studied to identify downstream regulatory targets of GliPR1 and to reveal its relation to specific cellular functional categories.

## Materials and methods

*Cell culture*. For the siRNA knockdown experiments, HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany). Medium was supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% antibiotic solution (penicillin and streptomycin; all from Gibco-BRL, Karlsruhe, Germany). Transfection of the cells was carried out in the absence of any antibiotics.

*siRNA transfection*. A negative control non-silencing siRNA with no known homology to mammalian genes (si-nons-Rho: 5'-UUC UCC GAA CGU GUC ACG Udtdt-3'; 5-prime labeled with rhodamine); and two different siRNAs specific to GliPR (si-GliPR-1, 5'-GGU GAA ACC AAC AGC CAG Udtdt-3' and si-GliPR-2, 5'-GGA CUA UGA CUU CAA GAC Udtdt-3') were used. All siRNAs were synthesized by Ambion (Darmstadt, Germany) and were purchased as annealed RNA-duplexes. Twenty-four hours before transfection, HeLa cells were plated in 24-well plates (Corning, Kaiserslautern, Germany) at $5x10^4$ cells/well in Dulbecco's minimal essential medium containing 10% FBS with no antibiotics. Transfections were performed with Lipofectamine® 2000 transfection reagent (Invitrogen) with siRNA at a final concentration of 20 nM according to the manufacturer's recommendations.

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After incubating for 6 h, the lipid/siRNA complexes were removed and replaced with fresh medium. For further analysis, cells were removed from the culture dish by trypsinization with 0.25% trypsin/0.02% EDTA in PBS (Cambrex, Verviers, Belgium) at different time points after transfection. Transfection efficiency was analyzed by flow cytometry 24 h after transfection. Data were acquired and analyzed on a FACScan instrument with Cell Quest software (Becton-Dickinson, Heidelberg, Germany). Effects on cellular viability after siRNA treatment were measured using the cell proliferation reagent WST-1 (Roche, Penzberg, Germany) according to the manufacturer's instructions.

*Real-time PCR quantification*. RNA was extracted using the RNeasy Mini kit (Invitrogen) including treatment with RNase-free DNase I (Qiagen). Synthesis of cDNA was carried out using random hexamer primers and Superscript II RNaseH-reverse transcriptase (Invitrogen) according to the manufacturer's specifications.

Real-time PCR was performed in duplicate reactions employing ABI PRISM 7700 (Applied Biosystems, Darmstadt, Germany) under standard conditions (50˚C for 2 min, 95˚C for 10 min and 40 cycles at 95˚C for 15 sec and 60˚C for 1 min). The 25 $\mu$l PCR included 2.5 $\mu$l cDNA, 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.2 $\mu$M TaqMan® probe, 0.2 $\mu$M forward primer and 0.2 $\mu$M reverse primer. Primers and probes were designed using Primer Express v.1.0 software (Applied Biosystems) and were synthesized by Thermo Hybaid (Ulm, Germany). In order to quantify GliPR1 and GAPDH cDNA, the following primers and probes were used: GliPR (sense, 5'-TGC CAG ACA AAG CAT GCG T-3'; antisense, 5'-GCT GTG TGT GAA TAA TTG GAG ACA A-3'; probe, 5'-FAM-TCA CAC TTG CTA CAA TAG CCT GGA TGG TTT C-3'-TAMRA) and GAPDH (sense, 5'-GAA GGT GAA GGT CGG AGT C-3'; antisense, 5'-GAA GAT GGT GAT GGG ATT TC-3'; probe, 5'-FAM-CAA GCT TCC CGT TCT CAG CC-3'-TAMRA). The probes were labeled with FAM at the 5' end and TAMRA at the 3' end. Copy numbers of the respective transcripts were calculated by plasmid standard curves, normalized by GAPDH housekeeping gene transcripts. Standard curves were obtained after amplification of log step dilutions between 10 to $10^6$ copy numbers of purified plasmids carrying the amplicons of human GliPR1 or GAPDH (12), respectively. The plasmid standard for the quantification of GliPR1 was prepared by inserting a PCR-generated fragment (sense, 5'-GGA TCC ATG CGT GTC ACA CTT GCT ACA ATA GC-3' and antisense, 5'-GTC GAC TTA GTC CAA AAG AAC TAA ATT AGG GTA CTT GAG C-3') into pCR2.1 (Invitrogen), which was amplified using HeLa cDNA as a template.

*Gene expression analysis by microarrays*. The microarray analysis of the effect of GliPR1 suppression by siRNA was performed using the HG-U133 Plus 2.0 microarray of Affymetrix (Santa Clara, CA, USA) according to the manufacturer's instructions (GeneChip® Expression Analysis Manual). This chip contains 47,000 transcripts which represent 39,000 annotated genes. The data analysis was carried out according to established standards for Affymetric microarrays using GeneChip® Operating software (GCOS; Affymetrix) and GeneSpring (Agilent Technologies).

*Overexpression of GliPR1-EGFP fusion protein*. Since GliPR1 has an N-terminal signal peptide that is important for its subcellular localization, EGFP was fused to the C-terminus of GliPR1. The plasmid p53-EGFP (Clontech, Heidelberg, Germany) was cut by restriction enzyme digestion with *Bam*HI and *Sac*II, in order to remove the p53 gene. The resulting gap was filled with the ORF of GliPR1 without the stop codon that had been amplified from HeLa mRNA by primers containing flanking *Bam*HI and *Sac*II restriction sites. The correct cDNA sequence of the GliPR1 insert was verified by sequencing.

*Immune fluorescence microscopy*. Intracellular localization of GliPR1 was determined by confocal fluorescence microscopy. Therefore $1x10^4$ to $5x10^4$ adherently growing HeLa cells were seeded onto a slide overnight (diagnostic slide with adhesive epoxy layer; Roth, Karlsruhe, Germany) in culture dishes. Cells were gently washed in PBS (37˚C), fixed for 10 min in 3% (v/v) formaldehyde in PBS at room temperature (RT) and permeabilized for 10 min in 0.1% (v/v) Triton X-100 in PBS. Prior to incubating with the antibodies, cells were blocked with Image-iT® FX Signal Enhancer reagent (Molecular Probes, Eugene, OR, USA) for 30 min at RT and after washing were incubated with the primary antibody [diluted in 3% (w/v) BSA in PBS] for 90 min at RT. After washing in PBS, cells were incubated with the respective fluorophore-coupled secondary antibody for 90 min at RT. Then nuclei were stained with 4',6-diamidino-2-phenylindol (DAPI; Molecular Probes) in dilution of 1:300 PBS for 5 min at RT followed by the overlaying of cells with cover medium (ProLong Mounting Medium; Molecular Probes). Slides were evaluated with the Axioplan II imaging microscope and AxioVision software (Zeiss, Göttingen, Germany) and displayed by Adobe Photoshop 6.01 software (Adobe Systems GmbH, Munich, Germany). Mouse IgG2a anti-protein disulfide isomerase (PDI) antibody (Acris Antibodies GmbH, Herford, Germany) was used as the primary antibody at a dilution of 1:200, and goat anti-mouse IgG (1:500) labelled with Alexa 594 (Molecular Probes, OR, USA; Invitrogen, Darmstadt, Germany) was employed as the secondary antibody for staining of the ER.

*Analysis of GliPR1 expression on Leukemia Gene Atlas (LGA) platform*. The LGA is a public platform which provides published gene expression datasets in the field of leukemia that can be used for further analyses in regard to specific genes of interest (13). The dataset published by Haferlach *et al* (14) (n=2,096 samples) was evaluated for differential GliPR1 expression among different leukemias. The same dataset was employed to compare GliPR1 expression between acute myeloid leukemia (AML) patients with different karyotypes (translocations) as well as between acute lymphoblastic leukemia (ALL) patients with different karyotypes. The dataset published by Verhaak *et al* (15) (n=461) was utilized to evaluate GliPR1 expression between the FAB classes of AML. Both datasets were generated on the Affymetrix HG-U133 Plus 2 platform that contains five probe sets for measuring the expression of GliPR1. Analyses were based on the mean of these probe sets. The Kruskal-Wallis test was applied to test for differential expression across several groups. The Wilcoxon test was applied to test for

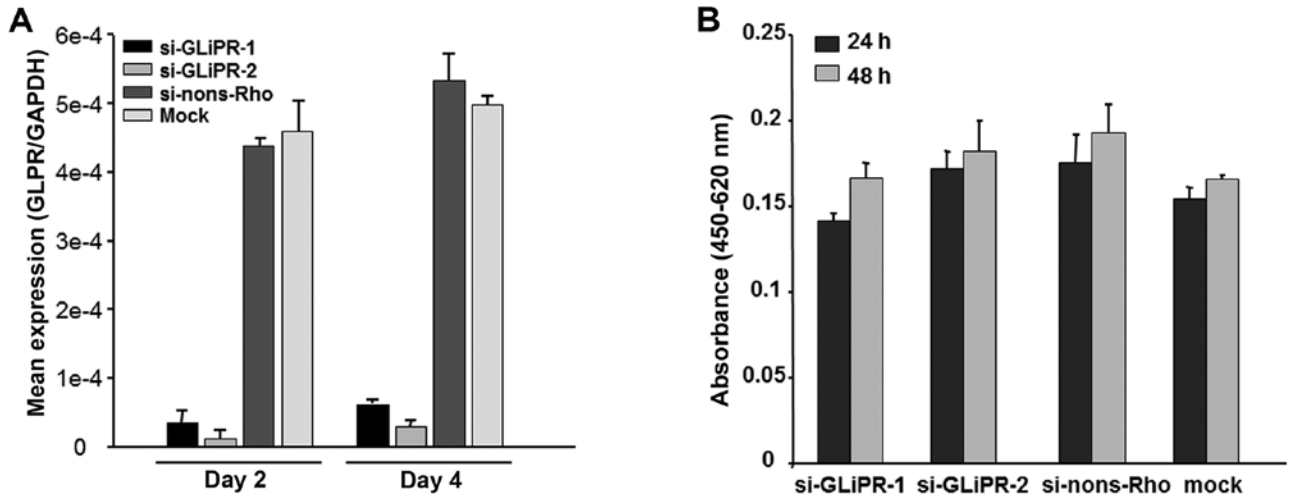


Figure 1. Suppression of glioma pathogenesis-related protein 1 (GliPR1) by siRNA and HeLa cell viability. (A) Quantitative PCR analysis of GliPR1 expression in HeLa cells transfected with 2 different siRNAs against GliPR1 or the control non-silencing siRNA labeled with rhodamine or mock-transfected. Results are presented as mean values of triplicate samples ± standard deviation of the mean (SD). (B) Cell viability was determined with the WST-1 assay 24 and 48 h after siRNA transfection. Results are expressed as absorbance ($OD_{450}$). Bars represent the mean ± standard deviation of the mean of determinations.

differences between a specific leukemia subgroup and the reference group (16).

## Results

*Suppression of GliPR1 mediated by short interfering RNA.* HeLa cells were transfected with siRNAs specific for GliPR1 or a non-silencing siRNA, which was 5-prime-labeled with rhodamine. Flow cytometric analysis of cells transfected with the non-silencing siRNA 24 h post transfection revealed transfection efficiencies on average of ~90%. Forty-eight hours after transfection, the relative levels of GliPR1 mRNA transcripts were decreased by more than 90%, compared to HeLa cells transfected with the non-silencing control siRNA as measured by quantitative PCR (Fig. 1A). Viability and the proliferation rate of HeLa cells transfected with siRNAs against GliPR1 or with the non-silencing siRNA remained unchanged as determined by the WST-1 cell proliferation assay (Fig. 1B).

In conclusion, GliPR1-directed siRNAs reduced the expression of GliPR1 effectively in HeLa cells without affecting cell viability in general.

*Differentially expressed genes after GliPR1 suppression.* In order to examine the effect of GliPR1 knockdown on cellular gene expression, a microarray analysis was performed to identify cellular target genes of GliPR1.

The microarray analysis showed a similar suppression of GliPR1 expression to 20% confirming the results of the quantitative PCR.

The knockdown of GliPR1 revealed 262 differentially expressed genes including 40 induced genes and 222 suppressed genes (≥3-fold increase or ≤0.3-fold decrease) as listed in Table I.

GliPR1-associated differentially expressed genes were grouped according to the Gene Ontology (GO) Databank categories. The GO Databank is structured hierarchically according to 3 different criteria: subcellular localization, type of biological process and molecular function. Each of the differentially regulated genes was annotated to one or more specific GO categories. Subsequently, it was evaluated, which GO categories showed an overrepresentation of the differentially expressed genes compared to a theoretical random annotation. Table II lists the relevant overrepresented GO categories with their differentially expressed genes. All of the genes in these overrepresented GO categories were found repressed except for one gene (MTM1) in the category dephosphorylation. The overrepresented GO categories included G protein signaling pathways (7 downregulated genes), regulation of cyclin-dependent protein kinase activity (4 downregulated genes), ER to Golgi vesicle-mediated transport (3 downregulated genes), axon guidance (10 downregulated genes) and dephosphorylation (1 upregulated gene; 6 downregulated genes). The factors by which these genes were downregulated or upregulated are included in Table I. The genes with a more marked downregulation by a factor ≤1/5 were APC, EGFR, DNAJC6, GNB5, APBB2 and phosphodiesterase 1A (PDE1A).

*Subcellular localization of GliPR1.* In order to ascertain a possible function of GliPR1, its subcellular localization was studied. Confocal immune fluorescence microscopy was employed to determine the subcellular localization of GliPR1. Therefore, GliPR1 was expressed as an EGFP fusion protein emitting green fluorescence (Fig. 2). The nuclei were contrasted with DAPI dye showing no nuclear localization of GliPR1. The ER was stained with a specific antibody against PDI and subsequently with a secondary antibody labeled with fluorophore Alexa-594. Exogenous expression of GliPR1 prompted apoptosis in ~25-35% of HeLa cells after 24 h according to Annexin V testing (data not shown). The confocal microscopy of cells without apoptotic morphology showed a diffuse EGFP staining in the cytosole with intensified staining around the nuclei (Fig. 2A). The PDI staining (marker of ER) in red showed a similar pattern. When the 3 fluorophores were analyzed by confocal microscopy simultaneously, the

Table I. Differentially expressed cellular genes after GliPR1 knockdown.

| Common gene | Fold-change | Common gene | Fold-change | Common gene | Fold-change |
|---|---|---|---|---|---|
| OGFRL1 | 21.99 | SEC23B | -3.08 | PEX10 | -3.49 |
| UBE2D1 | 20.93 | NASP | -3.09 | MRS2L | -3.50 |
| CGA | 13.54 | NEK7 | -3.09 | RBMS1 | -3.51 |
| PPP2R1B | 12.80 | MCFD2 | -3.10 | LRP5 | -3.52 |
| SLC7A11 | 12.41 | MGC14376 | -3.10 | PRKACB | -3.52 |
| DSC3 | 10.93 | CD59 | -3.11 | FNDC3B | -3.53 |
| TFF1 | 10.15 | ATG5 | -3.11 | PDE4B | -3.54 |
| PELI1 | 8.96 | CSGlcA-T | -3.12 | PRUNE | -3.55 |
| KIAA1199 | 8.22 | P15RS | -3.13 | MOBK1B | -3.55 |
| TPPP | 6.76 | MAST2 | -3.14 | PRKCA | -3.55 |
| VAMP4 | 6.71 | AGPAT2 | -3.17 | ACYP2 | -3.55 |
| HNRPH1 | 6.56 | EP400 | -3.17 | MAPK8IP3 | -3.55 |
| ARHGAP19 | 6.27 | EXTL2 | -3.18 | ZNF673 | -3.55 |
| KMO | 5.57 | PNPLA2 | -3.18 | CLASP2 | -3.58 |
| WBP4 | 5.26 | GNB1 | -3.18 | COL5A1 | -3.58 |
| C21orf55 | 3.96 | GALNT10 | -3.20 | MTCBP-1 | -3.58 |
| BICD2 | 3.95 | KIAA0143 | -3.22 | CACYBP | -3.59 |
| G3BP2 | 3.87 | CCND1 | -3.22 | LRP8 | -3.59 |
| GDAP1 | 3.84 | APITD1 | -3.22 | AQP3 | -3.60 |
| RABL4 | 3.78 | HIP1 | -3.22 | TNS3 | -3.60 |
| GPR107 | 3.70 | SEMA3F | -3.24 | EIF4G3 | -3.61 |
| CREM | 3.64 | MRPS30 | -3.24 | PLEKHA9 | -3.62 |
| RNF170 | 3.61 | DST | -3.25 | KIAA0143 | -3.64 |
| SMARCA5 | 3.60 | CKLFSF6 | -3.26 | LZTFL1 | -3.64 |
| PIM2 | 3.60 | EVL | -3.26 | CACYBP | -3.65 |
| SLC3A1 | 3.45 | RBMS1 | -3.26 | PITPNC1 | -3.69 |
| TPM4 | 3.41 | AQP3 | -3.28 | HPCAL1 | -3.70 |
| MLH3 | 3.41 | TAF6L | -3.28 | SEP9 | -3.71 |
| CPM | 3.38 | MBNL2 | -3.28 | C1orf22 | -3.72 |
| BMP2 | 3.36 | FER1L3 | -3.29 | HOXA9 | -3.72 |
| RCP9 | 3.28 | SYNPO | -3.30 | PTPRM | -3.74 |
| MTM1 | 3.24 | HPS5 | -3.31 | COL5A1 | -3.77 |
| MT1H | 3.24 | ATP2B4 | -3.31 | CRIM1 | -3.79 |
| PRO1853 | 3.23 | C6orf210 | -3.31 | GPM6B | -3.79 |
| FZD10 | 3.22 | SLCO3A1 | -3.31 | HPCAL1 | -3.80 |
| ZNF3 | 3.14 | PAWR | -3.31 | HTRA1 | -3.83 |
| MAP3K9 | 3.06 | KIF3A | -3.33 | ME3 | -3.84 |
| CDS2 | 3.01 | RAB26 | -3.34 | UBL3 | -3.85 |
| PMP22 | -3.02 | MAN1A2 | -3.35 | SEC23A | -3.86 |
| FLJ20280 | -3.02 | ARHGAP29 | -3.36 | HSPG2 | -3.86 |
| ANKRD15 | -3.02 | NXN | -3.37 | CCNE2 | -3.91 |
| TBC1D16 | -3.03 | SDC1 | -3.37 | SLC6A8 | -3.92 |
| ADARB1 | -3.03 | RNGTT | -3.37 | DNAJC1 | -3.94 |
| WWTR1 | -3.03 | SEC23A | -3.38 | NINJ2 | -3.95 |
| SDC2 | -3.05 | BTBD1 | -3.38 | ATXN10 | -4.02 |
| NSMAF | -3.05 | SNX10 | -3.42 | MKL2 | -4.04 |
| SFPQ | -3.05 | RCOR1 | -3.42 | SERPINI1 | -4.07 |
| C14orf78 | -3.06 | TFPI | -3.42 | PRKCA | -4.09 |
| PPP3CA | -3.06 | SEP11 | -3.45 | HEMK1 | -4.10 |
| GPR125 | -3.06 | ABHD3 | -3.47 | IGFBP6 | -4.10 |
| CAP2 | -3.06 | SAR1A | -3.47 | LMO7 | -4.12 |
| UBE2H | -3.08 | ZNF42 | -3.48 | EHD2 | -4.13 |

Table I. Continued.

| Common gene | Fold-change | Common gene | Fold-change | Common gene | Fold-change |
|---|---|---|---|---|---|
| ROBO1 | -4.13 | **GLiPR** | **-5.08** | GNB5 | -7.72 |
| DNAJB9 | -4.15 | HFE | -5.23 | CFDP1 | -7.74 |
| FLJ21687 | -4.15 | YAF2 | -5.26 | SNX24 | -7.91 |
| RANBP10 | -4.19 | COPS8 | -5.32 | MYT1L | -8.05 |
| ELP4 | -4.20 | SEP9 | -5.33 | ELOVL6 | -8.22 |
| YIPF4 | -4.22 | APC | -5.35 | MALT1 | -8.38 |
| MGC35048 | -4.24 | MAP2K1IP1 | -5.43 | MSX2 | -8.40 |
| MKRN1 | -4.26 | ELK3 | -5.60 | PDE6D | -8.85 |
| IRF2 | -4.28 | FLJ21168 | -5.67 | LOX | -8.86 |
| DHRS3 | -4.32 | SUPT7L | -5.81 | TPK1 | -8.89 |
| GMDS | -4.34 | SLC1A1 | -5.82 | ZCCHC10 | -9.13 |
| RBMS1 | -4.39 | KIAA1043 | -5.83 | MGC4707 | -9.66 |
| HSA9761 | -4.49 | CENTD1 | -5.89 | YIPF6 | -9.69 |
| LEPREL1 | -4.49 | RGS20 | -5.95 | OLFML1 | -9.69 |
| SAMD4 | -4.53 | GMDS | -6.00 | ZHX2 | -10.60 |
| NCOR1 | -4.55 | SFPQ | -6.13 | HCRT | -10.74 |
| ALPP | -4.58 | RBBP5 | -6.13 | FAM38B | -11.44 |
| CTNND1 | -4.58 | CLOCK | -6.24 | MBP | -12.35 |
| COL4A3BP | -4.60 | EGFR | -6.33 | PLEKHA5 | -12.39 |
| RAB17 | -4.60 | DNAJC6 | -6.43 | PDE1A | -12.44 |
| PTPRK | -4.61 | BF | -6.46 | MOBK1B | -12.59 |
| EPS8 | -4.64 | NCOA3 | -6.53 | SMYD3 | -12.79 |
| PEMT | -4.70 | ANKRD46 | -6.58 | APBB2 | -12.83 |
| BDH | -4.72 | PEX14 | -6.64 | UTX | -13.03 |
| ADK | -4.72 | PBX3 | -6.88 | SSX2IP | -13.28 |
| C9orf46 | -4.73 | GPHN | -6.91 | GPM6B | -15.02 |
| CSGlcA-T | -4.77 | RAB28 | -6.96 | MRS2L | -15.43 |
| SORBS2 | -4.79 | CENTG2 | -7.04 | SDC2 | -16.54 |
| LOC440669 | -4.80 | AQP3 | -7.08 | EVI5 | -17.15 |
| BTN3A3 | -4.83 | RABGAP1L | -7.09 | PDE6D | -17.54 |
| PORIMIN | -4.87 | ZNF307 | -7.19 | SDC2 | -20.23 |
| GLS | -4.90 | STS | -7.51 | APBB2 | -22.18 |
| KIAA1043 | -5.01 | SPHAR | -7.60 | | |
| SNTB2 | -5.07 | FTO | -7.62 | | |

Table I presents the list of genes, the expression of which was modified following GliPR1 knockdown in HeLa cells. The minus sign before the factor in the column 'Fold-change' indicates a decrease such that the respective expression level was the control expression level divided by the number. GliPR1, glioma pathogenesis-related protein 1.

red and green fluorescence became mostly yellow indicating a co-localization of GliPR1 with the ER. Another cell fraction revealed a vesicular pattern of green staining that was not superimposable with the red fluorescence (Fig. 2B). This pattern suggests a distribution of GliPR1 in cytoplasmic vesicles.

*Expression pattern of GliPR1 in leukemias*. We questioned whether GliPR1 expression is associated with specific leukemia types. The analysis was performed *in silico*. A dataset based on a microarray analysis of bone marrow samples of 2,096 hematological patients and controls in total (14) was employed. Bone marrow samples (n=542) of patients with AML prior to therapy revealed a significantly increased GliPR1 expression level compared to 73 subjects without leukemia and healthy bone marrow ($P<0.001$) (Fig. 3A). In contrast, 134 bone marrow samples of ALL at diagnosis prior to therapy had a significantly decreased level of GliPR1 expression compared with the control group ($P<0.001$). The subsets of patients with chronic lymphocytic leukemia (CLL)

Table II. GO categories overrepresented by the differentially expressed genes after GliPR1 knockdown.

| GO categories | Regulation | Gene ID |
|---|---|---|
| Regulation of cyclin-dependent protein kinase activity | ↓ | CCND1 (-3.2), CCNE2 (-3.9), APC (-5.4), EGFR (-6.3) |
| Dephosphorylation | ↑ | MTM1 (3.2) |
| | ↓ | PPP3CA (-3.1), RNGTT (-3.4), TNS3 (-3.6), PTPRM (-3.7), PTPRK (-4.6), DNAJC6 (-6.3) |
| ER to Golgi vesicle-mediated transport | ↓ | SEC23B (-3.1), SEC23A (-3.4), SAR1A (-3.5) |
| Axon guidance | ↓ | CAP2 (-3.1), SEMA3F (-3.2), EVL (-3.3), CLASP2 (-3.6), COL5A1 (-3.6), MAPK8IP3 (-3.6),PTPRM (-3.7), ROBO1 (-4.1), EGFR (-6.3), APBB2 (-12.8) |
| G protein signaling pathways | ↓ | PPP3CA (-3.1), GNB1(-3.2), PRKACB (-3.5), PDE4B (-3.5), PRKCA (-3.6), GNB5 (-7.7), PDE1A (-12.4) |

Table II lists the genes that are allocated in the GO categories overrepresented by the differentially expressed genes compared to random allocation. The upward arrow illustrates that this gene (MTM1) was induced in HeLa cells following knockdown of GliPR1, while the downward arrows illustrate that the other genes were suppressed. The positive numbers in parentheses indicate the magnitude (e.g. 3.2-fold) of induction. The numbers with a negative sign indicate the magnitude of suppression (e.g. the control expression divided by 3.2 equals the expression in the experimental culture). GO, Gene Ontology; GliPR1, glioma pathogenesis-related protein 1.

and chronic myeloid leukemia (CML) showed a slight decrease in GliPR1 expression (fold-changes, >0.7), although formally significantly different from the control group (CLL, P=0.008; CML, P=0.001). GliPR1 expression in the subset of patients with myelodysplastic syndrome (MDS) was not significantly different from the normal controls (P=0.136).

Furthermore, we investigated whether GliPR1 expression levels were associated with specific subgroups of AML. The dataset of Verhaak *et al* (15) revealed clear GliPR1 expression differences between different FAB types (P<0.001) (Fig. 3B). FAB types M4, M4E and M5 were characterized by high expression values compared to the other FAB groups. We employed the Haferlach *et al* (14) dataset again to study the effect of AML-specific chromosomal abnormalities on GliPR1 expression. Particularly, AML patients with translocation t(15;17) or inversion inv(16) showed an increased expression compared to the non-leukemia controls (P<0.001) (Fig. 3C), which is in line with the findings from the Verhaak *et al* (15) dataset.

Comparison of ALL samples (Fig. 3D) that had abnormal karyotypes with non-leukemia controls using the Haferlach *et al* (14) dataset revealed a significant decrease in GliPR1 expression in ALL samples with translocation t(1;19) (P<0.001) and in ALL samples with translocation t(12;21) (P<0.001). Hyperdiploid ALL samples did not show altered GliPR1 expression (14-16).

## Discussion

We characterized GliPR1 in regard to its cellular localization and effect on the cellular gene expression pattern. GliPR1 was found to be localized in the ER and in cytoplasmic vesicles. Notably, knockdown of GliPR1 resulted in downregulation of gene products related to the ER-to-Golgi vesicle-mediated transport, one of the GO categories over-represented by the allocated differentially expressed genes. The other over-represented GO categories included G protein signaling pathways, regulation of cyclin-dependent protein kinase activity, axon guidance and dephosphorylation. Of note, published microarray data from various leukemia types revealed an increased GliPR1 expression in AML and a decreased GliPR1 expression in ALL when compared to normal bone marrow samples.

GliPR1 was described to have an N-terminal signal peptide and a C-terminal transmembrane domaine (17). In conjunction with our data, it appears likely that GliPR1 is a transmembrane protein of the ER and vesicles emerging from the ER for transport to the Golgi system. The knockdown of GliPR1 resulted in downregulation of SEC23A, SEC23B and SAR1A expression, which are core components of the coat protein complex II (COPII) that is required by newly synthesized secretory proteins to exit the ER (18). It is conceivable that a knockdown of GliPR1 is associated with a downregulation of SEC23A, SEC23B and SAR1A expression, as these proteins are regulated in a coordinated manner and are functionally linked in the process of vesicle formation at the ER. Furthermore, myotubularin (MTM1) was the only upregulated gene among the overrepresented GO categories and is thus of specific interest. MTM1 has been shown to remodel the sarcoplasmic reticulum, the specialized ER of skeletal muscle cells (19). MTM1 has been reported to be involved in autophagy (vesicular trafficking pathway for protein degradation) (20). These observations may raise the question whether GliPR1 and MTM1 are involved in opposing cellular processes.

Expression of β-amyloid precursor protein-binding family B member 2 (APBB2, FE65L1) was most markedly decreased (1/12.8) following knockdown of GliPR1. APBB2 has been reported to be involved in the regulation of apop-

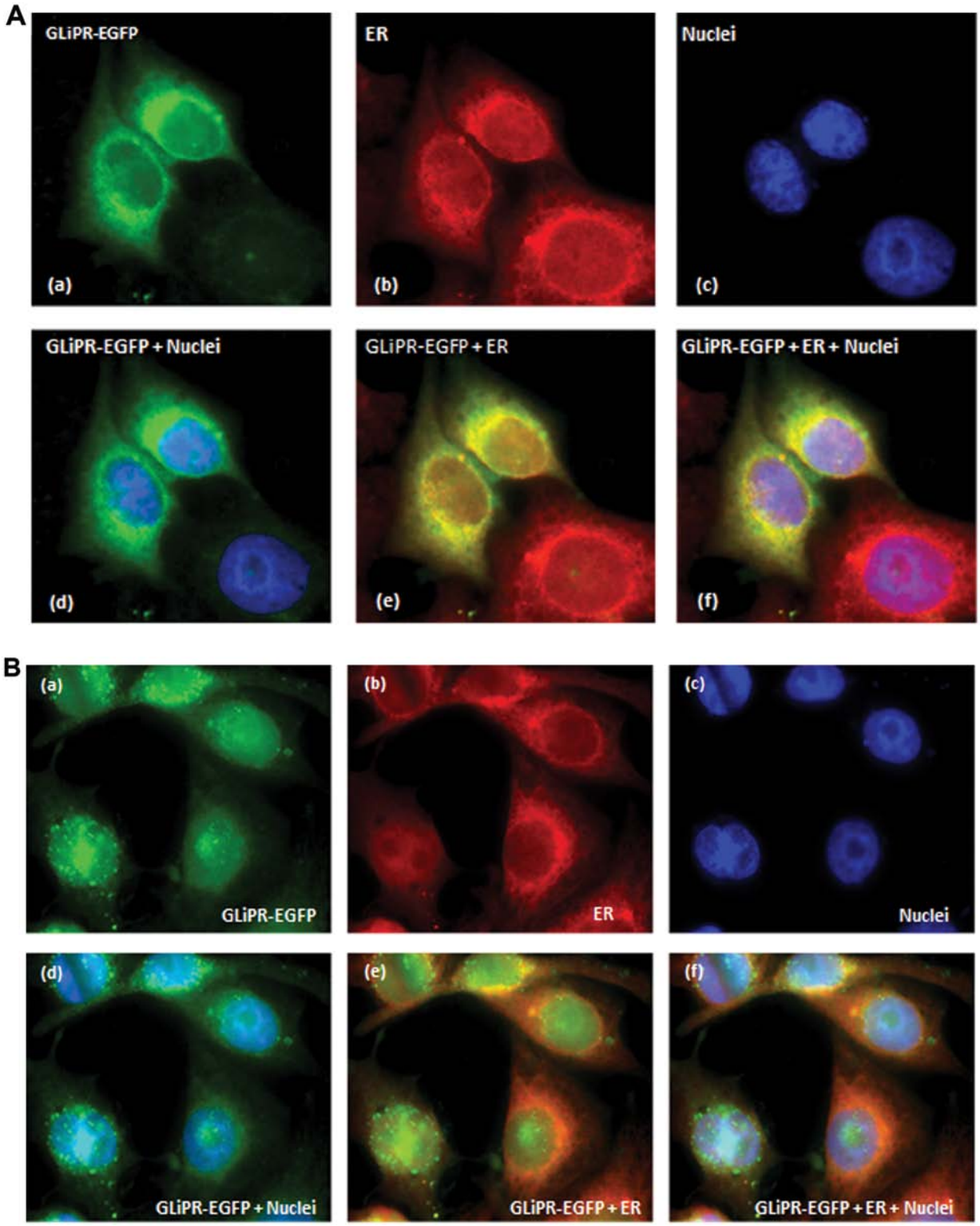


Figure 2. Intracellular localization of glioma pathogenesis-related protein 1 (GliPR1) by confocal microscopy. (a) HeLa cells transfected with expression plasmid for GliPR1-EGFP fusion protein were fixed to slides after 24 h showing GliPR1-EGFP subcellular localization by green fluorescence microscopy. (b) The endoplasmic reticulum was stained with a specific antibody against protein disulfide isomerase (PDI) and a secondary antibody labeled with the fluorophore Alexa-594 in red. (c) The nuclei were stained with DAPI in blue. Confocal microscopy tested whether there was a superimposition of (d) green and blue fluorescence, (e) green and red fluorescence or (f) all three fluorescence emitters. (A) Shows a cell subpopulation, which reveals a co-localization of GliPR1-EGFP and ER, while (B) does not show a co-localization but a vesicular cytoplasmic pattern of GliPR1-EGFP in green.

tosis (21) and differentiation of human embryonic stem cells into neuronal precursor cells (22). Furthermore, APBB2 has been associated with neurodegeneration such as in Alzheimer's disease (23). Perturbations of APBB2 splicing were found to be involved in cell cycle control and DNA damage response leading to cell transformation and tumorigenesis.

One of the most marked modifications of gene expression following knockdown of GliPR1 was the downregulation

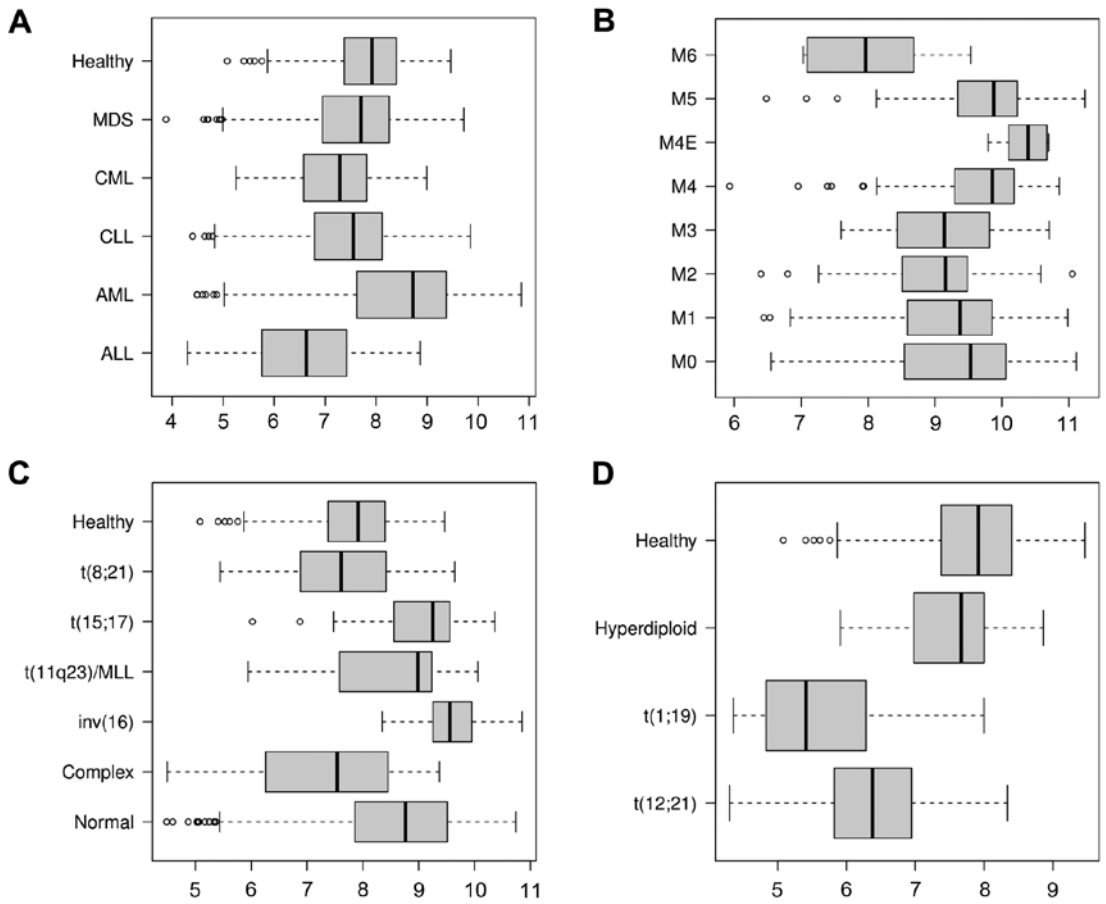


Figure 3. Glioma pathogenesis-related protein 1 (GliPR1) expression in leukemias, acute myeloid leukemia (AML) subgroups and acute lymphoblastic leukemia (ALL) subgroups. (A) GliPR1 expression was analyzed in the public Leukemia Gene Atlas platform in the dataset published by Haferlach *et al* (14) comparing ALL (n=134), AML (n=542), chronic lymphocytic leukemia (CLL; n=448), CML (n=76) and myelodysplastic syndrome (MDS; n=207) subgroups with non-leukemia bone marrow controls (n=73). (B) The dataset published by Verhaak *et al* (15) was evaluated in regard to GliPR1 expression in FAB classes M0 (n=16), M1 (n=95), M2 (n=106), M3 (n=24), M4 (n=79), M4E (n=5), M5 (n=104) and M6 (n=6). (C) The dataset published by Haferlach *et al* (14) was used to determine GliPR1 expression levels in AML subgroups with abnormal karyotypes t(8;21) (n=40), t(15;17) (n=37), t(11q23) (n=38), inv(16) or t(16;16) (n=28), complex karyotype (n=52) and normal karyotype (n=347). The healthy control group (n=73) was plotted as reference. (D) Boxplot shows the GliPR1 expression patterns of ALL patients with the abnormal karyotypes hyperdiploid (n=40), t(1;19) (n=36) and t(12;21) (n=58) compared to the healthy controls (n=73) from the Haferlach *et al* (14) dataset.

of PDE1A. It has been demonstrated that PDE1A inhibition exerts an anti-proliferative effect on melanoma cells (24). GliPR1 may be an upstream transcriptional effector of tumor proliferation in this respect.

It is intriguing that GliPR1 was shown to be upregulated and oncogenic in gliomas and glioblastomas (8), while being downregulated and a tumor suppressor in prostate cancer cells (4-7). The upregulation of GliPR1 (RTVP-1) was described to be regulated by PKC via SRF affecting glioma cell migration (25). We observed an overexpression of GliPR1 in AML bone marrow samples of newly diagnosed leukemia prior to chemotherapy. The upregulation was very specific to AML as compared to MDS, CML and CLL, which showed normal expression and as opposed to ALL, which showed downregulated expression of GliPR1. It may be hypothesized that GliPR1 exerts a pro-proliferative effect in the cell type of AML. It should be investigated whether knockdown of GliPR1 in AML cell lines is associated with inhibition of cell proliferation and induction of apoptosis. This future research direction may reveal whether GliPR1 should be characterized further as a potential target in AML. In addition, it may be of interest to elucidate the differential downregulation of GliPR1 in ALL samples.

In conclusion, GliPR1 is located in the membrane of the ER, and knockdown of GliPR1 was associated with transcriptional modifications particularly in the G protein signaling pathway and in cyclin-dependent kinases. Intriguingly, a leukemia gene expression database revealed upregulation of GliPR1 in AML and downregulation in ALL.

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