Disruption of Sphingosine 1-Phosphate Lyase Confers Resistance to Chemotherapy and Promotes Oncogenesis through Bcl-2/Bcl-xL Upregulation

Sandra Colié,1,2 Paul P. Van Veldhoven,4 Blandine Kedjouar,1,2 Carmen Bedia,1,2 Virginie Albinit,1,2 Sonia-Caroline Sorlí,1,2 Virginie Garcia,1,2 Mojgan Djavaheri-Mergny,5 Chantal Bauvy,1 Patrice Codogno,5 Thierry Levade,1,2,3 and Nathalie Andrieu-Abadie1,2

1INSERM U858; 2Université Paul Sabatier Toulouse III, Institut de Médecine Moléculaire de Rangueil; 3Laboratoire de Biochimie Métabolique, CHU Toulouse, Toulouse, France; 4LIPIT, Katholieke Universiteit Leuven, Leuven, Belgium; and 5INSERM U756, Chateauray-Malabry, France

Abstract
Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite involved in cancer development through stimulation of cell survival, proliferation, migration, and angiogenesis. Irreversible degradation of S1P is catalyzed by S1P lyase (SPL). The human SGPL1 gene that encodes SPL maps to a region often mutated in cancers. To investigate the effect of SPL deficiency on cell survival and transformation, the susceptibility to anticancer drugs of fibroblasts generated from SPL-deficient mouse embryos (Sgpl1−/−) was compared with that of cells from heterozygous (Sgpl1+/−) or wild-type (Sgpl1+/+) embryos. First, loss of SPL caused resistance to the toxic effects of etoposide and doxorubicin. Interestingly, heterozygosity for the Sgpl1 gene resulted in partial resistance to apoptosis. Secondly, doxorubicin-induced apoptotic signaling was strongly inhibited in Sgpl1−/− cells (phosphatidylserine externalization, caspase activation, and cytochrome c release). This was accompanied by a strong increase in Bcl-2 and Bcl-xL protein content. Whereas correction of SPL deficiency in Sgpl1−/− cells led to downregulation of antiapoptotic proteins, Bcl-2 and Bcl-xL small interfering RNA–mediated knockdown in SPL-deficient cells resulted in increased sensitivity to doxorubicin, suggesting that Bcl-2 upregulation mediates SPL protective effects. Moreover, SPL deficiency led to increased cell proliferation, anchorage-independent cell growth, and formation of tumors in nude mice. Finally, transcriptomic studies showed that SPL expression is downregulated in human melanoma cell lines. Thus, by affecting SPL metabolism and the expression of Bcl-2 members, the loss of SPL enhances cell resistance to anticancer regimens and results in an increased ability of cells to acquire a transformed phenotype and become malignant. [Cancer Res 2009;69(24):OF1–8]

Introduction
Sphingolipids are essential ubiquitous constituents of membranes and, notably, microdomains (1). However, like mediators derived from glycerophospholipids, sphingolipids are a source of an important family of bioactive signaling molecules that regulate numerous physiologic and pathologic processes (2). The importance of sphingolipids in human health and disease is illustrated by the dramatic consequences of inherited disruption of their normal metabolism (3) and also by their involvement as bioeffector molecules in cancer and hematologic malignancies (4–6). The best characterized sphingolipid mediators are ceramide and sphingo- sine 1-phosphate (S1P); whereas ceramide generally transduces antiproliferative responses, the lysolipid S1P has crucial roles in cell survival, cancer migration, and angiogenesis and as well as immune responses (7).

S1P exerts its effects either through autocrine and paracrine actions as a ligand for a family of specific G protein–coupled receptors (S1P1–5) as well as by intracellular functions as a second messenger involved in the control of cell growth and death signaling pathways (8, 9). Both intracellular and circulating levels of S1P depend on the activity of some membrane transporters and of three classes of enzymes that control its metabolism. Whereas sphingosine kinases 1 (SK1) and 2 produce S1P by phosphorylating sphingosine, two S1P phosphatases dephosphorylate S1P to generate sphingosine and S1P lyase (SPL) irreversibly degrades S1P to phosphoethanolamine and 2-trans hexadecenal (10). The critical role played by S1P as a “tumor-promoting” agent has been mostly evidenced through manipulation of the expression and biological activity of SK1. Indeed, SK1 is overexpressed in multiple types of cancers and upregulation of SK1 has been associated with tumor angiogenesis and resistance to radiation and chemotherapy. Conversely, pharmacologic or genetic inhibition of SK1 activity in vitro on different cultured tumor cell models and in vivo on transplanted animals has been shown to enhance drug-induced lethality (11, 12). Furthermore, S1P-generating enzyme SK1 acts as an oncogene because NIH3T3 fibroblasts overexpressing SK1 acquired a transformed phenotype and the capability to form tumors in nude mice (13).

Of the enzymes involved in determining S1P abundance, SPL represents an undeniable candidate as potential regulator of cell’s fate in response to stress (14). SPL is a microsomal pyridoxal 5′-phosphate–dependent aldehyde-lyase that catalyzes the irreversible cleavage of S1P in the final step of sphingolipid catabolism (15, 16). In mammalian cells, overexpression of SPL was shown to sensitize cells to apoptotic stress such as serum deprivation and chemotheraphy (17–19). SPL-induced apoptosis requires its enzyme activity and can be blocked by addition of exogenous S1P (17). In contrast, products of the lyase reaction had no effect on apoptosis. The cytotoxic effect of SPL appears to be dependent on the activation of proapoptotic signaling pathways involving...
p38 mitogen-activated protein kinase, p53, caspase-2, and caspase-3 (19). However, whereas SK1 overexpression was associated with upregulation of the antiapoptotic protein Bcl-2 (20, 21), the connection between SPL with Bcl-2 family members has never been investigated.

Interestingly, the SGPL1 gene, which encodes SPL, has been reported to be significantly downregulated in human colon cancer tissues compared with normal tissues (22). Moreover, the human SGPL1 gene maps to chromosomal region 10q21 (23), which is deleted or mutated in many human tumor types (24–26). Altogether, these observations suggest that loss of SPL expression might potentiate cancer cell proliferation and contribute to tumorigenesis; this, however, remains to be demonstrated.

Here, we report that the lack of SPL leads to upregulation of the antiapoptotic proteins Bcl-2 and Bcl-xL and consequently protects against apoptosis induced by chemotherapeutic agents but not autophagy. Moreover, SPL-deficient cells isolated from Sgpl1−/− mouse embryos showed an increase in cell growth rate in culture, colony formation in soft agar, and tumor progression in nude mice compared with cells isolated from wild-type embryos. Collectively, these observations suggest that loss of SPL expression might potentiate cancer cell proliferation and contribute to tumorigenesis; this, however, remains to be demonstrated.

Materials and Methods

Cell culture and genotyping. Sgpl1+/− mice were generated from OST58278 (27) gene-trapped ES cells, bought from OmniBank, by Lexicon Genetics on a fee basis, and inbred into a C57BL/6 background as reported (28).6 Heterozygous mice were mated and murine embryonic fibroblasts (MEF) were prepared at embryonic day 16 from the skin of wild-type (Sgpl1+/+), SPL heterozygous (Sgpl1+/−), and SPL-deficient (Sgpl1−/−) embryos. By dilution culturing, spontaneously immortalized MEFs were obtained. Cells were grown in DMEM containing 10% FCS (Invitrogen) at 37°C in 5% CO2 humidified incubators. Total genomic DNA was extracted and quantified using the Nanodrop ND-1000. Fragments derived from the β-geo and Sgpl1 sequences were amplified by duplex PCR using the following primers: Gal-2s 5′-CgATAATCTTCCGTATACAGC, Gal-2r 5′-ACCACACACTCATC-CAATCGACATG. Amplification was generated using the Nanodrop ND-1000. Fragments derived from the β-geo and Sgpl1 sequences were amplified by duplex PCR using the following primers: Gal-2s 5′-CgATAATCTTCCGTATACAGC, Gal-2r 5′-ACCACACACTCATC-CAATCGACATG. Amplification was carried out for 35 cycles using an annealing temperature of 60°C. DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel.

Vectors and cell transfections. The cDNA encoding mSPL (29) was amplified by PCR using Phusion polymerase (Finnzymes) and oligonucleotides matching the first and last bases, respectively, of the Sgpl1 sequence and containing ApII and Xhol restriction sites. The amplification product was cloned in the pcDNA5/TTO vector (Invitrogen) and its sequence was verified. Then, SPL-deficient cells were transfected with the pcDNA5TTO-mSPL vector using Lipofectamine 2000 (Invitrogen).

Transient interference was achieved by a pool of four small interfering RNAs (siRNA) specific for SPL, Bcl-2, or Bcl-xL or aleatory sequence scrambled siRNA. siRNA targeting SPL or Bcl-2 and Bcl-xL were transfected into wild-type or Sgpl1−/− MEFs, respectively, grown to 70% to 90% confluence using Lipofectamine 2000. After 48 h of transfection, doxorubicin was added to the medium and incubated for 24 h before evaluation of cell viability.

Cell viability, flow cytometry, and morphologic analyses. For cytotoxicity assays, cells were seeded in flat-bottomed 24-well plates (4 × 10^5 per well). After 24 h, the medium was replaced with fresh medium containing 5% FCS and etoposide or doxorubicin, and cells were incubated at 37°C for different times. Cell viability was assessed using the MTT assay (Euromedex).

Phosphatidylserine externalization was evaluated by flow cytometry after labeling with Annexin V-FITC (250 ng/mL) and propidium iodide (12.5 μg/mL; AbCys) using a FACScan (BD Biosciences) cytometer.
For analysis of cytochrome c release, cells seeded on glass coverslips at 2 × 10⁴/mL were incubated with doxorubicin, fixed with formaldehyde (4%), washed with PBS, permeabilized with Triton X-100 (0.1%), and stained with a monoclonal anti-cytochrome c antibody (BD Pharmingen) and a secondary Alexa 488-labeled antibody (Molecular Probes/Invitrogen).

DEVD cleavage enzyme assay. Cell lysates were incubated for 30 min with Ac-Asp-Glu-Val-Asp-aminomethylcoumarin (Alexis). The amount of the released fluorescent product was determined fluorometrically at 351 and 430 nm for the excitation and emission wavelengths, respectively. Protein concentrations were determined according to the Bradford method.

Western blot analyses. Equal amounts of proteins were electrophoresed on a 10% to 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Perkin-Elmer), and blotted with monoclonal anti-Bax and anti-poly(ADP-ribose) polymerase, and anti–β-actin antibodies (Cell Signaling Technology). Polyclonal anti-Bax and anti–Bcl-2 antibodies were from BD Pharmingen. Proteins were detected using an enhanced chemiluminescence detection system (Pierce). SPL was detected using a rabbit polyclonal anti-SPL antibody kindly provided by Drs. M. Ikeda and A. Kihara. S1P measurements. The amount of cellular S1P was evaluated as reported (31) using ω-erythro-[4,5-3H]dihydro-S1P (ARC/Isobio) as described (30).

SIP measurements. The amount of cellular SIP was evaluated as reported (31) using ω-erythro-[3-3H]sphingosine (0.45 μCi/mL; 1.5 μmol/L; Perkin-Elmer).

Anchorage-independent growth assay. Six-well plates were precoated with 2 mL DMEM containing 10% FCS and 0.6% agar. DMEM (2 mL) containing 10% FCS, 0.3% agar, and 5,000 cells were overlaid onto the precoated wells. After 2 weeks' incubation at 37°C in 5% CO₂ atmosphere, cell colonies were visualized by MTT staining (500 μg/mL) and photographed.

Tumor growth (allograft mouse models). Sgp1l−/− and Sgp1l+/− cells were trypsinized, washed, and resuspended at 5 × 10⁶/mL in sterile PBS. One hundred microliters were injected s.c. into the flank of 4-week-old female nude mice (NMRI-nu; Janvier) and tumors were allowed to develop for up to 40 days. Tumor volumes were calculated as described (32). All experiments were done in accordance with the principles and guidelines established by INSERM and were approved by the local animal care and use committee.

Quantitative real-time PCR. Nonconfluent human melanoma cell lines derived from adult skin NHEM-M2 or juvenile foreskin NHEM-f (Promo-Cell) were harvested and RNA was extracted (RNeasy kit; Qiagen) according to the manufacturer's protocol and treated with DNase (Qiagen). RNA quality was assessed by automated gel electrophoresis (Experion; Bio-Rad). RNA (2 μg) was reverse-transcribed (SuperScript II; Invitrogen) and used as a template for quantitative PCR. The reactions were done in duplicate on the StepOne instrument (Applied Biosystems) using SYBR Green PCR kit and primer assay (QuantiTect; Qiagen). The results were quantified using the system software, mRNA of TBP, TFRC, and β-actin were analyzed for normalization.

Statistical analyses. Results are expressed as mean ± SE. Student's t test was used for statistical comparisons among groups and differences were considered statistically significant when P < 0.05 (*, P < 0.01; ***, P < 0.001).
Results

Gene dosage effect of Sgpl1 on cell death induced by chemotherapeutic drugs. To address the effect of SPL deficiency on anticancer drug–induced cell death, we first used a genetic approach by testing mutant MEFs derived from heterozygous (Sgpl1+−/) and homozygous (Sgpl1−−/) Sgpl1-null mouse embryos. Sgpl1+−/ and Sgpl1−−/ cells carry a β-galactosidase-neomycin fusion gene trap cassette within the second intron of the Sgpl1 gene (Fig. 1A). Only in these mutant cells bacterial β-galactosidase activity was measurable (Supplementary Fig. S1). Moreover, Western blot analysis using an antibody raised against the murine SPL protein (Fig. 1B) and SPL enzymatic assay (Fig. 1C) confirmed that SPL expression and activity were decreased in Sgpl1−−/ cells and abolished in Sgpl1−−/ cells compared with wild-type cells. Disruption of Sgpl1 gene was accompanied by an intracellular increase of S1P levels (Fig. 1D; Supplementary Table S1).

The importance of SPL in stress-induced cell death was investigated by treating wild-type and SPL mutant cells with various concentrations of etoposide (Fig. 2A) and doxorubicin (Fig. 2B). These agents led to a dose- and time-dependent reduction in the viability of Sgpl1+−/ cells (Fig. 2C; Supplementary Fig. S2A). Under all concentrations tested, Sgpl1+−/ MEFs were significantly less sensitive to the lethal effects of the drugs than their normal counterparts, whereas Sgpl1−−/ MEFs were highly resistant. Interestingly, the chemoresistance of heterozygous and homozygous cells was also observed in primary MEFs (Supplementary Fig. S3) as well as SV40-transformed MEFs (data not shown). Furthermore, nuclear condensation observed in doxorubicin-treated Sgpl1+−/ cells by fluorescence microscopy was reduced in Sgpl1+−/ and completely abolished in Sgpl1−−/ cells (Supplementary Fig. S2B). Finally, Sgpl1+−/ MEFs transfected with a siRNA targeting SPL, which reduced SPL protein content by ~50%, became partially resistant to doxorubicin (Fig. 2D). Similar observations were made on NIH3T3 fibroblasts treated with mSPL siRNA (data not shown). These data point to a strong correlation between SPL expression and cell sensitivity to chemotherapy.

SPL is required for apoptosis induced by doxorubicin. To substantiate the importance of SPL in anticancer drug–induced cell death, the apoptotic cascade activated by doxorubicin in Sgpl1−−/ cells was analyzed. Of note, intracellular uptake of doxorubicin, analyzed by fluorometry, was similar in Sgpl1+−/ and Sgpl1−−/ cells (data not shown but illustrated in Fig. 3D by the red fluorescence of the nuclei). In wild-type but not in SPL-deficient cells, doxorubicin led to an increased proportion of Annexin V–positive and propidium iodide–negative cells, cells with externalized phosphatidylserine (Fig. 3A). These results are consistent with the absence of morphologic alterations such as nuclear condensation observed in mutant cells compared with control cells (Supplementary Fig. S2B) and suggest that apoptosis is impaired in SPL-deficient cells.

To test whether caspase activation was altered in SPL mutant cells upon doxorubicin treatment, we analyzed caspase activity by measuring cleavage of the fluorogenic tetrapeptide substrate Ac-Asp-Glu-Val-Asp-aminomethylcoumarin. Caspase activity (Fig. 3B) and processing of caspase-3 and caspase-7 as well as the cleavage of caspase-9 and the caspase substrate poly(ADP-ribose) polymerase (PARP) (Fig. 3C) were strongly impaired in SPL-deficient cells following doxorubicin treatment. As shown in Fig. 3D, translocation of cytochrome c from mitochondria to the cytosol was also blocked in Sgpl1−−/ cells, showing the involvement of SPL in the apoptotic cascade initiated by doxorubicin with a site of action likely lying upstream of mitochondria.
Autophagy is not altered in Sgpl1−/− cells. As the increase of S1P levels after SK1 overexpression was shown to stimulate autophagy and to protect cells from apoptotic death during nutrient starvation (33), we examined the effect of SPL deficiency on this phenomenon. As illustrated in Supplementary Fig. S4, starvation-induced apoptosis was reduced in Sgpl1−/− compared with Sgpl1+/+ cells. However, autophagy, monitored by measuring proteolysis of long-lived proteins, formation of LC3-II or LC3 translocation from the cytosol to aggregates into autophagic vacuoles, which are prerequisites for autophagosome formation and are regarded as autophagy markers (34, 35), was similar in wild-type and mutant cells. These data suggest that, under nutrient starvation, the resistance of SPL-deficient cells to apoptosis is not associated with alterations in autophagy.

Upregulation of the expression of antiapoptotic Bcl-2 family members contributes to the chemoresistance of SPL-deficient cells. Overexpression of prosurvival Bcl-2 proteins occurs in numerous tumors and correlates with resistance to antineoplastic drugs (36). To determine whether resistance to doxorubicin of SPL-deficient cells was accompanied with an alteration of the balance between proapoptotic and antiapoptotic members of the Bcl-2 family, the expression pattern of these proteins was investigated. A comparative Western blot analysis revealed that Sgpl1−/− cells displayed higher levels of both Bcl-2 and Bcl-xL than Sgpl1+/+ cells, whereas Bid and Bax were not modified, indicating a possible contribution of Bcl-2 antiapoptotic members to the chemoresistance of SPL-deficient cells (Fig. 4A; Supplementary Fig. S3C). To test the latter hypothesis, we investigated the expression of Bcl-2 and Bcl-xL in Sgpl1−/− cells transiently transfected with a cDNA encoding mSPL. As shown in Fig. 4B, SPL-corrected Sgpl1−/− cells displayed lower levels of both Bcl-2 and Bcl-xL than mutant cells transiently transfected with an empty vector. We next employed a knockdown approach to deplete Bcl-2 and Bcl-xL either individually or in combination in Sgpl1−/− cells. Effective downregulation of the target protein Bcl-2 or Bcl-xL was confirmed by Western blot analysis (Fig. 4C). Sgpl1−/− cells transfected with both Bcl-2 and Bcl-xL siRNA displayed enhanced sensitivity to doxorubicin compared with their control counterparts (Fig. 4D).

Lack of SPL expression leads to increased cell proliferation in vitro and oncogenesis in vivo. The S1P-generating enzyme SK1 behaves as an oncogene when overexpressed in nontransformed NIH3T3 fibroblasts (13). To investigate the effect of S1P-degrading SPL on cell transformation, we analyzed the growth properties of Sgpl1−/− and Sgpl1+/+ cells. We found that, in serum-free medium or in the presence of 1% serum, SPL-deficient cells exhibited higher rates of proliferation than their wild-type counterparts. In S1P-containing medium, full serum, both cell lines grew at identical rates (Fig. 5A). However, using the scratch wound closure assay, we observed that cell migration was not altered in SPL-deficient cells (data not shown). We then tested whether increased growth of Sgpl1−/− cells was accompanied by the ability to form colonies in vitro.
soft agar. As illustrated in Fig. 5B and Supplementary Table S2, SPL-deficient cells gave rise to numerous colonies, whereas wild-type cells showed much less anchorage-independent growth. To evaluate the in vivo transforming properties of SPL, we tested the ability of Sgpl1+/+ and Sgpl1−/− cells to grow in immunocompromised mice. When injected s.c. in female athymic nude mice, SPL-deficient cells induced rapidly growing tumors at the site of inoculation within 15 days, whereas mice grafted with Sgpl1+/+ cells did not develop tumors even 40 days postgraft (Fig. 5C). In all tumor-bearing mice sacrificed after 40 days, the bacterial β-galactosidase carried in the gene trap was fully active (Supplementary Fig. S5A). Histologic analysis showed that all tumors presented high cellularity and consisted of spindle cells, some with atypical nuclei, forming fascicles highly suggestive of a fibrosarcoma (Supplementary Fig. S5B). Altogether, these data indicate that expression of a functional SPL is required for inhibition of tumorigenesis.

**Downregulation of SPL expression in melanoma cells.** Mammalian genes frequently present allelic variants that differ in their expression levels and that, in the case of tumor suppressor genes, can be of relevance for cancer susceptibility. Because the human
SGPL1 gene maps to chromosomal 10q21 and that alteration of chromosome 10 is common in human cancers including melanoma (37), we evaluated the expression of SPL in a series of invasive or metastatic melanoma cells and compared with that of melanocytes derived from healthy individuals. As shown in Fig. 6, SGPL1 expression was significantly reduced in most melanoma cell lines tested, suggesting that this gene is downregulated during melanoma tumorigenesis.

Discussion

S1P is considered as a tumor-promoting agent for its role in the regulation of cancer cell growth, survival, adhesion, migration, metastatic potential, and angiogenesis (2, 7). Several antitumoral therapeutic approaches targeting S1P formation and signaling have been developed including the synthesis of sphingosine kinase inhibitors (38), the targeting of S1P receptors with the immunosuppressant FTY720 (39), and the use of an anti-S1P antibody (40). By controlling S1P catabolism, SPL emerges as a novel target in the generation of sphingolipid-based therapeutics (14).

Analysis of SPL-deficient genetic models revealed that SPL is crucial for mammalian survival because SPL knockout mice displayed severe lymphopenia and developed myeloid cell hyperplasia and also significant lesions in several nonlymphoid organs (28, 41). Here, using fibroblasts generated from SPL-deficient mouse embryos, we show that complete loss of SPL activity confers resistance to apoptosis in response to chemotherapeutic agents. Of particular interest was the finding that a gene dosage effect exists in the sensitivity to anticancer drugs because the loss of a single SPL allele (as seen in heterozygous cells) resulted in partial resistance to genotoxic stress. This observation was corroborated by the fact that siRNA-mediated knockdown of SPL in control fibroblasts also led to a partial resistance to doxorubicin-induced cell death. SPL deficiency blocked apoptotic events such as the cleavage of effector caspases and the caspase substrate poly(ADP-ribose) polymerase in accordance with data showing that caspase-3 activity was increased in SPL-overexpressing cells (17, 19). Moreover, we found that the release of mitochondrial cytochrome c into the cytosol and the cleavage of initiator caspase-9 were impaired in Sgpl1−/− cells, suggesting that SPL likely acts upstream of mitochondria and triggers the intrinsic apoptotic pathway.

Several Bcl-2 family proteins insert into mitochondrial membranes operating as guardians of these organelles (42). Here, we show that whereas loss of SPL activity contributes to upregulation of the oncoproteins Bcl-2 and Bcl-xL but not Bax and Bid, its restoration in SPL-deficient cells was able to diminish their expression and its effects are required to better understand its role in S1P-mediated transformation because the S1P-generating enzyme SK1 (13) has been shown that SPL promotes apoptosis via a p53-dependent pathway (19). The tumor suppressor p53 has been also associated to Bcl-2 because it could inactivate its antiapoptotic functions by promoting its phosphorylation (47). Moreover, p53 is inactivated in many cancers mostly through missense mutations that result in the abrogation of its activity and the acquisition of oncogenic functions (48).

S1P metabolism has also been linked to the oncogenic Ras-mediated transformation because the S1P-generating enzyme SK1 was activated in NIH3T3 cells transfected with an active mutant of Ras, whereas the transforming ability of Ras was inhibited in cells cotransfected with a dominant-negative mutant of SK1 (13). Interestingly, activation of the Ras pathway resulted in the rapid upregulation of Bcl-2 and Bcl-xL (49), and Bcl-2 and Ras mutant cooperated to give rise to malignant disease (50). Future studies are necessary to determine whether SPL loss and Bcl-2 overexpression are causally related to the Ras pathway or the expression of other oncoproteins.

Our data show that reduced SPL activity results in both resistance to apoptosis and oncogenesis, suggesting that SPL could restrict tumor development. The reduced expression of SPL in solid cancers would go along such a tumor suppressor role. Indeed, we observed that SGPL1 expression is downregulated in human melanoma. This finding is consistent with the diminished SPL expression reported on human colorectal carcinomas (19). A more in-depth analysis of the molecular mechanisms that regulate SPL expression and its effects are required to better understand its role in S1P-mediated oncogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Sandra Colié, Paul P. Van Veldhoven, Blandine Kedjouar, et al.

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