Laforin Confers Cancer Resistance to Energy Deprivation–Induced Apoptosis

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Abstract

A long-standing but poorly understood observation in experimental cancer therapy is the heterogeneity in cancer susceptibility to energy deprivation. Here, we show that the hexose kinase inhibitor 2-deoxyglucose (2-dG) preferentially kills cancer cells with defective laforin expression and significantly increases the survival of mice with aggressive lymphoma due to a genetic defect of the laforin-encoding *Epm2a* gene. Normal cells from *Epm2a*$^{-/-}$ mice also had greatly increased susceptibility to 2-dG. Thus, laforin is a novel regulator for cellular response to energy deprivation and its defects in cancer cells may be targeted for cancer therapy. [Cancer Res 2008;68(11):4039–44]

Introduction

An important hallmark of many poorly differentiated and rapidly growing malignant tumors is higher rates of glucose usage and glycolysis compared with corresponding normal tissues (1, 2). Increase in glucose uptake is the earliest change observed in cells after malignant transformation (3, 4). The abnormalities in energy metabolism of cancer cells have been targeted for cancer therapy with mixed success (5–8). The heterogeneity of cancer cell response to energy deprivation suggests that additional modifiers exist. As such, understanding the mechanism for cellular energy response may offer critical insights for cancer therapy. It is established that energy deprivation results in activation of AMP-activated protein kinase (AMPK), which in conjunction with glycogen synthase kinase 3β (GSK3β) activates the tuberous sclerosis complex (TSC) pathway to inhibit mammalian target of rapamycin (mTOR). Genetic defects of this pathway render cancer cells more susceptible to energy deprivation (9, 10).

Laforin is a dual-specific phosphatase encoded by *Epm2a*, which was initially identified as one of the causative genes for progressive myoclonus epilepsy (11). Its known substrates include GSK3β (12, 13) and carbohydrates (14). Recently, an elegant genetic study showed that laforin complements starch excess 4 mutations in the *Arabidopsis thaliana* (15). In immune compromised mice, *Epm2a* works as a tumor suppressor gene (13). It is unclear, however, whether laforin is involved in cellular response to energy deprivation. Here, we showed that laforin is a novel regulator for cellular response to energy deprivation and its defects in cancer cells may be targeted for cancer therapy.

Materials and Methods

Antibodies and reagents. Antibodies against the following proteins were used in this study: anti-Akt, phospho-Akt (Ser^{473}), phospho-GSK3β (Ser^{9}), phospho-AMPK, phospho-S6K70 (Thr^{389}), phospho-mTOR, and caspase-3 (Cell Signaling); anti-V5 and anti-Myc (Invitrogen Corp.); and anti-β-actin (I-19), TSC2, hexokinase, S6K, and VDAC (Santa Cruz Biotechnology, Inc.). Anti-laforin polyclonal antibody was produced by Genemed Synthesis, Inc. Rabbits were immunized with the synthetic peptide of 16 amino acid residues (YKFLQREPGGELHWEG, residues 85–100 in laforin protein, accession no. AAD26336) coupled with keyhole limpet hemocyanin in complete Freund's adjuvant. The antiserum was purified using peptide-conjugated Affigel column.

Plasmids and transfection. Full-length cDNA of *Epm2a* was amplified by reverse-transcription PCR using high-fidelity Taq enzyme (Invitrogen) from mouse spleen and cloned into vectors of pcDNA4-V5/His (Invitrogen) at restriction enzyme sites of *Hin*dIII/*Bam*HI to get fusion proteins with Myc or V5 tag fused in the C terminus of laforin. Human embryonic kidney HEK293 cells were transiently transfected or cotransfected with different expression plasmids for 24 h under a culture of Opti-MEM medium containing 10% fetal bovine serum (FBS) and the premixed mixture of the plasmids with Lipofectamine 2000 that was done according to the vendor protocol (Invitrogen).

***Epm2a* small interfering RNA constructs.** Oligonucleotides encoding small interfering RNA (siRNA) directed against *Epm2a* at COOH-terminal region of 934 to 954 nucleotides (5′-AAGGTGCAGTACTTCATCATG-3′) were inserted into a modified pLenti6/V5-D-TOPO vector (Invitrogen). Lentivirus stocks were produced in 293FT cells according to the manufacturer's protocol. Viral-transduced cells were selected with blasticidin.

MTT cell viability assay. One hundred microliters of cells (1×10^3 per mL) were plated out in triplicate into wells of a 96-well microplate, including three control wells of medium alone, to provide the blanks for absorbance readings. The cells were incubated under conditions appropriate for the cell line for 12 h and treated with different concentration of 2-deoxyglucose (2-dG, Sigma). Ten microliters of 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in PBS were added to each well, including controls. Plates were returned to cell culture incubator for 2 to 4 h. When the purple precipitate is clearly visible under the microscope, the cultures were removed from incubator and the resulting MTT formazan crystals were dissolved by adding 150 μL DMSO. Absorbance was spectrophotometrically measured at a wavelength of 490 nm.

Propidium iodide staining. 2-dG–treated or control cells were stained by propidium iodide (PI) using "PI/RNase Staining Buffer" from BD Biosciences according to product instruction. Briefly, cells were frozen in 70% ethanol overnight and washed with PBS thrice, and 0.5 mL staining buffer was added to each sample cells. The content of DNA in stained cells was determined by flow cytometry after 15-min room temperature incubation.

Results and Discussion

In analyzing laforin expression among cancer cell lines, we found great heterogeneity of its expression. For example, among four murine tumor cell lines tested, MC38 had a high level of laforin

Note: Y. Wang and Y. Liu are equal contributing first authors of the manuscript.

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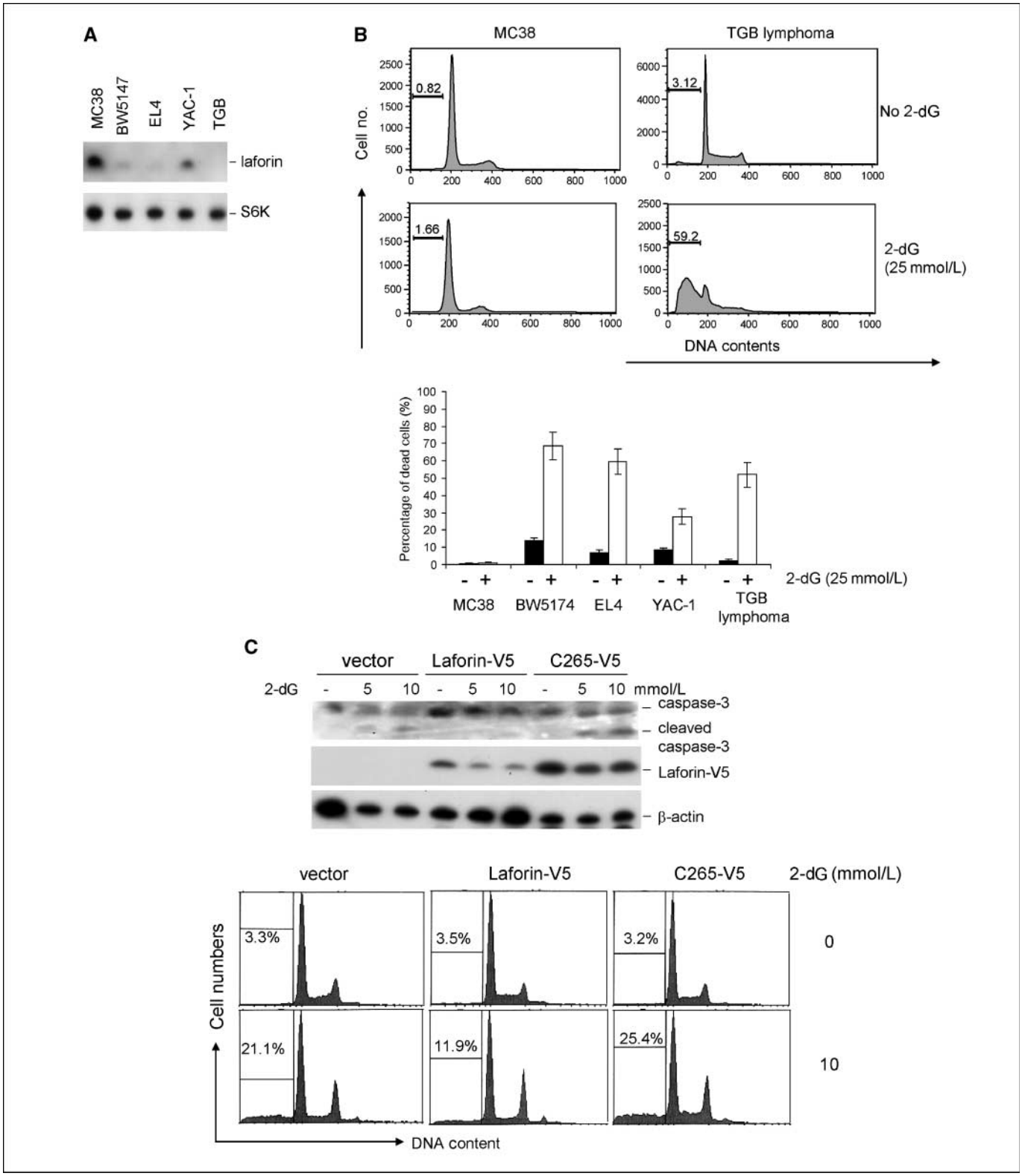

Figure 1. Laforin controls cellular response to energy deprivation. *A* and *B,* inverse correlation between laforin levels and cancer cell susceptibility to energy deprivation. *A,* levels of laforin in the colorectal cancer cell line MC38, thymoma cell lines BW5147 and EL4, T lymphoma YAC-1, and a cell line derived from TGB lymphoma, as determined by Western blot with anti-laforin antibody. *B,* correlation between laforin levels and susceptibility to energy deprivation. *Top,* representative fluorescence-activated cell sorting profiles of laforin^{hi} MC38 and laforin^{-} TGB lymphoma, depicting DNA contents. The numbers in the panels are percentage of cells with less than 2C DNA contents. *Bottom,* summary of data from two independent experiments. *C,* inducible expression of laforin confers resistance to energy deprivation. EL4 cells were transfected with Tet-off vector with laforin-V5 cDNA in the presence or absence of tetracycline for 36 h and treated with given doses of 2-dG for 24 h. The harvested cells were analyzed for caspase-3 activation (*top*) or DNA content (*bottom*). The percentage of cells with less than 2C DNA contents is listed in the panels. Data shown have been repeated thrice.

expression, whereas the lymphoma cell line TGB had no detectable laforin, and the remaining cell lines had low to intermediate levels (Fig. 1*A*). Given the function of laforin in regulating GSK3β activity and the role for GSK3β in the cellular response to energy, we compared these cell lines for their susceptibility to 2-dG treatment. Whereas the TGB lymphoma cells underwent extensive apoptosis, as judged by the percentage of cells with less than 2C DNA contents in response to 2-dG treatment, MC38 cells were largely resistant (Fig. 1*B, top*). When a panel of tumor cell lines expressing different levels of laforin were subjected to the same treatment, we observed a strong inverse correlation between the laforin levels and the percentage of apoptotic cells (Fig. 1*B, bottom*).

To directly show the protective effect of laforin to energy deprivation–induced apoptosis, we generated a Tet-off system in the EL4, a mouse T lymphoma cell line with low levels of endogenous laforin (13). As shown in Fig. 1*C*, removal of doxycycline resulted in a significant induction of the transfected laforin, regardless of the 2-dG in the medium. With the induction of laforin expression, the EL4 cells gained resistance to 2-dG treatment, as revealed by both activation of caspase-3 and the percentage of cells with sub-2C DNA contents (Fig. 1*C*). Thus, expression of laforin conveyed resistance to 2-dG.

An important issue is whether laforin is required for resistance of normal cells to 2-dG. To address this issue, we compared the sensitivity of T cells from wild-type (WT) and laforin-deficient mice for their sensitivity to 2-dG. As shown in Fig. 2, targeted mutation of the *Epm2a* gene drastically increased the sensitivity of the splenic T cells to 2-dG, as revealed by cellular viability (MTT assay; Fig. 2*A*) and DNA content (Fig. 2*B*). Taken together, our data showed that laforin plays a critical role in protecting cells from apoptosis induced by energy deprivation.

The AMPK-GSK3β-TSC-mTOR pathway plays an important role in regulating cellular energy response (9, 10). As a genetic test, we compared the effect of laforin knockdown in TSC2-deficient and TSC2-reconstituted EEF8 cells (Fig. 3*A*; refs. 9, 10). siRNA silencing of *Epm2a* significantly increased GSK3β phosphorylation, with the notable exception of 50 mmol/L 2-dG treatment when the cell viability was poor. This, however, did not appreciably affect mTOR activation as revealed by comparable levels of S6K P70 phosphorylation at the Thr^{389} site. As expected, activation of AMPK was also unaffected (Fig. 3*B*). Nevertheless, laforin knockdown did increase cellular susceptibility to 2-dG regardless of TSC2 expression (Fig. 3*C* and *D*). Thus, the laforin works independently of the TSC-mTOR pathway in regulating cellular response to energy deprivation.

The fact that a large proportion of both mouse and human lymphomas have depressed laforin expression (13) and that cells with defective laforin have increased susceptibility to energy deprivation suggests that 2-dG may be effective in treating this type of cancer. We reported that insertional mutation of one allele of the *Epm2a* gene in conjunction with epigenetic silencing caused lymphoma with rapid onset and 100% lethality within 1 year (13). This model allowed us to determine if energy deprivation can be therapeutic for lymphoma. Thus, we treated the TGB mice with 2-dG and followed the survival of the mice. As shown in Fig. 4*A*, regardless of the age of treatment, at either 8 to 12 weeks when some untreated mice had begun to become moribund, or at

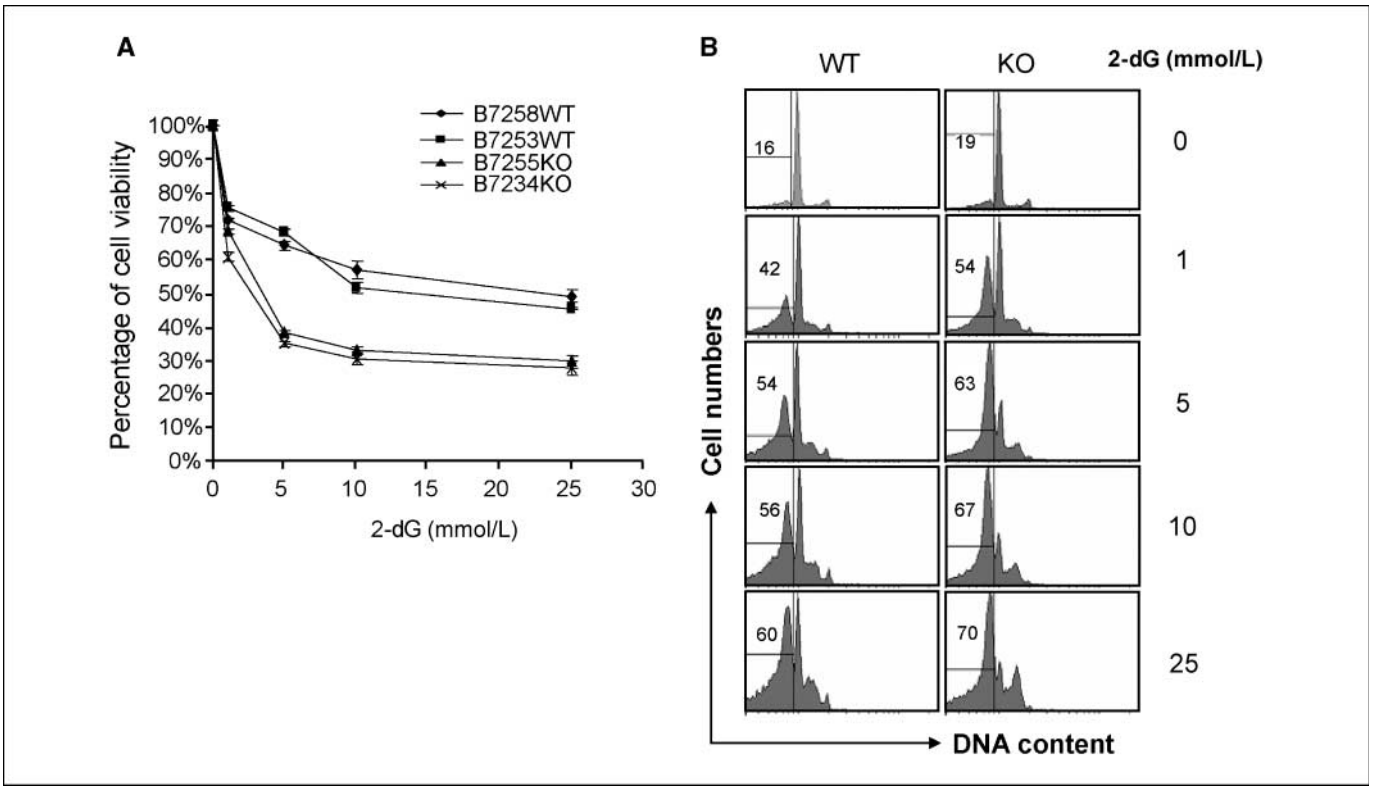

Figure 2. Targeted mutation of the *Epm2a* gene dramatically increases susceptibility of normal T cells. Spleen cells from the WT and $Epm2a^{-/-}$ mice (17) were stimulated with anti-CD3 (400 ng/mL) for 48 h. After washing with culture medium, the cells were cultured in the presence of given concentration of 2-dG for 24 h. The survival of T cells was determined either by MTT assay (*A*) using the absorbance of the untreated group as 100% or by DNA contents (*B*). *A, points,* mean of triplicates, T cells from two mice per group; *bars,* SD. These experiments have been repeated twice.

15 to 20 weeks of age when 25% to 50% of untreated mice had succumbed to cancer, 2-dG significantly prolonged mouse survival.

As we have reported previously (13), human lymphoma cell lines were heterogenous in laforin levels. This feature allowed us to determine whether laforin levels predict cancer susceptibility to 2-dG treatment. As shown in Fig. 4*B*, when a panel of eight lymphomas was tested, those with no or the lowest levels of laforin were significantly more susceptible to 2-dG treatment compared with cells displaying high levels of laforin. Notably, among the six lines with high levels of laforin and relatively more resistant to

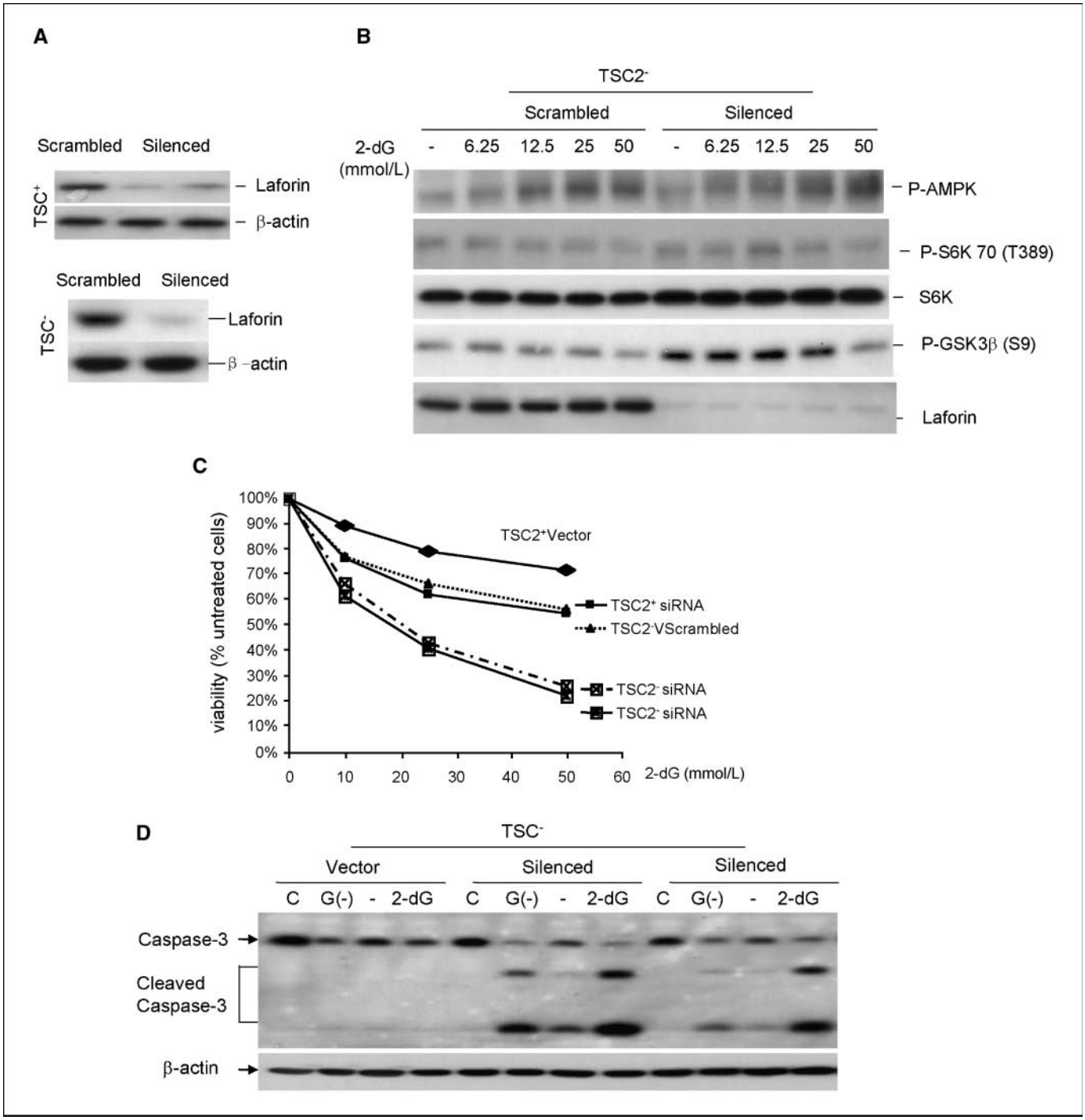

Figure 3. Laforin confers cellular resistance to energy deprivation by a TSC-mTOR–independent mechanism. *A,* efficacy of siRNA on laforin levels in TSC2$^+$ and TSC2$^-$ cell lines. *B,* in the TSC2$^-$ cell line, Epm2a silencing increases GSK3β phosphorylation without affecting activation of AMPK and S6K. The TSC2$^-$ vector and TSC2$^-$ siRNA transfectants were treated with given concentration of 2-dG for 0.5 h. The cell lysates were harvested and probed with antibodies specific for phosphorylated AMPK (*P-AMPK*; Thr^{172}), S6K70 (*P-S6K70*; Thr^{389}), and GSK3β (*P-GSK3β*; Ser^9) or total S6K and laforin. Data shown have been repeated four times. *C,* cell viability after treatment with given concentration of 2-dG (*top*) and apoptosis as revealed by caspase-3 activation (*bottom*). *D,* effect of *Epm2a* siRNA on 2-dG–induced apoptosis of TSC2$^+$ and TSC2$^-$ cells. The culture media used were the following: DMEM + 4.5 g/L glucose + 10% dialyzed FBS (*C*); MEM + 0 g/L glucose + 10% dialyzed FBS [*G*(−)]; DMEM + 1 g/L glucose (no FBS; -); and DMEM + 1 g/L glucose + 2-dG (no FBS; *2-dG*). Data have been repeated thrice.

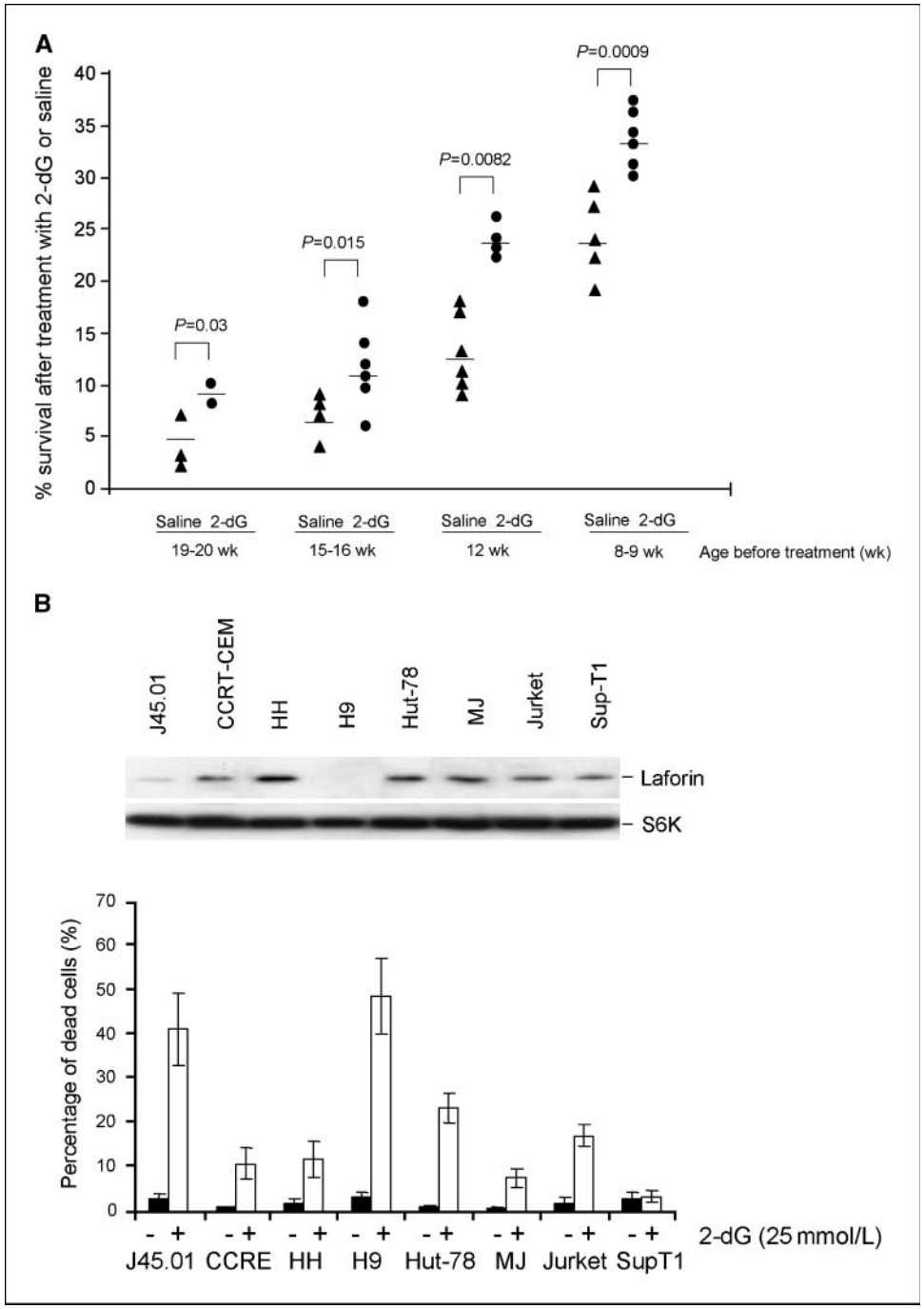

Figure 4. Laforin expression and cancer susceptibility to energy deprivation. *A,* 2-dG treatment significantly increases the life span of the TGB mice that develop lymphoma due to genetic and epigenetic defects of the *Epm2a* gene. TGB mice were grouped and paired according to age and treated with either PBS or 2-dG at the dose of 20 mg/mouse/3 d at the beginning week and then 20 mg/mouse/week in the following 1 mo until moribund or death. Data shown indicate the life span after initiation of treatment. *B,* inverse correlation between laforin levels and cellular response to 2-dG treatment. *Top,* levels of laforin as determined by Western blot using total S6K as a loading control; *bottom,* percentage of cells with less than 2C DNA contents as determined by flow cytometry. *Columns,* mean; *bars,* SD. Data have been repeated twice.

2-dG, there is no strict correlation between laforin levels and susceptibility to 2-dG. These data are best explained by involvement of other factors that may regulate cellular energy response (9, 10).

Taken together, we have shown laforin as a critical checkpoint for cellular susceptibility to energy deprivation–induced apoptosis and that laforin regulates cell survival under energy deprivation by a TSC-independent mechanism. Because down-regulation of laforin is quite widespread in human lymphoma (13), and because the human chromosome 6q24, where *Epm2a* resides, is often deleted in human cancer (16), targeting this defect may have a significant effect for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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