

# Activation of serum/glucocorticoid-induced kinase 1 (SGK1) underlies increased glycogen levels, mTOR activation, and autophagy defects in Lafora disease

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**ABSTRACT** Lafora disease (LD), a fatal genetic form of myoclonic epilepsy, is characterized by abnormally high levels of cellular glycogen and its accumulation as Lafora bodies in affected tissues. Therefore the two defective proteins in LD—laforin phosphatase and malin ubiquitin ligase—are believed to be involved in glycogen metabolism. We earlier demonstrated that laforin and malin negatively regulate cellular glucose uptake by preventing plasma membrane targeting of glucose transporters. We show here that loss of laforin results in activation of serum/glucocorticoid-induced kinase 1 (SGK1) in cellular and animals models and that inhibition of SGK1 in laforin-deficient cells reduces the level of plasma membrane-bound glucose transporter, glucose uptake, and the consequent glycogen accumulation. We also provide evidence to suggest that mammalian target of rapamycin (mTOR) activates SGK1 kinase in laforin-deficient cells. The mTOR activation appears to be a glucose-dependent event, and overexpression of dominant-negative SGK1 suppresses mTOR activation, suggesting the existence of a feedforward loop between SGK1 and mTOR. Our findings indicate that inhibition of SGK1 activity could be an effective therapeutic approach to suppress glycogen accumulation, inhibit mTOR activity, and rescue autophagy defects in LD.

## Monitoring Editor

Jonathan Chernoff  
Fox Chase Cancer Center

Received: May 17, 2013

Revised: Oct 7, 2013

Accepted: Oct 8, 2013

## INTRODUCTION

Lafora progressive myoclonus epilepsy, also known as Lafora disease (LD), is a fatal autosomal recessive disorder resulting from mutation in the *EPM2A* gene coding for laforin phosphatase or the *NHLRC1* gene coding for malin E3 ubiquitin ligase (Singh and Ganesh, 2009; Serratosa *et al.*, 2012). The symptoms of LD include epileptic seizures, ataxia, psychosis, and dementia. Disease onset is at around 15 yr of age, with death ~10–15 yr after onset (Ganesh *et al.*, 2006; Singh and Ganesh, 2009; Serratosa *et al.*, 2012).

One of the pathological hallmarks of LD is the presence of polyglucosan inclusions, called Lafora bodies, in various tissues, including muscle, liver, and brain (Ganesh *et al.*, 2006; Serratosa *et al.*, 2012). These inclusions are cytoplasmic and water insoluble and are composed of lesser-branched forms of glycogen (Yokoi *et al.*, 1968, 1975). Besides the finding of Lafora bodies, tissue glycogen level is higher in LD animal models that lack laforin or malin (Tagliabracci *et al.*, 2007; DePaoli-Roach *et al.*, 2010). Therefore the LD proteins laforin and malin have been proposed to play critical roles in glycogen metabolism, and some of their possible functions have been demonstrated. For example, the laforin–malin complex prevents glycogen synthesis by promoting the degradation of glycogen synthase and protein targeting to glycogen (PTG), the two proteins involved in glycogen metabolism, through the proteasome (Fernández-Sánchez *et al.*, 2003; Vilchez *et al.*, 2007). However, the LD mouse models did not show any change in the level of total and/or the active form of these proteins, and therefore they were excluded as possible players in the glycogen buildup seen in LD (Tagliabracci *et al.*, 2008; DePaoli-Roach *et al.*, 2010; Turnbull *et al.*, 2010). Laforin was shown to bind to glycogen/Lafora bodies

This article was published online ahead of print in MBoc in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-05-0261>) on October 16, 2013.

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Abbreviations used: DN, dominant negative; Glut, glucose transporter; LD, Lafora disease; mTOR, mammalian target of rapamycin; SGK1, serum/glucocorticoid-induced kinase 1.

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(Wang *et al.* 2002; Chan *et al.*, 2004; Ganesh *et al.*, 2004) and dephosphorylate glycogen and other complex carbohydrates (Tagliabracci *et al.*, 2007). Therefore laforin deficiency might result in the genesis of hyperphosphorylated glycogen as Lafora bodies. Besides their role in the genesis of Lafora bodies, laforin and malin were also shown to be involved in other cellular pathways, such as the ubiquitin–proteasome system (Mittal *et al.*, 2007; Garyali *et al.*, 2009), the endoplasmic reticulum stress response (Vernia *et al.*, 2009; Wang *et al.*, 2013), the heat shock response (Sengupta *et al.*, 2011), and RNA metabolism (Singh *et al.*, 2012b). Studies on LD animal models demonstrate an increased kinase activity for the mammalian target of rapamycin (mTOR) and autophagic defects (Aguado *et al.*, 2010; Criado *et al.*, 2012; Puri *et al.*, 2012). The possible links between these processes and their contributions to LD pathogenesis are unclear.

It is intriguing that suppression of PTG or glycogen synthase in laforin-deficient mice led to a normal level and form of glycogen (Turnbull *et al.*, 2011a, Pederson *et al.*, 2013). Considering that laforin is a glucan phosphatase and the suggestion that hyperphosphorylation of glycogen results in the genesis of Lafora bodies in LD tissues (Tagliabracci *et al.*, 2007, 2008; Turnbull *et al.*, 2010), one would expect that loss of laforin should favor the synthesis of abnormally hyperphosphorylated glycogen since these double mutants lack laforin—the glucan phosphatase. In this regard it is interesting to note that in LD tissues, besides Lafora bodies, tissue glycogen was also found at higher levels (Tagliabracci *et al.*, 2007; DePaoli-Roach *et al.*, 2010), suggesting that the loss of laforin may result in increased glycogen synthesis without altering the level and/or activity of glycogen synthase. Consistent with these observations, we showed that laforin and malin are negative regulators of glycogen synthesis and regulate cellular glucose uptake (Singh *et al.*, 2012a). On the basis of these findings, we hypothesized that increased availability of intracellular substrate (glucose) contributes to excessive glycogen synthesis, which might eventually result in the formation of Lafora bodies in the absence of LD proteins (Singh *et al.*, 2012a). With the discovery of autophagic defects in LD (Aguado *et al.*, 2010; Criado *et al.*, 2012; Puri *et al.*, 2012), and given its previously characterized role in glycogen degradation (Kotoulas *et al.*, 2004), it has been postulated that autophagic defects might contribute to glycogen accumulation in LD tissues (Puri and Ganesh, 2010; Polajnar and Zerovnik, 2011). The aim of the present study is to elucidate molecular mechanisms favoring glycogen accumulation under a laforin-deficient condition and investigate the possible links between mTOR activity, autophagy defects, and the excessive glycogen accumulation seen in laforin-deficient cells. We demonstrate here that loss of laforin results in the activation of the serum/glucocorticoid-induced kinase 1 (SGK1) and that its suppression reduces the level of plasma membrane-bound glucose transporter, glucose uptake, and consequent glycogen accumulation. We also provide evidence to suggest that SGK1 phosphorylation is rapamycin sensitive in laforin-deficient cells and perhaps mediated by mTOR. We further show that inhibition of SGK1 suppresses mTOR activity and restores autophagic defects in laforin-deficient cells. We propose SGK1 as a potential therapeutic target for the treatment of LD.

## RESULTS

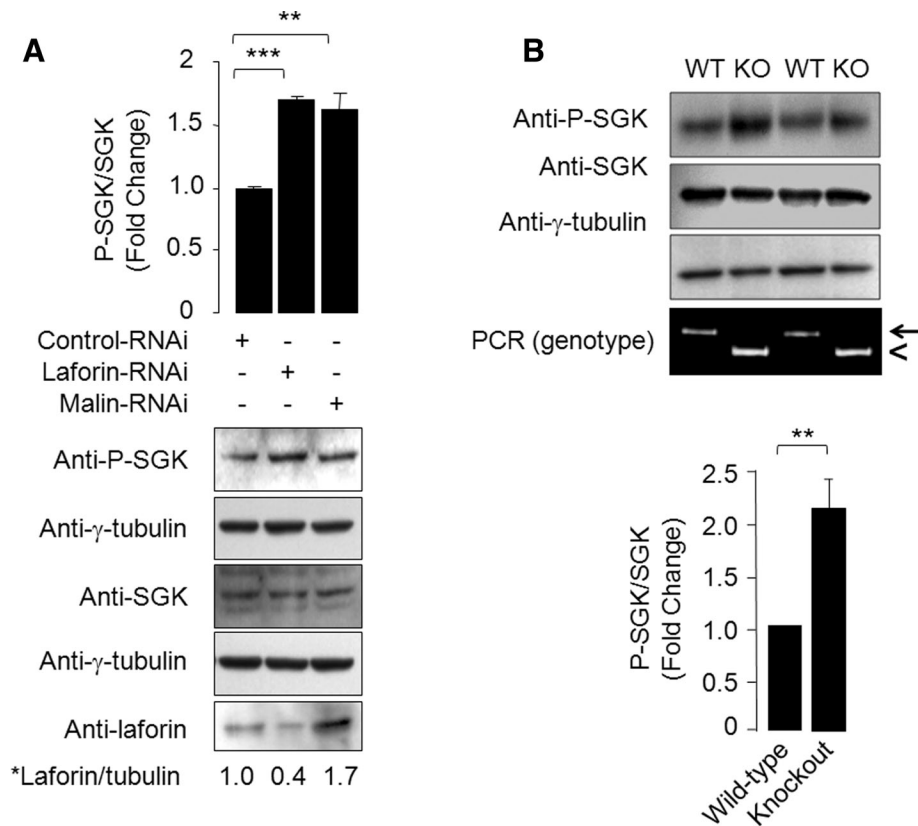
### Loss of laforin or malin results in activation of SGK1

We recently demonstrated that loss of laforin or malin results in increased abundance of glucose transporters in the plasma membrane, with resultant increase in glucose uptake (Singh *et al.*, 2012a). One of the factors known to regulate glucose transporters is the SGK1 (Palmada *et al.*, 2006; Jeyaraj *et al.*, 2007). SGK1 is fully active

when phosphorylated at Thr-256 (Kobayashi and Cohen, 1999), and the active SGK1 phosphorylates glucose transporters and promotes their membrane abundance (Palmada *et al.*, 2006; Jeyaraj *et al.*, 2007). We therefore explored the possible functional link between LD proteins and SGK1 by evaluating the phospho–Thr-256 level of SGK1 in the absence of these proteins. As shown in Figure 1A, knockdown of laforin or malin resulted in a significant increase in the level of the phosphorylated SGK1 as compared with the control set, whereas the total level of SGK1 is unchanged. The efficiency of the knockdown construct used is well established (Singh *et al.*, 2012a,b) and was further validated in a number of experiments performed in the present study (see Supplemental Figure S1 and Figures 1–6). Next we examined the level of SGK1 in laforin-deficient mice (Ganesh *et al.*, 2002). In our previous study, we demonstrated the enrichment of glucose transporter 1 (Glut1) and Glut4 proteins in the plasma membrane fraction of skeletal muscle tissue of laforin-deficient mice (Singh *et al.*, 2012a). Therefore we wanted to check the phosphorylation levels of SGK1 in laforin-deficient mice as well. As shown in Figure 1B, the phospho-SGK1 level was significantly higher in the skeletal muscle tissue of laforin-deficient mice, further suggesting that loss of laforin (or malin) would result in increased phosphorylation of SGK1 and that SGK1 might be overactive in LD tissues. The specificity of SGK1 antibodies (total and phospho forms) was established by knocking down SGK1 using short hairpin RNA interference (shRNAi; Supplemental Figure S1, E and F).

### Inhibition of SGK1 abrogates excessive glycogen buildup in laforin-deficient cells by decreasing level of plasma membrane-bound Glut1

Because loss of laforin/malin results in increased abundance of glucose transporters (Singh *et al.*, 2012a) and SGK1 appears to be hyperactive in the laforin-deficient condition, we tested whether inhibition of SGK1 activity in laforin/malin-deficient cells would reduce the level of the plasma membrane-bound form of glucose transporters. As shown in Figure 2A and reported earlier (Singh *et al.*, 2012a), knockdown of laforin or malin led to a significant increase in the level of Glut1 in the plasma membrane fraction in COS7 cells. We used a kinase-deficient, dominant-negative SGK1 variant (SGK1-DN) that blocks the kinase activity of SGK1 (Amato *et al.*, 2009) to test our hypothesis that blocking SGK1 activity prevents the enrichment of Glut1 in the plasma membrane in laforin-deficient cells. As expected, coexpression of SGK1 significantly reduced the level of Glut1 in the plasma membrane fraction of laforin-deficient cells (Figure 2A). However, no such effect was noted when SGK1-DN was expressed in malin-knockdown cells (Figure 2A). Similar observations were made when Glut1 was transiently coexpressed with SGK1-DN (Figure 2B). As reported earlier (Singh *et al.*, 2012a), no difference in level of Glut1 (endogenous or the overexpressed form) was observed when the whole-cell lysate was analyzed (unpublished data). SGK1 is one of the determinants of cellular glucose uptake (Palmada *et al.*, 2006; Jeyaraj *et al.*, 2007). Therefore we next explored whether the increased glucose uptake and glycogen accumulation observed in the laforin/malin-deficient cells depends on SGK1 activity. As shown in Figure 2C, coexpression of SGK1-DN prevented excessive glucose uptake and excessive glycogen synthesis seen otherwise in laforin-deficient cells. However, no such difference was noted in malin-knockdown cells (Figure 2C). To further confirm that the observed difference in the glycogen level is indeed due to the diminished activity of SGK1, we next used a pharmacological inhibitor for SGK1. SGK1 requires heat shock protein 90 (HSP90) for its activity, and inhibition of HSP90 with geldanamycin also inhibits



**FIGURE 1:** Loss of laforin or malin results in the activation of SGK1. (A) The level of SGK1 was measured with antibodies specific to its phospho form (anti-P-SGK1) and/or its total form (anti-SGK1) by immunoblotting upon partial knockdown of laforin or malin in COS7. The relative change the phospho level of SGK1 was plotted as a bar diagram (top) to represent the fold change vs. control knockdown by measuring signal intensities (densitometry analysis) of three independent sets. Densitometric values (fold change) of the laforin-specific band (normalized for tubulin) are listed below the blot (identified by an asterisk). (B) Immunoblot showing the change in the level of phospho-SGK1 (anti-P-SGK1) or its total level (anti-SGK1) in the skeletal muscle tissue of laforin-knockout mice (KO) as compared with their wild-type littermates (WT). The gel image shown below reveals the genotype of the animals as typed by allele-specific PCR (arrow, 620-base pair wild-type allele; arrowhead, 430-base pair knockout allele). Bar diagram (shown below) depicts the fold change in the level of phospho-SGK1 in laforin-deficient muscle tissues by measuring signal intensities (densitometry analysis). For both A and B,  $\gamma$ -tubulin level served as loading control (\*\* $p < 0.005$  vs. control; \*\*\* $p < 0.0005$  vs. control).

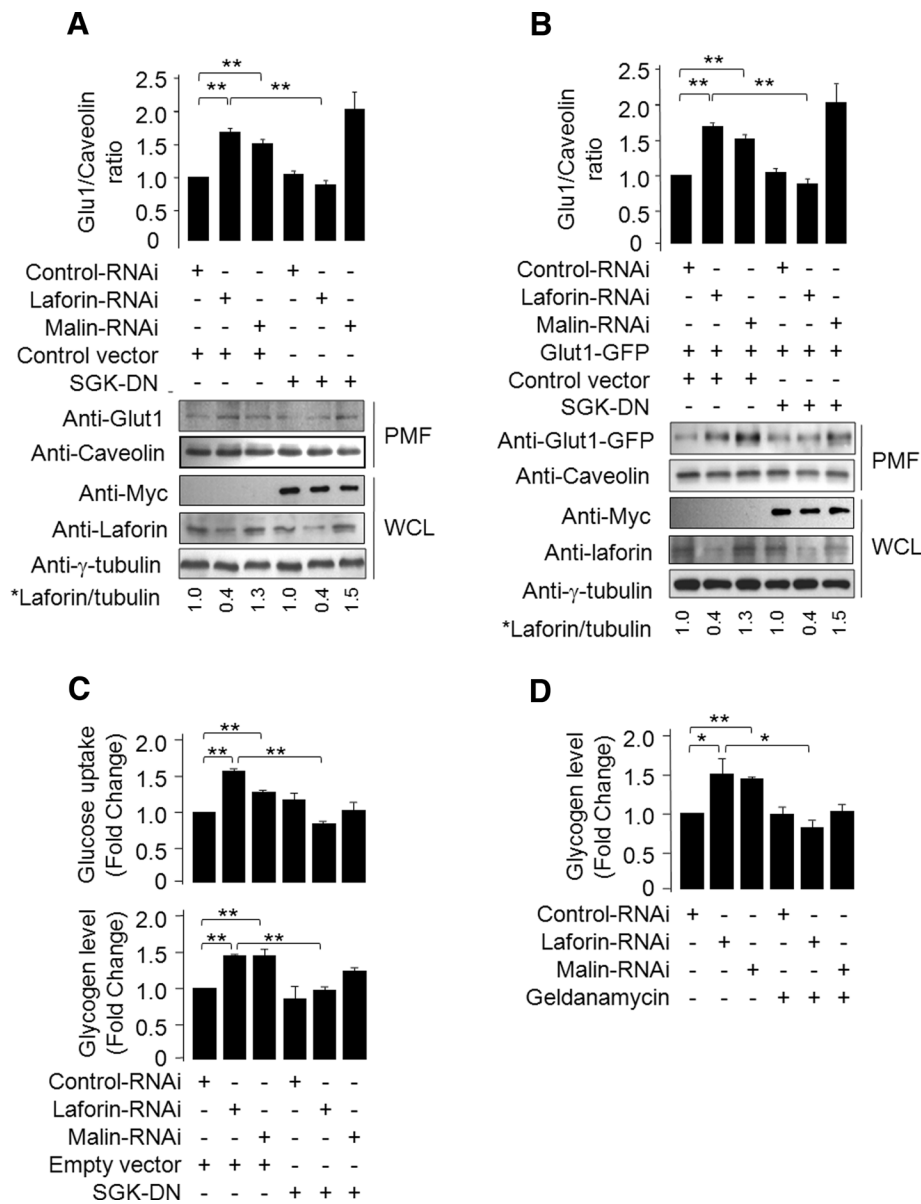
SGK1 kinase activity (Belova et al., 2008). Cells transfected with the knockdown constructs were therefore treated or not treated with geldanamycin (0.2  $\mu$ M, 12 h) and the cellular glycogen level estimated. As expected, geldanamycin treatment prevented excessive glycogen buildup but only under the laforin-knockdown condition (Figure 2D). Taken together, our results suggest that the hyperactive SGK1 might underlie excessive glycogen levels in laforin-deficient cells.

### Phosphorylation of SGK1 in the laforin-deficient condition is rapamycin sensitive

The increased SGK1 phosphorylation observed in the laforin-deficient state, in both cell and animal models, suggests that laforin can potentially be a SGK1 phosphatase. To test this possibility, we transiently expressed wild-type or catalytically inactive laforin (or malin) in COS7 cells for 36 h, either alone or in combination (Supplemental Figure S2), and evaluated the phospho-Thr-256 level of SGK1 by immunoblotting. None of these proteins, upon overexpression, altered the phospho level of endogenous SGK1 (Supplemental

Figure S2). Our repeated attempts to coimmunoprecipitate SGK1 with the transiently expressed laforin or malin were not successful, suggesting that SGK1 might not directly interact with LD proteins (unpublished data). It is likely therefore that loss of laforin might indirectly activate SGK1, possibly through one of the upstream kinases of SGK1. Studies identified mTOR as one of the regulatory kinases of SGK1 (Garcia-Martinez and Alessi, 2008; Hong et al., 2008), and the mTORC1 inhibitor rapamycin inhibits SGK1 phosphorylation at Thr-256 (Hong et al., 2008). Intriguingly, increased mTOR activity was noted in laforin-deficient mice and in fibroblasts derived from laforin-mutant patients (Aguado et al., 2010). We therefore explored whether mTOR could be the kinase that phosphorylates SGK1 in the absence of laforin. To investigate this possibility, we first checked whether, in our cellular model, knockdown of laforin activates mTOR. For this, we evaluated the phosphorylation status of the Thr-389 residue in p70S6 kinase (p70S6K), one of the downstream substrates of mTOR and a conventional marker of mTOR activity (Aguado et al., 2010). As shown in Figure 3A, knockdown of laforin did not change the total level of p70S6K but led to an increase in the level of its Thr-389 phospho form, suggesting the activation of mTOR in laforin-deficient cells in our experimental conditions as well. We next explored the potential role of mTOR in SGK1 activation in laforin-deficient cells. For this, COS7 cells transiently expressing the laforin-knockdown construct (or a nonsilencing construct) were either treated or not treated with rapamycin (0.2  $\mu$ M; 1 h), and the Thr-256 phospho level of SGK1 was evaluated. As shown in Figure 3B, rapamycin treatment led to a reduction in the level of phospho-SGK1 even

in the laforin-deficient condition, suggesting that the activation of SGK1 in the laforin-deficient condition is likely to be mediated by mTORC1, one of the two functional complexes of mTOR that is known to be sensitive to rapamycin (Laplante and Sabatini, 2012). The 1-h rapamycin treatment, however, did not inhibit glycogen accumulation in laforin-deficient cells (Figure 3C), but when cells were treated with rapamycin for 24 h there was a significant reduction in the level of both Thr-256 phospho SGK1 and cellular glycogen content, in control and as well as in laforin-knockdown conditions (Figure 3, D and E). A reason for this could be that rapamycin requires a longer time to regulate physiological processes such as glycogen metabolism, and hence no change in glycogen could be detected at the end of 1-h treatment. Nonetheless, both treatments (1 and 24 h) led to reduction in the level of phospho p70S6K (Figure 3F), suggesting that mTOR was suppressed in both conditions. Taken together, our results suggest that mTOR activation in the laforin-deficient state could be the primary event leading to the activation of SGK1 and the increased glycogen content seen in LD tissues.

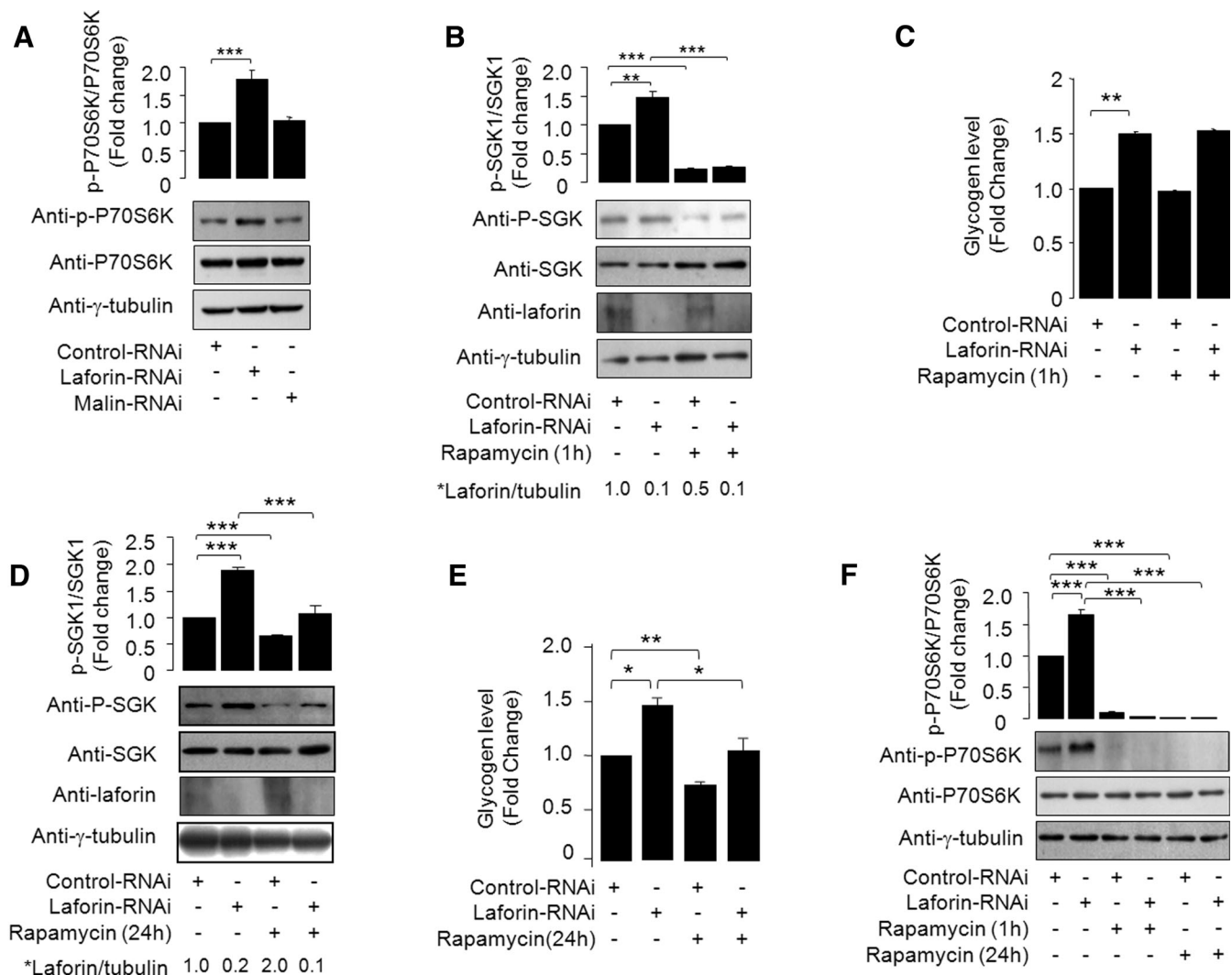


**FIGURE 2:** SGK1 inhibition reduces plasma membrane targeting of Glut1, glucose uptake, and glycogen accumulation in laforin-deficient cells. Immunoblot showing the enrichment of endogenous Glut1 (A) or transiently expressed, green fluorescent protein-tagged Glut1 (B) in plasma membrane fractionation (PMF) of COS7 cells transfected with various constructs as indicated. Probing for caveolin, a plasma membrane protein, served as loading control. Expression of the dominant-negative SGK and knockdown of laforin were confirmed by probing the whole-cell lysate (WCL) with anti-Myc (for DN-SGK) or anti-laforin (for endogenous laforin) antibodies. Efficiency of malin knockdown was confirmed by semiquantitative reverse transcription-PCR (RT-PCR) (Supplemental Figure S1C). However, the notable increase in the level of laforin in malin-knockdown cells indirectly confirms the efficiency of malin knockdown, since laforin is an established substrate of malin. Top, bar diagram depicting the fold change in the level of Glut1 in the PMF by measuring signal intensities (densitometry analysis from three independent sets;  $**p < 0.005$  vs. control). Densitometric values (fold change) of the laforin-specific band (normalized for tubulin) are listed below the blot (identified by an asterisk). (C) Bar diagram showing the fold change in the levels of cellular glucose uptake (top) and intracellular glycogen level (bottom) in COS7 cells transfected with the construct coding for the dominant-negative form of SGK (SGK1-DN) and the knockdown construct for laforin or malin, as indicated. (D) Bar diagram showing the fold change in the intracellular glycogen level in cells treated or not treated with the SGK1 inhibitor geldanamycin in cells transiently transfected with the knockdown constructs as indicated. For both C and D, each bar represents the average of three independent experiments ( $*p < 0.05$ ,  $**p < 0.005$  vs. control; Student's *t* test).

### Glycogen accumulation in laforin-deficient state is autophagy independent

Loss of laforin inhibits autophagosome formation (Aguado *et al.*, 2010). Therefore it has been suggested that the autophagic defect could also contribute to glycogen buildup in laforin-deficient cells (Puri and Ganesh, 2010), since glycogen is cleared via autophagy (Kotoulas *et al.*, 2004). mTOR is a negative regulator of autophagy, and studies have shown that rapamycin treatment inhibits mTOR activity and consequently induces autophagy (Ravikumar *et al.*, 2004). Thus reduction in the glycogen level observed in rapamycin treated laforin-deficient cells could be due to its enhanced degradation via the autophagic pathway. To test this possibility, we first looked at the autophagic process in our cellular model. We evaluated the level of LC3-II, a marker for autophagosome formation. As shown in Figure 4A, loss of laforin led to a reduction in the formation of autophagosome, as judged by the decreased level of the LC3-II. We next tested whether rapamycin treatment could induce autophagy in laforin-deficient cells. Surprisingly, whereas rapamycin treatment led to the induction of autophagy in control cells, no significant effect was seen in cells transfected with the laforin-knockdown construct (Figure 4B). Therefore it could be suggested that rapamycin may not induce autophagy in the laforin-deficient state and that the observed reduction in cellular glycogen level in rapamycin-treated, laforin-deficient cells could be due to the inhibition of SGK1-mediated glucose uptake. To further strengthen this notion, we used bafilomycin A1 (BafA1), an antibiotic compound known to perturb the fusion between autophagosome and lysosome. BafA1 treatment did not significantly alter the cellular glycogen level in rapamycin-treated, control knockdown cells as compared with rapamycin-treated, laforin-deficient cells (Figure 4C), further confirming that the observed effect of rapamycin on cellular glycogen content appears to be autophagy independent.

We showed earlier that glucose deprivation (a glycogenolytic condition) led to glycogen breakdown even in laforin-deficient cells, suggesting that the glycogen degradation pathway is not altered upon loss of laforin (Singh *et al.*, 2012a). Glucose deprivation is a known inducer of autophagy (Kim *et al.*, 2011), and therefore we were interested in investigating whether glycogen degradation during glucose deprivation is mediated by autophagy. For this, the cells were glucose starved and treated or not

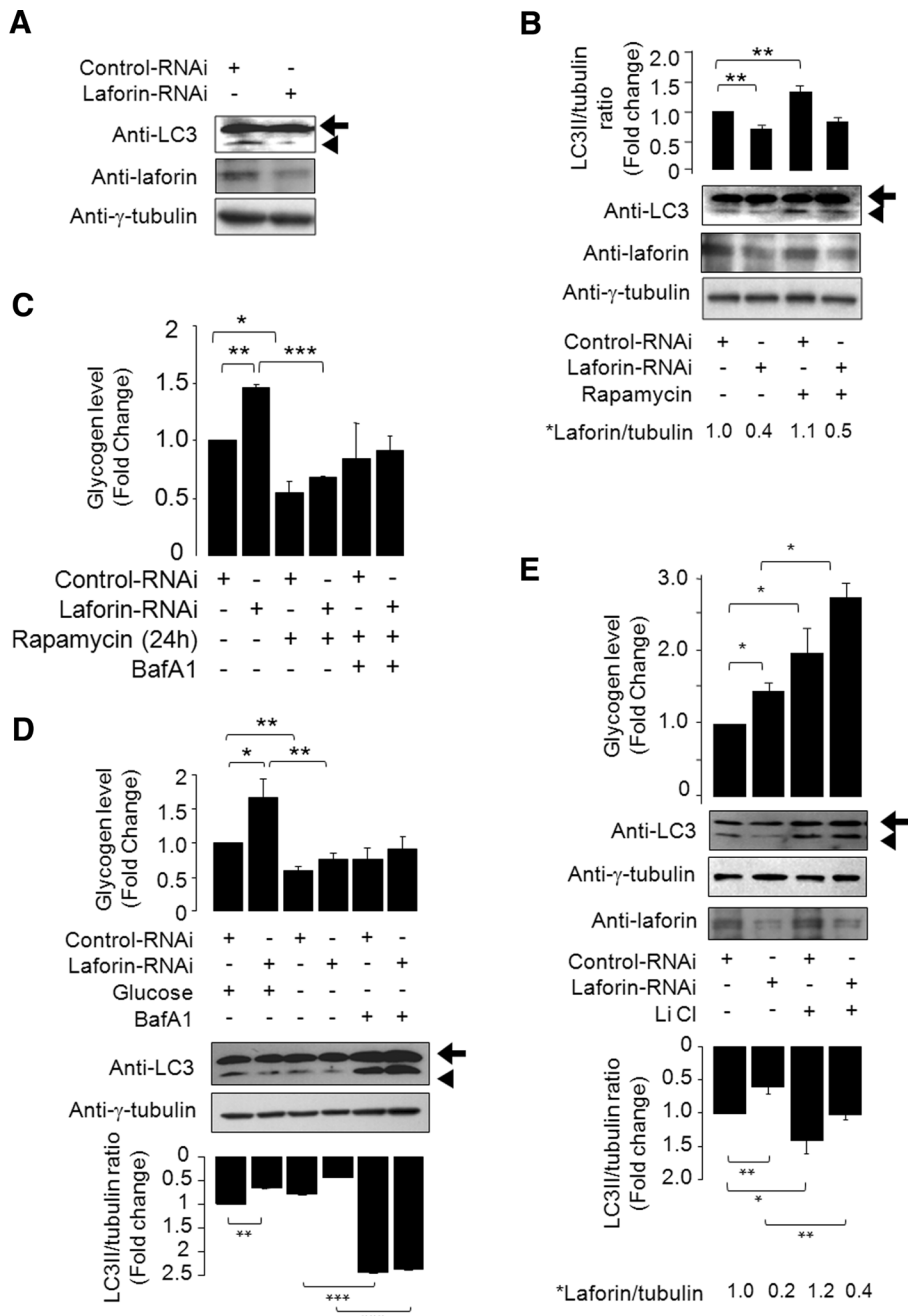


**FIGURE 3:** Loss of laforin indirectly regulates SGK1 phosphorylation. (A) Immunoblot showing phospho and total levels of P70S6K in COS7 cells transfected with the control and laforin-knockdown construct. (B) Immunoblot showing the level of total and phospho-SGK1 levels in COS7 cells transfected with the control or the laforin-knockdown construct and treated or not treated with rapamycin (0.2  $\mu$ M) for 1 h, as indicated. The knockdown efficiency was established by probing the blot with anti-laforin antibody. Top, bar diagram showing the fold change in the phospho SGK ( $N = 3$ ;  $**p < 0.005$  vs. control). (C) Bar diagram depicting cellular glycogen content under similar conditions as in B ( $N = 3$ ;  $**p < 0.005$  vs. control). (D) Total and phospho-SGK1 level and (E) glycogen content in control and laforin-knockdown cells treated with rapamycin for 24 h. Results are representative of three independent experiments and show fold change in glycogen level vs. control knockdown ( $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.0005$  vs. control; Student's *t* test). (F) Immunoblot showing the phospho and total levels of P70S6K in COS7 cells transfected with the knockdown construct and treated or not treated with rapamycin, as indicated. Tubulin served as loading control for all immunoblots. Each bar represent the average of three independent experiments ( $*p < 0.05$ ,  $**p < 0.005$  vs. control; Student's *t* test). The densitometric values (fold change) of the laforin-specific band (normalized for tubulin) are listed below B and D (identified by an asterisk).

treated with BafA1, and the cellular glycogen level was measured (Figure 4D). As shown in Figure 4D, glucose deprivation did significantly reduce the cellular glycogen level in both control and laforin-knockdown cells. However, blockade of autophagy with BafA1 treatment (as judged by increased level of LC3-II) under the same conditions did not significantly prevent reduction in glycogen level, suggesting that changes in glycogen level in laforin-deficient cells are not directly connected to defects in the autophagic process.

Autophagy is also induced by mTOR-independent mechanisms (Fleming *et al.*, 2011). We therefore tested whether an

mTOR-independent mechanism could rescue the autophagy defect in laforin-deficient cells. We used LiCl, which induces autophagy by inhibition of inositol monophosphatase independently of mTOR inhibition (Sarkar *et al.*, 2005). Of interest, as shown in Figure 4E, LiCl treatment (10 mM, 24 h) restored the level of LC3-II in laforin-knockdown cells. However, the same treatment led to an increase in the level of glycogen in both control and laforin-deficient conditions (Figure 4E), suggesting that autophagic defects may not have a causal role in glycogen accumulation in laforin-deficient cells.

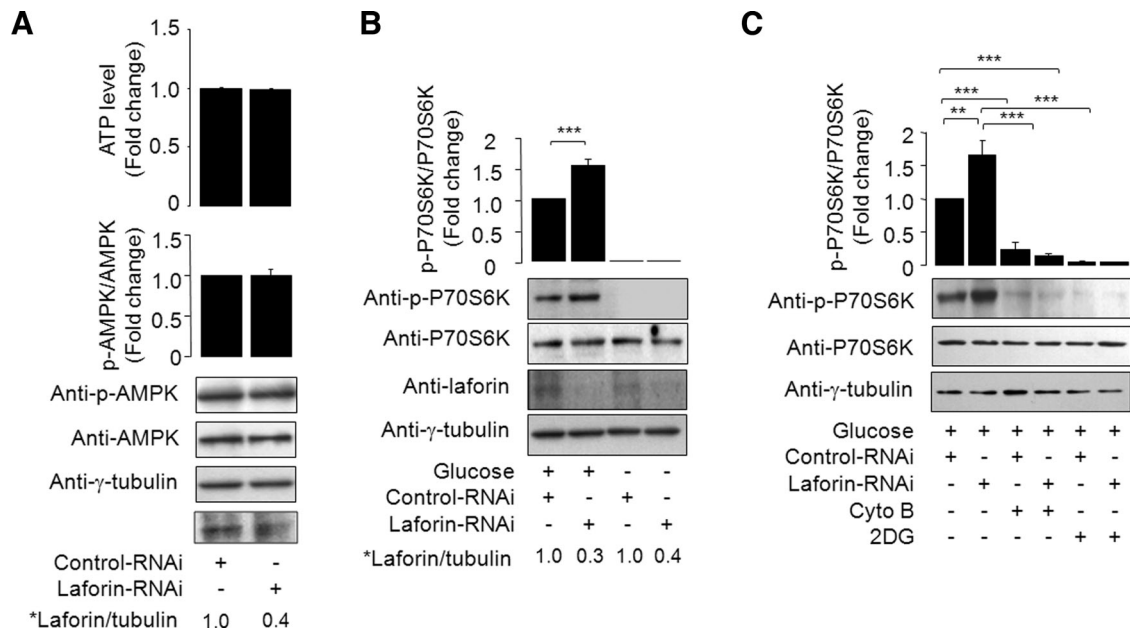


**FIGURE 4:** Glycogen accumulation in laforin-deficient cells is rapamycin sensitive but autophagy independent. Immunoblots showing LC3II level under control and laforin-knockdown conditions when cells were left untreated (A) or treated with rapamycin (B). (C) Bar diagram showing fold change in glycogen level in control and laforin-knockdown COS7 cells and treated with rapamycin for 24 h either alone or in the presence of the autophagy inhibitor bafilomycin A1 (BafA1), as indicated. (D) Glycogen level (top, bar diagram) in COS7 cells transfected with control or laforin-knockdown construct and glucose starved for 12 h at 36 h posttransfection in the presence or absence of bafilomycin A1 treatment, as indicated. The lysates were also probed with anti-LC3 antibody (middle) to show autophagy blockade. The bar diagram (below) indicates the fold change of the LC3II band as measured by densitometry ( $N = 3$ ;  $**p < 0.005$  vs. control; Student's  $t$  test). (E) Immunoblot showing LiCl-mediated partial restoration of autophagy blockade in laforin-deficient cells. COS7 cells transiently transfected with the control or laforin-knockdown construct were treated or not treated with LiCl (10 mM, 24 h), and the level of LC3II was evaluated with immunoblotting. The bottom bar diagram shows the fold change in the level of the LC3II band, and the top bar diagram represents the fold change in glycogen content under similar conditions. Each bar in both figures represents the average of three independent experiments ( $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.0005$  vs. control; Student's  $t$  test). The densitometric values (fold change) of the laforin-specific band (normalized for tubulin) are listed below B and E (identified by an asterisk).

### mTOR activation in the laforin-deficient condition is glucose dependent

We were next interested in understanding the cellular mechanism(s) that activate mTOR in the laforin-deficient state. mTOR regulates cellular homeostasis by modulating various anabolic and catabolic processes. The activity of mTOR is regulated by several upstream signals, including the nutrient, growth factor, and the energy source (Hay and Sonenberg, 2004; Sengupta *et al.*, 2010). Growth factors regulate mTOR activity via the phosphoinositide 3-kinase (PI3K)/AKT pathway, whereas nutrients (glucose and amino acids) regulate mTOR activity through various GTPases and the AMP-activated protein kinase (AMPK; Efeyan *et al.*, 2013). No alteration in PI3K/AKT pathway was observed in laforin-deficient cells (Supplemental Figure S1D) or laforin-deficient mice (Puri *et al.*, 2009). We therefore wanted to investigate whether a nutrient-dependent mechanism could be the trigger for mTOR activation in laforin-deficient cells. Glucose availability regulates mTOR activity by both AMPK-dependent and AMPK-independent mechanisms (Kimura *et al.*, 2003; Buller *et al.*, 2011). Because the intracellular AMP/ATP ratio regulates AMPK-dependent mTOR activity (Kimura *et al.*, 2003), we first investigated whether loss of laforin perturbs the cellular AMP/ATP ratio and the AMPK activity. As shown in Figure 5A, neither cellular ATP content nor AMPK activity (as measured by level of phospho-Thr-172 AMPK) showed any difference in laforin-deficient cells, suggesting that mTOR activation in laforin-deficient cells is AMPK independent.

Intracellular glucose availability and glycolytic flux regulate mTOR activity independently of the AMPK and tuberous sclerosis complex (Lee *et al.*, 2009; Buller *et al.*, 2011). We therefore investigated whether increased cellular glucose availability in laforin-deficient cells could be a potential reason for mTOR activation. To test this, we maintained cells transiently transfected with a laforin-knockdown construct or a non-silencing construct in the presence or absence of glucose for 12 h and evaluated the level of phospho-p70S6K (Thr-389). Indeed, as shown in Figure 5B, glucose deprivation led to a significant reduction in phospho-p70S6K level even in the absence of laforin, suggesting that activation of mTOR in laforin-deficient state could be due to increased intracellular glucose level. To further confirm this, we treated cells transiently transfected with the laforin-knockdown construct with cytochalasin B (a glucose uptake inhibitor) or 2-deoxyglucose (a glycolytic inhibitor) for 12 h and checked the



**FIGURE 5:** mTOR activation in the absence of laforin is glucose dependent. (A) Cellular ATP content was measured in COS7 cells transfected with either the control or laforin-knockdown construct, and the fold change is presented in the top bar diagram. Similarly, the relative levels of phospho (Thr-172) AMPK and its total form were evaluated by immunoblotting (bottom) in cells transfected with a knockdown construct for laforin or a control vector, and the relative ratio is plotted as a bar diagram (middle). (B, C) Phospho and total levels of p70S6K were estimated by immunoblotting in lysates of cells that were transiently transfected with laforin-knockdown construct or a nonsilencing control construct and grown in the presence of absence of glucose (B) or upon treatment either cytochalasin B or 2-deoxy glucose (2DG) for 12 h (C), as indicated. The bar diagrams (top) show the fold difference in the phospho form as measured by densitometry analysis of the signal ( $N = 3$ ,  $**p < 0.005$ ,  $***p < 0.0005$  vs. control; Student's  $t$  test). The densitometric values (fold change) of the laforin-specific band (normalized for tubulin) are listed below A and B (identified by an asterisk).

p70S6K phosphorylation level. As shown in Figure 5C, both treatments led to a significant reduction of phospho-p70S6K level even in the absence of laforin. Cumulatively these observations suggest that activation of mTOR in laforin-deficient cells is suppressed by restricting cellular glucose availability, and therefore increased cellular glucose level could be a contributing factor toward mTOR activation in laforin-deficient cells.

### Overexpression of dominant-negative SGK1 inhibits mTOR activity and restores autophagy defects in laforin-deficient cells

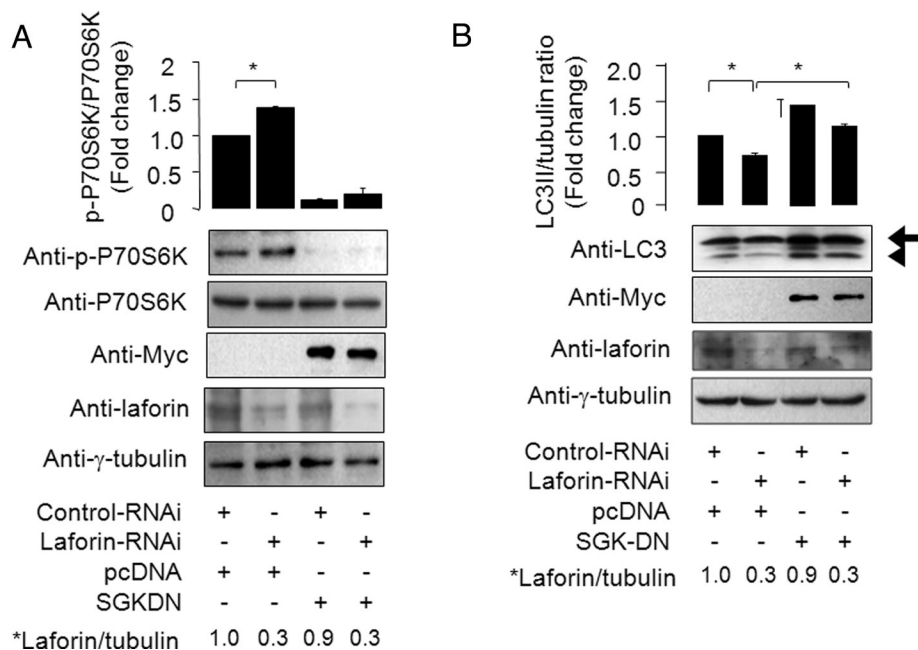
With the observation that cellular glucose levels modulate mTOR activity and that inhibition of SGK1 suppresses excessive glucose uptake in laforin-deficient cells, we were next interested in testing whether suppression of SGK1 activity would inhibit mTOR activity in laforin-deficient cells. Therefore the phospho-p70S6K level was evaluated in cells transfected with laforin-knockdown construct and a construct that codes for the dominant-negative SGK1. As shown in Figure 6A, dominant-negative SGK1 significantly reduced phosphorylation of p70S6K in laforin-deficient cells, as well as in control cells, suggesting the existence of a positive feedback loop between SGK1 and mTOR activity. In this regard it is interesting to note that a very similar mechanism was proposed in diabetic glomeruli, in which enhanced expression of Glut1 resulted in mTOR activation (Buller *et al.*, 2011).

To evaluate the therapeutic potential of SGK1 inhibition in LD, we next checked whether inhibition of SGK1 would also rescue the autophagy defect in the laforin-deficient condition. We measured the level of LC3II by immunoblotting in cells that expressed the

laforin-knockdown construct and dominant-negative SGK1. As shown in Figure 6B, coexpression of dominant-negative SGK1 was able to significantly enhance autophagic flux even in laforin-deficient cells, suggesting that wild-type SGK1 is an inhibitor of the autophagic process. Taken together, our results suggest that the autophagic defects in LD tissues could be due to activation of SGK1.

### DISCUSSION

The laforin and malin complex appears to function at more than one step in regulating glycogen metabolism. For example, a role for laforin in removing erroneously added phosphate during the glycogen synthesis process has been proposed, and therefore loss of laforin could result in hyperphosphorylated glycogen in LD tissues (Tagliabracci *et al.*, 2011), although the specific reason for the same in malin-deficient mice (Turnbull *et al.*, 2010) is not yet established. Suppression of PTG—a regulatory subunit of protein phosphatase 1—prevented the formation of Lafora bodies in laforin-deficient mice (Turnbull *et al.*, 2011a), although why did these animals had normal form of glycogen was not well understood, as these double mutants should have favored the synthesis of abnormally hyperphosphorylated glycogen since they lack laforin. In the present study, we extended our previous finding of laforin- and malin-regulated glycogen metabolism at the level of glucose uptake and show that the SGK1 could be the connecting link between the LD proteins and glycogen accumulation. It is intriguing to note that a majority of Lafora bodies in muscle are present in fast-twitch (type II) muscle fiber (Turnbull *et al.*, 2011b), a site that shows predominant SGK1 expression (Andres-Mateos *et al.*, 2013).



**FIGURE 6:** Dominant-negative SGK1 restores the autophagic defect seen in the laforin-deficient state. (A) Immunoblot showing the relative levels of phospho and total p70S6K in COS7 cells transfected with the dominant-negative SGK1 (SGK-DN) and the knockdown construct as indicated. The bar diagram (top) depicts the fold change in the ratio of phospho to total p70S6K levels in respective lanes and plotted with respect to the control (densitometry analysis from three independent sets; \* $p < 0.05$ , Student's  $t$  test). (B) Immunoblot showing the relative levels of LC3II species (arrowhead) in COS7 cells transfected with knockdown constructs with or without dominant-negative SGK-DN. Tubulin served as loading control. Each bar represents the average of three independent experiments (\* $p < 0.05$ ; Student's  $t$  test). The densitometric values (fold change) of the laforin-specific band (normalized for tubulin) are listed below A and B (identified by an asterisk).

SGK1, a gene originally discovered to be induced by serum and glucocorticoids in tumor cells (Firestone *et al.*, 2003), is one of the downstream players in the PI3K signaling cascade and regulates the cell surface localization and function of several metabolite transporters and ion channels (Lang *et al.*, 2006; Lang and Shumilina, 2013). For example, SGK1 activates glucose transporters SGLT1, GLUT1, and GLUT4 via phosphorylation and by targeting them to the plasma membrane (Palmada *et al.*, 2006; Jeyaraj *et al.*, 2007). It was also shown that SGK1-regulated cellular glucose uptake is salt sensitive (Boini *et al.*, 2006). The activity of SGK1 is regulated by the serum growth factor and insulin via their downstream target kinases such as PDK1 and mTOR, which are known to phosphorylate SGK1 (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Garcia-Martinez and Alessi, 2008; Hong *et al.*, 2008). Our observations that suppression of SGK1 prevented the plasma membrane targeting of glucose transporters, excessive glucose uptake, and glycogen accumulation upon loss of laforin suggest that SGK1 activation in laforin-deficient cells might underlie some of these metabolic changes. Intriguingly, suppression of SGK1 did not show any such effect in the malin-deficient condition, although SGK1 appeared to be overactive upon loss of malin. One possible explanation is that the process of activation of SGK1 could be different upon loss of malin as compared with loss of laforin. For example, the activity of SGK1 also depends on its subcellular localization (Firestone *et al.*, 2003). Thus loss of malin may alter the subcellular localization of SGK1 in addition to its activity, and the approaches used in the present study to suppress SGK1 might be ineffective in its new environment. This suggestion, however, is purely speculative, and the cellular process that activates

SGK1 in malin-deficient cells needs to be studied further.

There are at least two possible ways by which the loss of laforin could activate the SGK1: 1) a direct mechanism, in which laforin acts as an SGK1 phosphatase, and 2) an indirect mechanism, in which loss of laforin activates one of the upstream kinases of SGK1. The first possibility does not seem likely since we could not observe an interaction between laforin and SGK1 in our pull-down assays. However, in accordance with the second possibility, we found that mTOR is a likely link between SGK1 and laforin. Our observations that mTOR inhibition suppresses SGK1 activity and, conversely, that dominant-negative SGK1 inhibits mTOR activity suggest the existence of a feedforward loop between SGK1 and mTOR in laforin-deficient cells; the activation of one results in the activation of the other. PI3K signaling is known to regulate glucose/glycogen metabolism (Ueki *et al.*, 1998; Farese, 2001), and AKT is one of its targets (Alessi *et al.*, 1996). Because no change in the total level or in the Ser-473 phospho level of AKT was observed in the laforin-deficient mice (Puri *et al.*, 2009), malin-deficient mice (DePaoli-Roach *et al.*, 2012), or cell lines upon knockdown of laforin or malin (Supplemental Figure S1D), we hypothesize that SGK1 and mTOR activation might occur through a PI3K-independent pathway. Note that intracellular calcium ions (Imai *et al.*, 2003; Gulati

*et al.*, 2008; Lang *et al.*, 2013) and the small GTPase Rac1 (Shelly and Herrera, 2002; Sacci *et al.*, 2011) activate both SGK1 and mTOR. Conversely, SGK1 regulates calcium signaling as well (Lang *et al.*, 2013). Therefore it will be of interest to see whether these two signaling cascades are altered in LD tissues.

mTOR is a serine/threonine kinase and a central component of nutrient-sensitive signal pathways that regulate several cellular processes, such as cell growth and metabolism (Wullschlegel *et al.*, 2006; Laplante and Sabatini, 2012). For example, mTOR directly regulates the expression of nesfatin-1, a peptide hormone that suppresses appetite and the production of body fat in mammals (Li *et al.*, 2012). mTOR exists as two distinct complexes: mTOR complex 1 (mTORC1) and mTORC2. These two complexes are distinguished by unique accessory proteins—raptor in mTORC1, and rictor in mTORC2 (Laplante and Sabatini, 2012)—and their sensitivity toward rapamycin, with mTORC1 being more sensitive than mTORC2 (Laplante and Sabatini, 2012). mTORC1 activity is regulated by input from various signals, including the growth factor, nutrient (glucose and amino acids), and energy. For example, nutrient deprivation stabilizes the interaction between mTOR and raptor, resulting in inhibition of mTORC1 (Kim *et al.*, 2002), and, conversely, glucose availability and higher glycolytic flux trigger Rheb, an adaptor protein, to bind and activate mTORC1 (Lee *et al.*, 2009). Although abnormal mTOR activation is known in laforin-deficient mice (Aguado *et al.*, 2010), the specific signal that activates mTOR in laforin-deficient state was not elucidated. We show here that restricting glucose availability or inhibiting glycolytic flux reduces mTOR activity. Therefore excessive glucose is likely to be one of the



triggers that activate mTORC1 in laforin-deficient cells. Considering the fact that mTORC1 is a critical regulator of protein translation, it would be interesting to see whether mTOR regulates the function of LD proteins in posttranscriptional gene regulation (Ganesh *et al.*, 2000; Singh *et al.*, 2012b).

The activation of mTORC1 was proposed to cause the autophagic defects in laforin-deficient mice (Aguado *et al.*, 2010). However, we found that the autophagy defect in the laforin-deficient condition is perhaps mTOR independent, as it is not rescued by rapamycin treatment. This is supported by the observation made in malin-deficient mice, in which autophagy defects are seen without any alteration in mTOR activity (Criado *et al.*, 2012). This was strengthened by our observation that lithium treatment, which activates autophagy via an mTOR-independent pathway, restored autophagic blockade in laforin-deficient cells. Lithium inhibits inositol monophosphatase to deplete the free inositol level (Sarkar *et al.*, 2005). Of interest, hyperglycemia-induced uptake of inositol has been found in rat mesangial cells (Guzman and Crews, 1992), and therefore it is possible that a similar mechanism might operate in LD and contribute to autophagy defects. Our demonstration that the autophagy defect does not lead to glycogen accumulation in laforin-deficient cells suggests that mTOR activation, autophagy defect, and glycogen accumulation are independent outcomes of laforin deficiency. Similarly, our demonstration that partial suppression of SGK1 restores the autophagic process and suppresses mTOR activity in laforin-deficient cells reveals a novel function for SGK1 and that these defects could be secondary to the activation of SGK1 in LD tissues. While this article was being written, Andres-Mateos *et al.* (2013) documented that the activation of starvation-induced autophagy is compromised in a transgenic mice line that overexpressed constitutively active SGK1, which independently established a critical role for SGK1 in autophagy induction. Taken together, our findings propose that suppression of SGK1 could be an effective therapeutic approach to suppressing glycogen accumulation, inhibiting mTOR activity, and rescuing autophagy defects in LD. Given that SGK1 inhibitors are known (Ackermann *et al.*, 2011), it would be of significance to test their therapeutic potential in LD animal models.

## MATERIALS AND METHODS

### Cell culture, transfection, expression constructs, and animal models

COS7 cells were obtained from the American Type Cell Culture (ATCC, Manassas, VA) and grown in DMEM (Sigma-Aldrich India, Bangalore, India) with 25 mM of glucose and 10% (vol/vol) fetal calf serum (HyClone, Logan, UT). Transfection was done using Turbofect (Thermo Fisher Scientific India, Mumbai, India) as recommended by the manufacturer. All of the chemicals were purchased from Sigma-Aldrich India unless stated otherwise. The mammalian expression constructs used for the expression of wild type or the mutant forms of laforin, malin, and the glucose transporters were described previously (Singh *et al.*, 2012a). The expression construct that codes for the dominant-negative SGK1 mutant (Amato *et al.*, 2009) was generously provided by Nicola Perrotti (University Magna Graecia, Catanzaro, Italy). The shRNA knockdown constructs for laforin, malin, and the control nonsilencing RNAi vector were purchased from Open Biosystems (Katy, TX) and were validated in our previous studies (Garyali *et al.*, 2009; Sengupta *et al.*, 2011; Singh *et al.*, 2012a,b). The shRNA construct for the knockdown of SGK1 was a gift from Emory H. Y. Lee (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). Skeletal muscle tissues of the laforin-knockout mice and their wild-type

littermates were generously provided by Kazuhiro Yamakawa (RIKEN Brain Science Institute, Wako, Japan).

### Immunoblotting and antibodies

Immunoblotting was done essentially as described previously (Singh *et al.*, 2012a). In brief, after transfer of protein onto a nitrocellulose membrane (MDI, Ambala, India), it was blocked with either a 5% solution of nonfat dry milk powder (G-Biosciences, St. Louis, MO) or bovine serum albumin (Sisco Research Laboratories, Mumbai, India) in 1× Tris-buffered saline and Tween 20 for 1 h. Subsequently, the membrane was probed with primary antibody, followed by secondary antibody tagged with horseradish peroxidase as recommended by the manufacturer. Proteins were visualized using a chemiluminescent detection kit (SuperSignal West PICO; Pierce, Rockford, IL). The following antibodies were used: anti-Myc and anti-green fluorescent protein were from Roche India (Mumbai, India); anti-Glut1, anti-SGK1, and anti-phospho (Thr-256) SGK1 were from Millipore India (Bangalore, India); anti-LC3, anti-AKT, anti-phospho (Ser-473) AKT, anti-p70S6K, anti-phospho (Thr-389) p70S6K, anti-AMPK, anti-phospho (Thr-172) AMPK, and anti-caveolin were from Cell Signaling Technology (Danvers, MA). The anti-tubulin antibody was from Sigma-Aldrich India. Anti-laforin antibody was described in previous studies (Puri *et al.*, 2009; Singh *et al.*, 2012a). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

### PCR and semiquantitative PCR

Semiquantitative PCR to check the efficiency of malin-knockdown plasmid was done essentially as detailed earlier (Singh *et al.*, 2012a). For PCR-based genotyping of laforin-deficient animals, the following primers were used: forward (for both genotypes), 5'-GCATCG-GCTGTAAGTTAGCC-3'; reverse (wild type), 5'-CGTGTGTCCAT-TCTCCAGAA-3'; reverse (knockout allele), 5'-AGCGTATTCAATAAC-CCTTAAT-3', using a multiplex reaction. Amplification from the wild-type allele produces a 620-base pair fragment, and the knockout allele produces a 430-base pair fragment.

### Plasma membrane fractionation

Plasma membrane fractionation was performed essentially as described previously (Singh *et al.*, 2012a). Briefly, cells were harvested at 36 h posttransfection, and after washing twice with phosphate-buffered saline, the cells were incubated in a detergent-free cell lysis buffer (10 mM Tris, pH 7.5, 137 mM NaCl, 2 mM phenylmethylsulfonyl fluoride) including a cocktail of phosphatase and protease inhibitors. The cells were kept on ice for 10 min and mildly sonicated. The cell lysate was cleared by low-speed centrifugation as 12,000 × g for 5 min, and a small aliquot of the supernatant was saved as the total cell lysate. The supernatant was then subjected to ultracentrifugation at 100,000 × g (Sorvall MTX 150 microultracentrifuge; rotor S55-S) for 1 h. The final pellet fraction, enriched in membrane proteins, was washed twice with lysis buffer and then resuspended in lysis buffer containing 1% Triton X-100.

### Glucose uptake assay

This was done essentially as described previously (Singh *et al.*, 2012a). The cells were incubated with Krebs-Ringer-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer for 2 h and then in 100 μM 2-NBDG (Invitrogen) for 10 min at 37°C. The cells were then washed and lysed, and a small aliquot was saved for protein estimation (Bradford method). The fluorescence intensity in the remaining cell lysate was measured using a fluorescence spectrometer (LS 55; Perkin Elmer-Cetus). Nonspecific fluorescence was determined by measuring the fluorescence intensity of

cells treated with an inhibitor of glucose uptake, cytochalasin B (50  $\mu$ M). Fluorescence intensity was normalized based on protein content, and the data are presented as the fold change.

### Glycogen measurement

To measure cellular glycogen levels, cells were scraped in ice-cold 30% KOH and lysed by heating the suspension to 100°C for 20 min. A small aliquot of the lysate was saved for protein estimation, and the rest was spotted onto filter paper (Whatman 31-ET CHR). This paper was first washed in ice-cold 66% ethanol for 10 min, followed by two more washes in the same solution at room temperature, and the membrane was kept at 37°C overnight for drying. To digest glycogen, the membrane was incubated in an amyloglucosidase solution (0.5 mg/ml in 0.02 M sodium acetate, pH 4.8) for 2 h at 37°C. The released glucose was measured, and the quantity of glycogen was expressed as amount of released glucose per milligram of total protein.

### Statistical analysis

Experiments were repeated at least in triplicate. Data were analyzed by two-tailed, unpaired Student's *t* test using GraphPad (La Jolla, CA) software. Differences were considered significant at *p* < 0.05 and denoted by \**p* < 0.05, \*\**p* < 0.005, and \*\*\**p* < 0.0005, respectively.

### ACKNOWLEDGMENTS

We thank Kazuhiro Yamakawa for generously providing muscle tissue samples from laforin-deficient mice, Nicola Perrotti for SGK1 expression constructs, Emory H. Y. Lee for SGK1 shRNA plasmid, and Ashwani Kumar Thakur (Indian Institute of Technology, Kanpur, India) for sharing his laboratory facilities. We also thank our colleagues Anupama Rai and Rashmi Parihar for technical support. This work was supported by a research grant from the Department of Science and Technology, Government of India, to S.G, in the form of a Ramanna Fellowship. S.G is a Gill-Joy Chair Professor and DAE-SRC Outstanding Research Investigator. P.K.S. and S.S. received a research fellowship from the Council of Scientific and Industrial Research, Government of India.

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