***StARD13(Dlc-2)* RhoGap Mediates Ceramide Activation of Phosphatidylglycerolphosphate Synthase and Drug Response in Chinese Hamster Ovary Cells**

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To identify genes involved in etoposide drug response, we used promoter trap mutagenesis to isolate an etoposide-resistant Chinese hamster ovary (CHO) cell line. This resistant CHO-K1 line, named E91, showed cross-resistance to C₂-ceramide (N-acetyl sphingosine). The promoter trap retrovirus was found integrated into intron 1–2 of the *Dlc-2* (*Stard13*) RhoGap gene. The E91 cells showed elevated guanosine triphosphate (GTP)-bound RhoA levels compared with the parental line, suggesting that retrovirus integration had inactivated one of the *Dlc-2* RhoGap alleles. To test whether E91 cells were impaired in an intracellular ceramide-regulated process not directly related to cell killing, we measured mitochondrial phosphatidylglycerolphosphate (PGP) synthase and phospholipase A2 enzyme activities in cells after C₂-ceramide addition. Parental cells showed elevated enzyme activities after treatment with C₂-ceramide or tumor necrosis factor α , but not the E91 cells. These results suggested that intracellular ceramide signaling was defective in E91 cells due to increased levels of active GTP-bound RhoA. RNA knockdown experiments of the *Dlc2* RhoGap resulted in increased GTP-bound RhoA and reduced induction of PGP synthase after C₂-ceramide addition compared with controls. Expression of a dominant-negative RhoA in the E91 cell line allowed induction of PGP synthase by ceramide. The RNA interference knockdown cell line also showed increased etoposide resistance. This study is the first report for the regulation of a phospholipid biosynthetic enzyme through RhoGap expression.

INTRODUCTION

Ceramides are involved in the regulation of cellular growth, differentiation, and apoptosis (for reviews, see Spiegel and Merrill, 1996; Gomez-Munoz, 1998; Mathias *et al.*, 1998). Ceramides are generated de novo or from the hydrolysis of sphingomyelin (SM) on the outer leaflet of the plasma membrane and from intracellular SM in many different cell types. Recently, the use of short chain cell-permeable ceramides (e.g., C₂-Cer) has greatly facilitated research in ceramide-induced cell signaling processes. These short chain cell-permeable ceramides rapidly enter into the cell, and they mimic the generation of intracellular ceramide. Several studies support the existence of a signal-transducing pool of SM that is distinct from the pool of SM accessible to exogenous SMase (for review, see Gomez-Munoz, 1998). Incubation

with bacterial SMase, which hydrolyzes SM on the outer leaflet of the plasma membrane, produces an extracellularly derived pool of ceramide for cell signaling. The use of cell-permeable ceramides has enabled researchers to distinguish the effect of intracellularly derived ceramides on metabolic processes from ceramide generated at the external surface of the plasma membrane. For example, in U987 cells the biological actions of ceramide were shown to depend upon its topology of production (Wiegmann *et al.*, 1994). Ceramide generated from plasma membrane-associated neutral SMase-activated pathways are independent of ceramide generated from endosomal acidic SMase. When Molt-4 leukemia cells were transfected with *Bacillus cereus* SMase, the resultant increase in SMase activity and intracellular levels of ceramide resulted in cleavage of poly(ADP-ribose) polymerase and hence apoptosis (Zhang *et al.*, 1997). In contrast, addition of exogenous *B. cereus* SMase to these cells did not induce apoptosis. Tumor necrosis factor (TNF) α binding to its receptor activates both a membrane-associated neutral SMase, and an endosomal acidic-SMase resulting in distinct subcellular topology of intracellular ceramide production (Wiegmann *et al.*, 1994).

Some chemotherapeutic drugs and radiation can increase endogenous ceramide levels in cells, and this may play a role in cell killing. Daunorubicin can increase ceramide levels through stimulation of the SMase activity in the cell (Jaffrezou *et al.*, 1996) or an increase in ceramide synthase activity (Bose *et al.*, 1995). Mouse and human cells deficient in acid SMase showed resistance to radiation induced apo-

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Abbreviations used: C₂-Cer, N-acetyl sphingosine; CHO, Chinese hamster ovary; CL, cardiolipin; CDP-DG, cytidine-5'-diphosphate-1,2-diacyl-sn-glycerol; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPA, lysophosphatic acid; LTR, long terminal repeat; PG, phosphatidylglycerol; PLA2, phospholipase A2; SM, sphingomyelin; siRNA, short interfering RNA, shRNAi, short hairpin interfering RNA.

ptosis (Santana *et al.*, 1996). The topoisomerase inhibitor etoposide can also increase de novo ceramide synthesis through the activation of serine palmitoyltransferase activity (Perry *et al.*, 2000) and sphingomyelin hydrolysis by neutral SMase induction (Sumitomo *et al.*, 2002).

Cardiolipin (CL) is an important structural and functional phospholipid in eukaryotic and prokaryotic cells (for reviews, see Hostetler, 1982; Hatch, 2004). The committed step of CL biosynthesis involves the conversion of cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) and *sn*-glycerol-3-phosphate to phosphatidylglycerolphosphate (PGP) in a reaction catalyzed by PGP synthase (EC 2.7.8.5). The PGP synthase gene has been identified in *Saccharomyces cerevisiae* (Chang *et al.*, 1998), and the enzyme has also been purified from *Schizosaccharomyces pombe* (Jiang *et al.*, 1998). A mammalian temperature-sensitive Chinese hamster ovary (CHO) cell mutant with a thermolabile PGP synthase had been isolated previously (Ohtsuka *et al.*, 1993). This mutant was defective in both phosphatidylglycerol (PG) and CL production when grown at the nonpermissive temperature. These mitochondrial abnormalities were corrected by expression of the cDNA for PGP synthase in the temperature-sensitive CHO mutant cells (Kawasaki *et al.*, 1999). CL levels can also play an important role in targeting of the cleaved proapoptotic protein Bid (tBid) to mitochondrial membranes (Lutter *et al.*, 2000).

Little information concerning the signaling processes involved in the regulation of PGP synthase in mammalian cells is currently available. Previously, we demonstrated that PGP synthase activity in H9c2 cardiac myoblast cells could be regulated by ceramide signaling (Xu *et al.*, 1999). Addition of exogenous C₂-Cer or TNF- α -mediated generation of endogenous ceramide resulted in elevation of mitochondrial PGP synthase activity. Transfection of cells with bacterial SMase or additions of cell-permeable ceramides are useful means to elevate intracellular ceramide levels. However, one problem in the study of ceramide signaling has been the lack of cell mutants in which the generated ceramide signal could be blocked or attenuated. If such ceramide-signaling mutants existed then one could probe for the effect of ceramide signaling on various ceramide-regulated cellular processes. In a study to isolate etoposide-resistant mutants by using a promoter trap retrovirus, we found one cell line that showed cross resistance to C₂-Cer. In the present study, we show that this mutant CHO cell line shows mutation of the *Stard13* (*Dlc2*) RhoGap gene due to retroviral integration and that this gene plays a role in ceramide signaling to the RhoA pathway and control of the PGP synthase activity.

MATERIALS AND METHODS

MATERIALS

[U-¹⁴C]Glycerol-3-phosphate, [5-³H]cytidine 5'-triphosphate (CTP), and [³²P]orthophosphate were obtained from GE Healthcare (Oakville, ON, Canada) or DuPont Canada (Mississauga, ON, Canada). Phosphatidyl[U-¹⁴C]glycerol was synthesized from [U-¹⁴C]glycerol-3-phosphate as described previously (Xu *et al.*, 1999). Etoposide, tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), crystal violet, C₂-Cer, and all lipid standards were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture media and reagents were products of Invitrogen (Burlington, ON, Canada). Thin-layer chromatographic plates (silica gel G) were from Fisher Scientific (Edmonton, AB, Canada). Antibodies to cleaved caspase-3 (catalog no. 9661) and poly (ADH-ribose) polymerase (PARP) (catalog no. 9548) were obtained from Cell Signaling Technology (Pickering, ON, Canada).

Selection of the Etoposide-C₂-Cer-resistant Cell Line E91

The CHO-K1 ceramide-resistant cell line E91 was isolated from a pool of etoposide resistant clones that had been previously infected with the

U3NeoSV1 promoter trap retrovirus vector (Hicks *et al.*, 1995). The CHO-K1 clone 22 cells also contain a transfected ecotopic retrovirus receptor gene (Hubbard *et al.*, 1994). Cells were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin G, 10 g/ml streptomycin, and 0.25 g/ml amphotericin. CHO-K1 cells were initially infected with helper virus-free U3NeoSV1 promoter trap retrovirus at a multiplicity of infection of 0.1. This virus vector contains the G418 resistance gene (*Neo*) in the U3 long terminal repeat (LTR) region of the virus (Hicks *et al.*, 1995). Pools of G418-resistant colonies were then replated in the presence of the topoisomerase II inhibitor etoposide at a concentration of 5 μ M dissolved in dimethyl sulfoxide (DMSO) for 2 d, and the media were then changed. Etoposide-resistant colonies were picked and replated in 10 μ M etoposide in 24-well microtiter plates. In parallel experiments starting with the same population of uninfected parental CHO-K1 cells (5×10^5) and identical plating conditions, no etoposide-resistant colonies were obtained. Etoposide-resistant colonies were tested for parallel resistance to C₂-Cer dissolved in absolute ethanol.

Cell Viability Assays

Short-term cell viability was determined by the MTT tetrazolium salt assay as described previously (Campling *et al.*, 1991). For long-term survival, a clonogenic assay using crystal violet staining and dissolution was modified as follows (Essmann *et al.*, 2004). Briefly, 1000 cells were seeded on six-well plates, and cells were treated with drug or DMSO for 48 h. Media were removed, and cells were allowed to recover with media changes every 2 d for 1 wk. Adherent cells were stained with 0.5% aqueous crystal violet and washed, and then the stain was dissolved in 0.1% acetic acid in 50% ethanol. Triplicate samples were read on SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 538 nm. SOFTmax Pro3.1.2 software (Molecular Devices) was used to compile and analyze the data.

De Novo Phospholipid Biosynthesis Studies

Cells at confluence were made quiescent by incubation with a serum-free medium for 12 h before the addition of chemical. All cell incubation procedures were performed at 37°C. Cell viability was assessed by trypan blue exclusion. Cells were incubated for 4 h with [³²P]orthophosphate (20 μ Ci/dish) in the absence or presence of 30 μ M C₂-Cer. Then, cells were harvested, and radioactivity incorporated into phospholipids was determined as described previously (Hatch, 1994; Hatch and McClarty, 1996).

Preparation of Mitochondrial Fractions and Assay of PGP Synthase Activity

Cells were cultured as described above, and all isolation procedures were performed at 4°C. Cells in 100-mm tissue culture dishes were incubated with 3 ml of medium in the absence or presence of 30 or 50 μ M C₂-Cer for 4 or 24 h. The medium was removed, and the cells were then washed twice with 5 ml of fresh medium. The cells were then harvested with 4 ml of ice-cold phosphate-buffered saline (PBS) by using a rubber policeman, and they were placed into glass tubes. These cells were centrifuged at 1000 $\times$ g for 5 min. The PBS was removed, and a 10% homogenate prepared in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.145 M NaCl, and 1 mM EDTA by using 40 strokes of a tight-fitting Dounce A homogenizer. The homogenate was centrifuged at 1000 $\times$ g (RC-5 centrifuge with SS-34 rotor; Sorvall, Newton, CT) for 10 min. The resulting supernatant was centrifuged at 10,000 $\times$ g for 15 min. The pellet was resuspended in 1 ml of stabilization buffer (50 mM Tris-maleate, pH 6.5, 10% glycerol by volume, 0.1 M KCl, 10 mM MgCl₂, and 0.5% Triton X-100 by volume) by using 15 strokes of Dounce A homogenizer. The mitochondrial fraction was used to assay PGP synthase, phospholipase A₂ (PLA₂) and CDP-DG synthetase, CL synthase, and PA:CTP cytidyltransferase activities as described previously (Hatch and McClarty, 1996; Xu *et al.*, 1999). Marker enzyme analysis indicated that the mitochondrial fraction was contaminated with ~5% microsomal particles.

Rho Activation Assay

Rho activity in CHO cell lines was analyzed using the active Rho-GTP pull-down assay kit available from Cytoskeleton (Denver, CO) (Ren *et al.*, 1999). CHO cells were grown in 60-mm dishes, and then they were serum starved for 12 h followed by addition of 25 ng/ml lysophosphatidic acid (LPA) for 30 min or 50 μ M C₂-Cer for 4 h. At the end of the treatment, cells were rinsed with ice-cold PBS, and 500 μ l of lysate buffer was added to each dish. Cells were scraped, and cell lysates were prepared according to manufacturer's protocol. After centrifugation at 10,000 $\times$ g for 10 min, supernatants were incubated with glutathione transferase (GST)-tagged rhotekin Rho binding domain (RBD) peptide immobilized on agarose, and activated GTP-Rho bound to rhotekin-agarose was detected by Western blot with anti-RhoA antibody and compared with total Rho in the extract.

Plasmid Rescue and 5' Rapid Amplification of cDNA Ends (RACE) Polymerase Chain Reaction (PCR)

To identify the flanking sequences of the U3NeoSV1 provirus from E91 cells, genomic DNA was cut with EcoRI or BstEII, ligated and transformed into

DH5⁺ cells, and selected with kanamycin and ampicillin as described previously (Hicks *et al.*, 1995). Plasmids were sequenced using primers NeoA, 5'-ATT GTC TGT TGT GCC CAG TCA-3' and NeoB, 5'-CGA ATA GCC TCT TCC ACC CAA-3'. To amplify the fused Dlc-2-Neo transcript, total RNA was extracted from E91 cells with RNeasy mini kit (QIAGEN, Mississauga, ON, Canada) and resuspended in 30 μ l of RNase-free water as described by the manufacturer. First-strand cDNA was synthesized with 2 μ l of total RNA by using Superscript RNaseH⁻reverse transcriptase (Invitrogen) followed by the Smart RACE cDNA amplification kit protocol (Clontech, Mountain View, CA). 5' RACE PCR was performed using Smart universal primer mix and Neo GSP1 primer 5'-3'-GAA TAC TTT CTC GGC AGG AGC AAG GTG A. A second nested PCR was performed using the nested Smart and NeoB primers (see above). After amplification, all PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced using the ABI Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) by using Stard13 RhoGap primer or NeoA primers. Sequencing reactions were analyzed using the ABI Prism 310 genetic analyzer (Applied Biosystems). The nucleotide sequence for *Stard13* fusion cDNA has been deposited in the GenBank database under accession no. DQ198148.

Generation of Cell Lines Defective in RhoGAP and Rho Signaling

To knockdown the Dcl2 gene activity, we used both vector-mediated short hairpin RNA interference (shRNAi) and synthetic small interfering RNAs (siRNAs). The pSUPER, pSUPER.puro, and pSUPER.neo+gfp and pSuperior.puro plasmids (OligoEngine, Seattle, WA) were used to express shRNAi against the target gene (Brummelkamp *et al.*, 2002). The nucleotide sequence of 19 base pairs specific to Dcl2 (*Stard13*) RhoGap and green fluorescence protein (GFP) genes were designed using the Ambion-siRNA finder program and Blast searched to determine specificity (see below). The *Dcl2* gene sequence was mutated at three positions to generate a negative control. The target sequences formed part of a large 64-base pair cassette when inserted in both the sense and antisense orientation within the context of a stem loop sequence structure as per OligoEngine manual design specifications. The 64-base pair oligonucleotides were synthesized in the forward and reverse orientation, annealed, and ligated into the pSuper vector backbone. The presence of the insert was determined by sequencing. The corresponding RNAi oligonucleotide sequences are as follow: *Dcl2* gene targeted sequences: A, 5'-3'-AAT TGA GGC GAA GGA AGC AT; B, 5'-3'-AAC ACA GCC AGC AGT GAG AG; *Dcl2* mutated control sequence, 5'-3'-CAC AGT*CAG CC*G TA*A GAG-3' (*, mutated nucleotide).

To inhibit Rho signaling, the dominant-negative pZIP-RhoA19N (RhoA^{S19N}) vector or empty pZIPneo vector plasmids (kindly provided by Dr. James Fiordalisi, University of North Carolina at Chapel Hill) were stably transfected into Cl22 or E91 cell lines (Fiordalisi *et al.*, 2006). For transient expression, 0, 250, or 750 ng of pZIP-RhoA19N plasmid was transfected into the E91 cell line 48 h before treatment. The pZIPneo vector was added to equalize the total amounts of DNA added per well to 750 ng. For these experiments, PGP synthase determinations were carried out in 3% FBS because serum starvation was lethal in cells expressing the dominant-negative RhoA.

Design and Synthesis of siRNA

The stealth small interfering RNAs (StealthRNAi; Invitrogen) were designed to target at the 740-base pair site of *Stard13* RhoGap mRNA, synthesized by Invitrogen. The sequences were as follows: siRNA1, sense 5'-3'-GGG CCA AGU CCU UUC UGA AAC GCA U and antisense 5'-3'-AUG CGU UUC AGA AAG GAC UUG GCC C; siRNA2 mutation control, sense 5'-3'-GGG UGA AUU CCG UCU CAA AGC CCA U and antisense 5'-3'-AUG GGC UUU GAG ACG GAA UUC ACC C. Hybridized siRNA oligonucleotides were transfected into CHO-Cl22 cells by using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's protocol. Control cells were treated with Lipofectamine without added siRNA. Total RNA was extracted between 72 and 96 hours after transfection, and the cells were collected from the six-well plates in each group by using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. A relative quantitative RT-PCR was performed to determine knockdown of expression.

Relative Quantitative and Multiplex Reverse Transcription (RT)-PCR

A cDNA equivalent of 400 ng of total RNA was quantified relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal standard, in which two sets of primers are used in the same PCR reaction: one set specific to the *Stard13* cDNA and the other set specific to the GAPDH gene. GAPDH primers used were as follows: forward, 5'-3'-TGG CAA GTT CAA AGG CAC AGR CAA GG and reverse, 5'-3'-CTT CAG CGT CCT CTG TTG GAC CAG GA. For the *Stard13* RhoGap gene (accession no. BC027830), exon 5 primers RhoGap Forward1 (5'-3'-CCA GTC TTT TCA CCC CAA GA) and Reverse1 (5'-3'-CTG CCA ATG TGC TGT GAC TT) were used. Fragment size of the internal standard GAPDH was 699 bp for and 832 bp for *Stard13*.

For multiplex RT-PCR, 400 ng of total RNA was reversed transcribed, and primers for the CHO *Stard13* exon 1 RhoGap Forward2 (5'-3'-TGC GGC TGC TAC TAT CTA AAC C) and exon 5 RhoGap Reverse2 (5'-3'-CTT TCG CTG TGA ATA GAG CAG AC) along with NeoB and GAPDH primers were used (see above). The PCR product size for *Stard13* is 575 base pairs.

Quantitative Real-Time RT-PCR Analysis

Amplification of each target cDNA was performed with QuantiTect SYBR Green PCR Master Mix (QIAGEN) by using a Real-Time PCR Detection System iQ5 cyclor (Bio-Rad, Hercules, CA) according to the protocols provided. The PCR cycling was programmed as 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles. PCR primers used were as follows: PGP synthase forward, 5'-GAC AAC AAC GTC GTC TTG AGT G-3' and reverse, 5'-GAA GTC TGC AAT CTC AGC ACA G-3'; and for GAPDH, forward, 5'-CGA AGG TGG AAG AGT GGG AG-3' and reverse, 5'-TGA AGC AGG CAT CTG AGG G-3'. Relative gene expression was determined using the $2^{-\Delta\Delta C_T}$ method normalized to the GAPDH levels (Livak and Schmittgen, 2001). Real-time PCR data quality control, $2^{-\Delta\Delta C_T}$ and analysis of covariance calculations were run on the SAS program as described (Yuan *et al.*, 2006).

Other Determinations

The protein concentration of lysates was determined with the bicinchoninic acid protein assay (Sigma-Aldrich) or by the method of Lowry (Lowry *et al.*, 1951). Western blotting was done by standard techniques (Harlow and Lane, 1988). Statistical methods used included the Student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for comparison of means and the linear regression curve fitting tool from the Origin program (OriginLab, Northampton, MA). Survival curves were compared by the logrank test by using the LIFETEST procedure in SAS software (SAS Institute, Cary, NC).

RESULTS

Etoposide-resistant E91 Cell Line Shows Cross-Resistance to C₂-Ceramide

In a study to identify genes that may be involved in etoposide drug response, we used the U3NeoSV1 promoter trap retrovirus as mutagen and genetic tag. Virus-infected cells were selected for resistance to etoposide (see *Materials and Methods*). One etoposide-resistant clone we isolated, named E91, also showed cross-resistance to C₂-ceramide compared with parental CHO-K1 Cl22 and Neo610 cell lines (Figure 1, A and B). The Neo610 cells are U3NeoSV1-infected cells that were not selected for etoposide resistance. This line showed a slight resistance compared with the parental CHO-K1 Cl22 cells but less than the E91 line. This indicates that expression of the Neo resistance gene did not have a major effect on etoposide response. In contrast, the E91 line showed reduced killing with etoposide up to a 300 μ M concentration compared with CHO-K1 Cl22 and Neo610 cells in MTT cell growth assays. CHO-K1, Neo610, and E91 cells were also tested for parallel resistance to C₂-Cer. Cells were incubated for 48 h with various concentrations of C₂-Cer, and the cell viability was determined by MTT assay (Figure 1B). Approximately 60–70% of CHO-K1 and Neo610 cells incubated with 10 μ M C₂-Cer for 48 h underwent cell death. In contrast, only 10% of E91 cells incubated with 10 μ M C₂-Cer for 48 h underwent cell death.

To determine whether this difference in cell survival was reflected in changes in apoptosis induction, we looked at cleavage of caspase-3 and PARP after various times and doses of etoposide (Figure 2, A and B). After 24 h of drug treatment, the E91 cells showed slightly higher levels of caspase-3 compared with the Cl22 cell line. However, after 48 h of treatment, the E91 cells showed a slight reduction in the level of cleaved caspase-3 compared with the Cl22 cells. Both cell lines showed similar levels of PARP cleavage at all time points. These results suggest that the survival difference between E91 and Cl22 cells is not due to major changes in the level of apoptosis induction.

Figure 1. Etoposide and C_2 -ceramide survival curves in CHO-K1 clones. (A) Etoposide sensitivity was determined by the MTT assay 48 h after drug addition. Data points are the mean of five determinations $\pm$ SEM. (B) C_2 -ceramide survival was determined 48 h after lipid addition by the MTT assay as outlined in *Materials and Methods*. Data points are the mean of three experiments $\pm$ SD.

E91 Cells Are Defective in Ceramide Signaling

The above-mentioned studies suggested that ceramide signaling, as related to cell death, was impaired in E91 cells. To determine whether intracellular ceramide signaling was impaired in E91 cells, we examined a ceramide-regulated process not directly related to cell death. Previously, we had shown that addition of exogenous C_2 -Cer or $\text{TNF-}\alpha$ to H9c2 cells resulted in elevated CL de novo biosynthesis via activation of PGP synthase and PLA2 activities (Xu *et al.*, 1999). Therefore, we examined PGP synthase and PLA2 activities in CHO-K1 Cl22, Neo610, and E91 cells after incubation with exogenous C_2 -Cer. PGP synthase activities were elevated 57 and 46% in Cl22 CHO-K1 and Neo610 cells, respectively, after C_2 -Cer treatment (Table 1). In contrast, C_2 -Cer treatment of E91 cells did not elevate PGP synthase activity above the constitutive level. PLA2 activities were elevated 58% in CHO-K1 and 44% in Neo610 cells when treated with C_2 -Cer (Table 2). In contrast, C_2 -Cer treatment of E91 cells did not elevate PLA2 activity. Thus, ceramide signaling was impaired in the E91 cell line.

Figure 2. Apoptosis induction in Cl22 and E91 cells lines after etoposide treatment. Cells were treated with various doses of etoposide for 24 and 48 h. Cell lysates were run on SDS-polyacrylamide gel electrophoresis and immunoblotted using antibodies specific for cleaved caspase-3 (A) or cleaved PARP (B) and β -actin. The arrow indicates a nonspecific cross-reacting protein.

Table 1. Effect of C_2 -Cer on PGP synthase activity in CHO-K1, Neo610, and E91 cells

Treatment	Enzyme activity (pmol/min/mg)		
	CHO K1	Neo610	E91
No addition	191 $\pm$ 19	183 $\pm$ 22	192 $\pm$ 15
+ C_2 -Cer	299 $\pm$ 45*	268 $\pm$ 34*	197 $\pm$ 17

* $p < 0.05$.

CHO-K1 Cl22, Neo610, and E91 cells were incubated with 30 μM C_2 -Cer for 4 h. The mitochondrial fraction was then isolated, and PGP synthase activity was determined as described in *Materials and Methods*. Results represent the mean of three experiments $\pm$ SD.

Table 2. Effect of C_2 -Cer on PLA2 activity in CHO-K1, Neo610, and E91 cells

Treatment	Enzyme activity (pmol/min/mg)		
	CHO K1	Neo610	E91
No addition	100 $\pm$ 15	97 $\pm$ 12	117 $\pm$ 20
+ C_2 -Cer	158 $\pm$ 17*	139 $\pm$ 19*	122 $\pm$ 11

* $p < 0.05$.

CHO-K1, Neo610, and E91 cells were incubated with 30 μM C_2 -Cer for 4 h. The mitochondrial fraction was then isolated, and PLA2 activity was determined as described in *Materials and Methods*. Results represent the mean of three experiments $\pm$ SD.

We next examined PGP synthase and PLA2 activities in CHO-K1, Neo610, and E91 cells incubated with exogenous $\text{TNF-}\alpha$. PGP synthase activities were elevated 64% in CHO-K1 and 53% in Neo610 cells treated with $\text{TNF-}\alpha$ for 1 h, but they returned to baseline levels by 4 h (Figure 3A). $\text{TNF-}\alpha$ treatment of E91 cells did not affect PGP synthase activity at either time examined. PLA2 activities were elevated by 61% in CHO-K1 and 59% in Neo610 cells treated

Figure 3. Effect of TNF- α on PGP synthase and PLA2 activities in CHO-K1, Neo610, and E91 cells. Cells were incubated with 30 nM TNF- α for 1 or 4 h. The mitochondrial fraction was then isolated, and PGP synthase (A) and PLA2 (B) activities determined as described in *Materials and Methods*. Open bars, no addition; solid black bars, 1 h incubation with TNF- α ; and gray bars, 4-h incubation with TNF- α . Results represent the mean of four experiments $\pm$ SD, * $p < 0.05$. (C) Cardiolipin synthesis in ceramide-treated cells. Cells were incubated with 20 μ Ci of [32 P]orthophosphate in the absence or presence of C₂-Cer, and radioactivity was incorporated into cardiolipin was determined as described previously (Hatch, 1994). The closed bars are controls and the open bars are cells treated for 4 h with 30 μ M C₂-Cer. The results are the mean of four determinations $\pm$ SD (* $p < 0.05$).

with TNF- α for 1 h but not after 4 h (Figure 3B). PLA2 levels were also not affected at either time examined in E91 cells after TNF- α treatment. The elevation in PGP synthase and PLA2 activities at 1 h of TNF- α treatment corresponds with the rapid generation of intracellular ceramide levels due to the activation of intracellular sphingomyelinases followed by a temporal attenuation (Kim *et al.*, 1991; Xu *et al.*, 1999).

These changes in PGP synthase enzyme activity result in a significant elevation in cardiolipin synthesis after ceramide treatment of Cl22 cells (Figure 3C). In contrast, the E91 cells showed no increase in cardiolipin synthesis above control levels after C₂-ceramide treatment.

As a control for enzymes in the CL biosynthetic pathway not affected by intracellular ceramide signaling (Xu *et al.*, 1999), we examined CDP-DG synthetase and CL synthase activities in CHO-K1, Neo610, and E91 cells. CDP-DG synthetase activities were 25 ± 4 pmol/min/mg protein and CL

synthase activities were 2.9 ± 0.5 pmol/min/mg protein in CHO-K1 cells, and they were similar in the Neo610 and E91 cells. We previously demonstrated that addition of exogenous bacterial SMase to H9c2 cardiac cells, which would mobilize ceramide from the cell surface SM, did not affect PGP synthase or PLA2 activities (Xu *et al.*, 1999). In agreement with our previous study, treatment of cells with exogenous bacterial SMase did not affect these enzyme activities in CHO-K1, Neo610, or CHO-E91 cells (data not shown). These data indicate that the elevation in PGP synthase and PLA2 activities in CHO-K1 and Neo610 cells were mediated by intracellular generated ceramide. The above-mentioned data suggest that ceramide insensitivity in E91 cells may be due to a defect in intracellular ceramide signaling.

E91 Cells Show a Defect in RhoGap Activity

We next determined where the promoter trap retrovirus was integrated in E91 cells and whether this contributed to the ceramide signaling defect. The Neo resistance gene located in the U3 LTR region on this virus can be activated by integration proximal to a promoter region or by splicing of the mRNA into the cryptic 3' splice site in the Neo gene when integrated into an intron (Osipovich *et al.*, 2004). Initially, we determined the flanking sequences of the U3NeoSV1 provirus through plasmid rescue as outlined in *Materials and Methods*. Sequencing of the flanking sequences revealed that the virus had integrated into intron 1–2 of the CHO *Stard13* gene in E91 cells near the exon 1 (data not shown). This sequence is the orthologue of the mouse *Stard13* RhoGap gene found on mouse chromosome 5 (GenBank accession no. BC027830). Next, we determined how the Neo gene in the provirus was activated using 5' RACE. The sequences of this cDNA and predicted protein product are shown in Figure 4A. This sequence shows a fusion transcript of exon 1 of the *Stard13* gene spliced into the cryptic 3' splice site of the Neo gene. This fusion is similar to what has been described previously for activation of the Neo gene in the U3NeoSV1 retrovirus when integrated into introns of other genes (Osipovich *et al.*, 2004).

To determine the expression of the other *Stard13* allele in E91 cells, multiplex RT-PCR was carried out using primers that would recognize the normal and fusion transcripts along with GAPDH primers (Figure 4B). This gel shows RT-PCR products of 575 and 699 base pairs from CHO-K1 Cl22 RNA that are expected sizes of the normal *Stard13* and GAPDH transcripts, respectively. In contrast, the E91 cells showed three products with a reduced level of the normal *Stard13* PCR product and a 170-base pair fragment, which is the expected size of the fusion transcript of exon 1 with the Neo gene. These results indicate that the normal *Stard13* transcript is found at a reduced level in E91 cells compared with CHO-K1 Cl22 cells.

To determine whether E91 cells show reduced RhoGap activity, we measured the level of active GTP bound Rho protein by using the GST-RBD rhotekin pull-down assay (Ren *et al.*, 1999) (Figure 5, A–C). These results show higher levels of the active GTP bound RhoA in the E91 cells compared with the parental CHO-K1 Cl22 cells after both LPA and C₂-ceramide treatments (Figure 5, A–C). This is consistent with there being reduced RhoGap activity in E91 cells. The parental CHO-K1 Cl22 cells showed only a slight increase or a reduction in active RhoA after treatment, consistent with higher RhoGap activity.

RhoGAP Mediates Ceramide Activation of PGP Synthase

To directly test whether reduced expression of *Stard13* is involved in induction of PGP synthase, we transfected

Figure 4. Fusion of exon 1 of *Stard13* into the cryptic *Neo* 3' Splice Site in the E91 cell line. (A) cDNA sequence of the fusion transcript. The cDNA sequence was generated by 5' RACE and sequenced using NeoA- and *Stard13*-specific primers. Amino acid single-letter codes are shown for the *Stard13* exon 1 sequence fused to the *Neo* coding sequence. The arrows indicate the location of the spliced transcript that uses the *Neo* 3' SS and the *Stard13* exon 1 and Neo boundaries. (B) Multiplex RT-PCR of RNA from CHO-K1 CI22 and E91 cell lines. RT-PCR was performed using primers for *Stard13* exon 1–5, *Neo* gene, and *GAPDH* in the same reaction. MW, molecular weight marker.

CHO-K1 CI22 cells with plasmids expressing shRNAi to knock down *Stard13* expression. The level of RNA knockdown was determined by RT-PCR and compared with

Figure 5. Increased Rho-GTP in *Stard13* shRNAi-expressing cells. Cell lines were serum starved for 12 h, and C₂-ceramide was added for 4 h (A) or LPA was added for 30 min (B). Rho-GTP levels were measured using pull-down assays (see *Materials and Methods* for details). Blots were probed with monoclonal antibody for RhoA. Representative immunoblots are shown with RBD-GST bound Rho (top) and total Rho from a portion of the same extract (bottom). (C) The graph shows the average ratio of GTP-bound Rho to the total amount of Rho as determined in the pull-down assays. Results are the average three experiments $\pm$ SD (* p < 0.05 vs. untreated control).

GAPDH as an internal control (Figure 6). These results show good RNA knockdown for cell lines RSC5 and -6 (Figure 6A, lanes 4–6). The RSC1 line showed a faint *Stard13* band. Under these PCR conditions, E91 cells showed very low expression of the normal *Stard13* allele. There was also evidence of some off target effects on expression because the control cell lines expressing both the mutated RhoGap (RhoGapMT; Figure 6A, lane 7) or shRNAi to GFP (both specific and mutant shRNAi; Figure 6B, lanes 3 and 4) showed a slight reduction in both *Stard13* and *GAPDH* levels compared with CI22 or CI22 cells expressing GFP alone. These lines were used for further study.

The active Rho levels in the *Stard13*, shRNAi knockdown cell lines were measured by the Rho-GTP pull-down assay (Figure 5, A–C). The RSC5 and -6 lines expressing *Stard13*-specific shRNAi showed significant increases of GTP bound

Figure 6. Multiplex RT-PCR of cell lines expressing shRNAi plasmids. (A) Multiplex RT-PCR was carried out using primers for *Stard13* and *GAPDH* cDNAs as outlined in *Materials and Methods*. Lane 1, molecular weight marker; lane 2, CHO-K1 CI22 cell line; lane 3, E91 cell line; lane 4, RSC1 cell line; lane 5, RSC5 cell line; lane 6, RSC6 cell line; and lane 7, RhoGapMT cell line. Cell lines RSC1 and -6 contain the pSuper plasmid expressing *Stard13* shRNAi. Cell line RSC5 contains the pSuper.puro plasmid expressing *Stard13* shRNAi, which is tetracycline inducible and pcDNA6/TR containing the Tet repressor. This line was grown in the presence of tetracycline. Cell line RSC6 contains the pSuper.neo + GFP plasmid expressing *Stard13* shRNAi. RhoGapMt contains pSuper expressing a mutated *Stard13* shRNAi. (B) RT-PCR of control cell lines: lane 1, marker; lane 2, CHO-GFP contains pEGFP plasmid; lane 3, CHO-GFP RNAi contains pEGFP + pSuper expressing GFP shRNAi; and lane 4, CHO-GFPMt contains pGFP + pSuper plasmid expressing a mutated GFP shRNAi.

Figure 7. (A) PGP synthase activity in CHO-K1 lines expressing shRNAi plasmids after C₂-ceramide addition. CHO-K1, CI22, E91, RSC1, RSC5, RSC6, and RhoGapMt cell lines were incubated in the absence (solid bars) or presence (open bars) of 50 μ M C₂-Ceramide for 24 h, and PGP synthase activity was then determined as described in *Materials and Methods*. Data represents the mean of three experiments $\pm$ SD, * p < 0.01 versus medium-treated control for each line as determined by Student's t test. (B) Relative-fold induction of the PGP synthase mRNA expression in CHO-K1 CI22 lines expressing shRNAi plasmids after C₂-ceramide addition. Cells were incubated in the absence or presence of C₂-Ceramide for 24 h, and RNA was isolated. Real-time RT-PCR was carried out using primers to PGP synthase and GAPDH mRNA as described in *Materials and Methods*. Relative-fold induction was calculated using the $\Delta\Delta C_T$ method. The plotted $2^{-\Delta\Delta C_T}$ results are based on the mean of three replicates at three RNA concentrations $\pm$ SD (* p < 0.0001 and ** p < 0.001, as determined by ANOVA compared with the untreated control). (C) Correlation of PGP synthase RNA induction versus the active GTP-bound Rho after ceramide induction. The active Rho

RhoA compared with nontreated cells, which was comparable with the E91 cells after treatment with LPA or C₂-ceramide. The RSC1 line showed increased active Rho compared with nontreated cells, but this did not reach significance. The control cell line expressing a mutated Stard13 shRNAi (RhoGapMT) showed reduced levels of active Rho activity comparable with the parental CHO-K1 CI22 cells after addition of LPA or C₂-ceramide.

These shRNAi-expressing cell lines were then tested for induction of the PGP synthase enzyme activity after C₂-Cer treatment (Figure 7A). CHO-K1 CI22 and shRNAi control line RhoGapMT showed significant increases in PGP synthase activity after C₂-Cer addition (33.2 and 32%, respectively). The RSC1 and RSC5 shRNAi-expressing lines showed smaller nonsignificant increases of PGP synthase activity after C₂-Cer treatment (10 and 13%, respectively) compared with CHO-K1 CI22 cells. In contrast, the E91 and RSC6 lines showed a reduction of PGP synthase activity after ceramide treatment compared with untreated cells (−16 and −26%, respectively), with the RSC6 cells showing a significant reduction.

These cells were then tested for changes in PGP synthase mRNA expression after C₂-Cer treatment by using real-time RT-PCR (Figure 7B). The CHO-K1 CI22 and RhoGapMT lines showed significant 2.5- and 3.2-fold induction of PGP synthase mRNA, respectively, after C₂-Cer treatment. E91 cells exhibited only a 1.2-fold induction of PGP synthase mRNA expression after ceramide treatment. The *Stard13* knockdown cell lines RSC1 and RSC5 showed significant induction at 2.0- and 1.7-fold; however, this was less than that seen in the CI22 and RhoGapMT cells. The induction of PGP synthase mRNA in the line RSC6 lines showed a nonsignificant 1.3-fold induction of PGP synthase mRNA after C₂-ceramide addition, similar to the E91 line. Because there was variation in knockdown of *Stard13* as reflected in the levels of active GTP-RhoA (Figure 5C), we wanted to determine whether PGP synthase RNA induction was correlated with the level of active GTP bound Rho in cells after C₂-ceramide treatment. This analysis (Figure 7C) shows that there is a linear relationship between active GTP-bound Rho and PGP synthase mRNA induction after ceramide treatment. Thus, the higher the GTP-Rho levels in the cell the lower the PGP synthase mRNA levels, i.e., a smaller $\Delta\Delta C_T$ value. We have also confirmed these results by using siRNAs targeted to *Stard13* mRNA with a mutated *Stard13* control transfected into CI22 cells (Supplemental Figure 1).

The previous results suggest that reduced *Stard13* RhoGap activity in cells results in increased active RhoA, which in turn interferes with ceramide induction of PGP synthase. To further test the role of Rho, CI22 and E91 cell lines stably expressing a dominant-negative RhoA (S19N) or vector control (pZIPneo) were isolated, and PGP synthase activity after C₂-ceramide induction was determined (Figure 8). In these experiments, 3% fetal calf serum was included in the medium because serum starvation of the RhoA(S19N)-expressing cells was lethal. The results in Figure 8A show that CI22 cells with stable expression of dominant-negative RhoA increased the induction level of PGP synthase activity after C₂-ceramide compared with the empty vector control cells (135 vs. 76%). E91 cells expressing RhoA19N also showed a significant 29% increase in PGP synthase activity

ratio data were taken from Figure 5 and plotted against the $\Delta\Delta C_T$ values. The line and 95% confidence bands was determined by linear regression analysis ($R = 0.93795$ and $p = 0.00566$).

Figure 8. (A) PGP synthase activity in CHO-K1 cell lines stably transfected with dominant negative pZIP-RhoA19N (RhoAS19N) or empty vector control (pZIPneo). PGP synthase activity was measured as described in *Materials and Methods*. The results are the mean of three determinations $\pm$ SD, * $p < 0.01$ versus medium control. (B) PGP synthase activity in E91 cell line transiently transfected with increasing concentrations of pZIP-RhoA19N (RhoAS19N). The pZIPneo vector was added to equalize the total amounts of DNA added per well to 750 ng. PGP synthase activity was determined at various times after C₂-ceramide addition as indicated. The results are the mean of nine determinations $\pm$ SEM (* $p < 0.01$ vs. control 0 h, 750 ng; ** $p < 0.01$ vs. control 0 h, 250 ng, as determined by one-way ANOVA).

after ceramide addition in contrast to the vector control cells that showed a slight reduction (–5%).

The constitutive level of PGP synthase in the RhoA(S19N)-expressing E91 cells was significantly reduced compared with the vector only controls cells. Therefore, we wanted to confirm these results in E91 cells transiently expressing the dominant-negative RhoA(S19N) plasmid (Figure 8B). These results show that the constitutive levels of PGP synthase were slightly reduced compared with the vector control cells, but this did not reach significance. C₂-treated cells transfected with 750 ng of RhoA(S19N) showed a significant 24% increase in PGP synthase activity after 24 h compared with the transfected but nonceramide-treated cells. This is in contrast to the vector-only transfected cells that showed a nonsignificant reduction (–3.2%) in PGP synthase activity after ceramide treatment compared with untreated cells. These results demonstrate that interference with Rho signaling in E91 cells could reverse the inhibition of PGP synthase induction after ceramide treatment.

Figure 9. Clonogenic survival assay of CI22, E91, and shRNAi-expressing cell lines treated with etoposide. Cell survival was measured by crystal violet staining and dissolution assay as outlined in *Materials and Methods*. The results are the mean of five experiments $\pm$ SD.

Knockdown of *Stard13* RhoGap Alters Drug Response

Long-term clonogenic assays after etoposide treatment of the *Stard13* shRNAi-expressing cells are shown in Figure 9. E91 cells showed a significant increase in survival compared with the parental CI22 cells ($p < 0.0001$, by log rank test). All the shRNAi-expressing cell lines, including the RhoGapMT control line, showed significant resistance compared with the CI22 cells ($p < 0.0001$), indicating some nonspecific effects of RNAi expression. However, the RSC1 line did not show a significant difference from the RhoGapMT control line ($p = 0.7109$). In contrast, the RSC6 line showed a significant resistance compared with the RhoGapMT control line ($p < 0.0001$). The level of resistance in the RSC6 line was comparable with the E91 cell line. As shown previously, the E91 and RSC6 lines showed the highest levels of the active GTP form of RhoA (Figure 5C).

We were not able to unequivocally demonstrate a role for *Stard13* knockdown in ceramide resistance due to off-target effects of shRNAi expression in these lines. In these experiments, the control shRNAi-expressing cells lines showed significant sensitivity to ceramide in the MTT assays compared with the parental CI22 cells (Supplemental Figure 2).

DISCUSSION

This study is the first report for the regulation of a phospholipid biosynthetic enzyme through RhoGap expression. The results of this study support the concept that ceramide may alter RhoA activity via interaction with the *Stard13* RhoGap. Elevated GTP-bound RhoA levels found in cells with a mutated *Stard13* gene or knock down of its mRNA can dampen ceramide's induction of PGP synthase gene expression. Also, cells with elevated active RhoA show partial resistance to the drug etoposide.

Stard13 is unique as a RhoGap in that it also contains a steroidogenic acute regulatory protein (StAR)-related lipid transfer domain (START) and a SAM2-interacting domain (Soccio and Breslow, 2003). The prototypical START-containing protein is the StAR, which functions to transport cholesterol from the outer to inner mitochondria membranes

in steroidogenic cells (Stocco, 2001). Whether the START domain of *Stard13* directly interacts with ceramide awaits future experiments. However, there is a precedent for ceramide interacting with a Start lipid domain. The CERT (*Stard11*) protein, which contains both a START lipid transfer and a phosphatidylinositol-4-monophosphate binding domains, functions to shuttle ceramide from the endoplasmic reticulum to the Golgi (Hanada *et al.*, 2003). Also of relevance is the recent finding that the START domain of *Dlc-2* plays a role in its localization to mitochondria and proximity to lipid droplets found in the cytoplasm (Ng *et al.*, 2006).

Three RhoGap proteins with START domains have been described in mammals. These are *Dlc-1* (also named p122RhoGap, Arhgap7), *Stard13* (*Dlc-2*), and *StarD8* (KIAA0189 RhoGAP) (Soccio and Breslow, 2003). The *Stard13* (*Dlc-2*) gene is frequently found deleted in hepatocellular and breast carcinoma cells (Ching *et al.*, 2003; Nagaraja and Kandpal, 2004), and along with the closely related *Dlc-1* is a tumor suppressor gene (Yuan *et al.*, 1998; Ng *et al.*, 2000).

The experiments presented here suggest that the START lipid binding domain of the *Stard13* may be interacting with endogenous ceramide to stimulate its RhoGap activity and then down-regulate RhoA activity. This in turn would alter RhoA's downstream effectors and result in a change in CL metabolism through alteration of the PGP synthase and PLA2 activities. E91 and *Stard13* RNAi knockdown cells showed reduced ceramide activation of these enzymes, suggesting that active RhoA inhibits this process partially through control of transcription of the PGP synthase gene. Because the magnitude of PGP synthase activity did not fully reflect the mRNA induction levels, this would suggest that there is a posttranslational control of PGP synthase as well. Posttranslational control of PGP synthase by inositol has been described in yeast, and this negative regulation is mediated through changes in phosphorylation of the protein (He and Greenberg, 2004). Our results predict that increased ceramide levels would result in lower RhoA activity in normal cells through stimulation of RhoGap activity, and this may be mediated through ceramide binding to the START lipid domain. With these mutant cells, we will be testing this hypothesis and try to understand which downstream Rho effectors are involved in this control.

The E91 cells with a retrovirus integrated into one allele of the *Stard13* gene showed reduced expression of the other allele and increased levels active GTP-bound RhoA after ceramide and LPA treatment. This may be due to the fact that CHO cells are functionally hemizygous for many loci due to either increased promoter methylation or chromosomal rearrangements (for reviews, see Siminovitch, 1976; Holliday, 1991). This was the reason why we chose CHO cells for the promoter trap experiments because only one allele would need to be inactivated for many genes.

Our results showed an association with etoposide resistance in cells with knockdown or mutation of *Dlc2*. The RSC6 line showed resistance at levels comparable with the E91 cells, and it also showed the high levels of active RhoA, whereas the RSC1 line that showed only slight resistance and lower levels of active RhoA. This may indicate that there is a critical threshold of active RhoA in cells that is needed to manifest etoposide resistance.

The results testing ceramide sensitivity were ambiguous. Although the RNAi knockdown of *Stard13* mRNA resulted in increased ceramide resistance compared with shRNAi controls, this did not reach significance except for one cell line. The shRNAi control cells showed increased sensitivity to ceramide compared with the parental Cl22 cells. This may

be due to possible off target effects of RNAi such as the production of interferon (Bridge *et al.*, 2003). These off target effects of RNAi did not seem to affect the ceramide signaling to PGP synthase to the same extent. These results also emphasize the importance of appropriate controls for RNAi experiments especially with respect to drug response. We will have to wait the generation of knockout mice to better test the role of *Stard13* in ceramide sensitivity.

Others have found association of increased Rho activity and drug resistance. One group found that dominant-negative RhoA expression could interfere with resistance to etoposide induced apoptosis caused by CD44 overexpression (Fujita *et al.*, 2002). Another study found that HMG-CoA reductase inhibitors simvastatin and lovastatin could reverse the cell adhesion-mediated drug resistance to drugs such as malphalan, treosulfan, and doxorubicin in human multiple myeloma cells (Schmidmaier *et al.*, 2004). This reversal of resistance by statins seemed to be due to interference with Rho protein geranylgeranylation (Schmidmaier *et al.*, 2004). Elevated expression of the Lymphoid blast crisis protooncogene, a Rho guanine-nucleotide exchange factor, in hepatocarcinoma cells results in resistance to doxorubicin (Sterpetti *et al.*, 2006). Our results suggest that tumors with mutation of the *Stard13* (*Dlc-2*) gene may be intrinsically resistant to some drugs.

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