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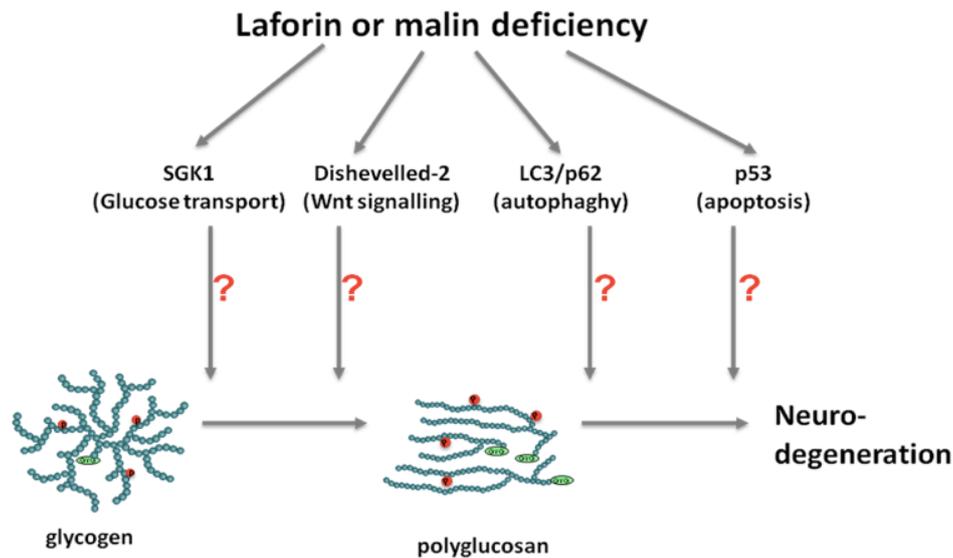
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## SGK1 (glucose transport), dishevelled2 (wnt signaling), LC3/p62 (autophagy) and p53 (apoptosis) proteins are unaltered in Lafora disease

Peixiang Wang<sup>1</sup>, Lori Israelian<sup>1,2</sup>, Yunlin Xue<sup>1</sup>, Siyuan Song<sup>3</sup>, Liliana Attisano<sup>3</sup> and Berge A. Minassian<sup>1,2,4,5</sup>

<sup>1</sup>Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON, Canada. <sup>2</sup>Institute of Medical Sciences, University of Toronto, 1 King's College Circle, Toronto, ON, Canada. <sup>3</sup>Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada. <sup>4</sup>Division of Neurology, Department of Paediatrics, The Hospital for Sick Children, Toronto, ON, Canada. <sup>5</sup>Correspondence: [berge.minassian@sickkids.ca](mailto:berge.minassian@sickkids.ca)

**Graphical Abstract:** Multiple disparate functions have been attributed to the Lafora disease gene products laforin and malin with separate hypotheses of pathogenesis of neurotoxic polyglucosans (Lafora bodies) and neurodegeneration in this disease. We tested four of these hypotheses in Lafora disease mouse models. We were unable to confirm these hypotheses.



**Abstract:** Glycogen forms through the concerted actions of glycogen synthase (GS) which elongates glycogen strands, and glycogen branching enzyme (GBE). Lafora disease (LD) is a fatal neurodegenerative epilepsy that results from neuronal accumulation of hyperphosphorylated glycogen with excessively long strands (called polyglucosans). There is no GBE deficiency in LD. Instead, the disease is caused by loss-of-function mutations in the *EPM2A* or *EPM2B* genes, encoding, respectively, a phosphatase, laforin, and an E3 ubiquitin ligase, malin. A number of experimentally derived hypotheses have been published to explain LD, including: *The SGK1 hypothesis* - Phosphorylated SGK1 (pSGK1) raises cellular glucose uptake and levels, which would activate GS. Based on observing increased pSGK1 in LD mice it was proposed that raised pSGK1 leads to polyglucosan generation through GS hyperactivation. *The Dishevelled2 hypothesis* - Downregulating malin in cell culture was reported to increase levels of dishevelled2, which through the wnt/glycogen synthase kinase-3 pathway would likewise overactivate GS. *The Autophagic defect hypothesis* - Polyglucosans may be natural

byproducts of normal glycogen metabolism. LD mice were reported to be autophagy-defective. LD would arise from failed autophagy leading to failed polyglucosan clearance. Finally, *the p53 hypothesis* - laforin and malin were reported to downregulate p53, their absence leading to increased p53, which would activate apoptosis, leading to the neurodegeneration of LD. In the present work we repeat key experiments that underlie these four hypotheses. We are unable to confirm increased pSGK1, dishevelled2, or p53 in LD mice, nor the reported autophagic defects. Our work does not support the above hypotheses in understanding this unique and severe form of epilepsy.

**Keywords:** Lafora Disease; polyglucosan; glucose transport; Wnt pathway; autophagy; apoptosis

## Introduction

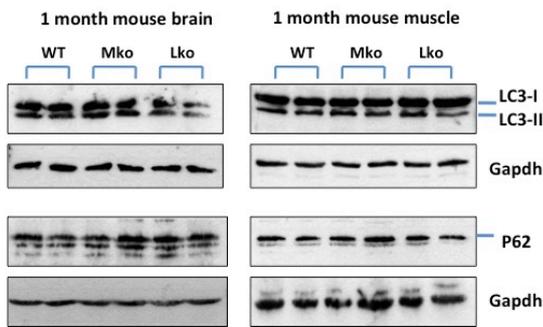
Glycogen is synthesized by glycogen synthase (GS) and glycogen branching enzyme (GBE). GS activity is downregulated by phosphorylation, mainly by glycogen synthase kinase-3 (GSK3), and allosterically activated by the main intracellular form of glucose, glucose 6-phosphate (G6P). GBE deficiency results in an abnormal form of glycogen (called polyglucosan) which is poorly branched (i.e. has chains that are too long) and which precipitates, accumulates, and in neurons clogs axons causing Adult Polyglucosan Body Disease (which clinically resembles amyotrophic lateral sclerosis). GS overactivity likewise generates polyglucosans. In horses, an overactivating muscle GS gene mutation, and in mice, transgenic overexpression of muscle GS, lead to muscle polyglucosan accumulations. In humans, deficiency of the irreversible glycolytic enzyme muscle phosphofructokinase impairs glycolysis, which raises G6P levels, which hyperactivates GS and leads to muscle polyglucosans and disease<sup>1</sup>.

Lafora disease (LD) is an epilepsy that onsets in teenagers, progresses to intractability, and gradually to death within 10 years<sup>2</sup>. Pathologically, LD is characterized by gradual accumulation in neuronal somatodendritic compartments of masses of polyglucosans (Lafora bodies; LB) that have the particularity of being hyperphosphorylated<sup>3</sup>. LB are most likely pathogenic: it was shown that preventing their formation through knockout (ko) or downregulation of GS in LD mouse models eliminates LB and rescues the disease<sup>4-6</sup>. LD is caused by loss-of-function mutations of the *EPM2A* or *EPM2B* genes encoding the interacting laforin and malin enzymes respectively<sup>7, 8</sup>. Laforin is a glycogen phosphatase, and malin a ubiquitin E3 ligase whose ubiquitination targets, at least in cell culture experiments, include GS<sup>3, 7-11</sup>. Multiple hypotheses have been advanced to explain LD, including: (I) Laforin deficiency leads to glycogen hyperphosphorylation, which, through unknown mechanisms, results in conversion of normal glycogen to polyglucosan<sup>3</sup>. (II) Malin deficiency

leads to reduced ubiquitin-mediated proteasomal degradation of GS, and the resultant increased GS drives polyglucosan generation<sup>9, 11</sup>. (III) Laforin or malin deficiency lead, through unknown mechanisms, to increased phosphorylated serum/glucocorticoid-induced kinase-1 (pSGK1), which increases glucose uptake, potentially hyperactivating GS to drive polyglucosan formation<sup>12</sup>. (IV) Malin deficiency upregulates dishevelled2, which upregulates the wnt pathway, which downregulates GSK3, which would upregulate GS, leading to polyglucosans<sup>13</sup>. (V) Polyglucosans may be byproducts of normal glycogen metabolism, which would need to be cleared, e.g. by autophagy. Laforin or malin deficiency downregulates autophagy, which would prevent clearance of polyglucosans and allow their accumulation<sup>14-16</sup>. (VI) Laforin or malin deficiency upregulates p53, which is proapoptotic and would lead to neurodegeneration<sup>17</sup>.

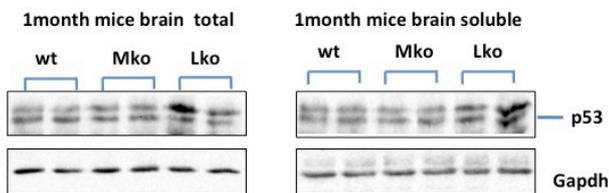
The first two hypotheses garnered the greatest attention because of the direct mechanistic connections between the disease gene products and corresponding substrates, glycogen for laforin's phosphatase activity and GS for malin ubiquitination. This led to substantial new glycogen metabolism knowledge, including the specific locations of phosphorylation in glycogen<sup>18, 19</sup>. However, both hypotheses were undermined by recent results. It was shown that expressing a laforin mutant lacking the laforin phosphatase activity in laforin ko mice rescues the disease, including eliminating LB<sup>20</sup>, suggesting that laforin's glycogen phosphatase function is dispensable, and some other laforin function is critical in LD. Secondly, it was shown that laforin or malin ko mice do not have increased amounts of soluble GS or GS activity<sup>3, 21, 22</sup>, bringing into question the relevance of the malin action on GS seen in cell culture experiments. Given these new weaknesses of the leading hypotheses we decided to take a fresh look at the other four, and as a first step repeated experiments that had been fundamental to generating each of them, and performed new experiments. In each case we were unable to replicate





**Figure 3.** LC3-II and p62 are unchanged in LD mice (anti-LC3 antibody from Novus Biologicals, and anti-p62 from Sigma). One month-old brain and muscle tissue lysates from wild-type, Mko and Lko mice, were used, respectively.

*Hypothesis VI (p53)* – Based on cell culture experiments, and observations in laforin and malin ko mouse, the authors concluded that laforin or malin deficiency lead to increased levels of p53, which leads to apoptosis, which could explain the neurodegeneration of LD<sup>17</sup>. We cannot confirm increased p53 levels in laforin or malin ko mice (Figure 4).



**Figure 4.** p53 protein level is unchanged in LD mice (anti-p53 antibody from Santa Cruz Biotechnology). Western blot results for anti-p53 antibody were obtained from total protein and soluble protein of wild-type, Mko and Lko mice, respectively.

Conversion of glycogen to toxic polyglucosans in the diseases associated with GBE deficiency or phosphofructokinase deficiency is readily explained by classical glycogen metabolism. This is not so in LD, where heretofore veiled and until recently unsuspected facets of glycogen metabolism need to be uncovered. Identification of the LD genes and enzymatic activities of their products, glycogen dephosphorylation (laforin) and GS ubiquitination (malin), were critical steps forward and gave rise to the leading ‘glycogen hyperphosphorylation’ and ‘GS hyperactivation’ hypotheses of LD. As mentioned, recent observations weakened these hypotheses and led us in this work to revisit four others. We are unable to confirm in LD mouse models the disturbance in dishevelled2 suggested by cell culture overexpression experiments and are unable to replicate results critical to the SGK1, autophagy, and increased p53-apoptosis hypotheses. A possible explanation for the difference between our mouse data with previous results is murine background. Our malin ko mice<sup>24</sup> were generated separately from others<sup>15</sup>. The laforin ko mice, on

the other hand, are the same in all LD labs<sup>23</sup>, although they have been available in the different labs for over a decade and might have diverged in genetic background. The ‘defective autophagy’ hypothesis is additionally indirectly contradicted by the following. One of the groups that advanced this hypothesis found a disturbance in LC3 in cell lines but themselves could not confirm this in mouse brain<sup>16</sup>. Secondly, a recent experiment showed that what disturbances there are in markers of autophagy in LD mice disappear with GS downregulation. In this experiment, malin ko mice were bred with GS ko mice to eventually generate malin ko animals heterozygous for GS (GS activity is reduced by 50% in these mice) or completely lacking GS. In both situations, LB were dramatically reduced, the LD neurological phenotype was completely rescued, and what autophagic marker dysregulations there were corrected<sup>4</sup>, confirming that autophagic disturbance in LD, if or when present, is secondary to the disturbance in glycogen metabolism, and is not a primary cause of LD<sup>4</sup>.

But what then are the early pathogenic steps linking laforin or malin deficiency to polyglucosan formation and LD? There are additional LD hypotheses invoking laforin/malin actions via the neurodevelopmental protein neuronatin<sup>25</sup> and others, all of which will need to be revisited. However, in our view, the original two major hypotheses should continue to be explored. After all, laforin is a confirmed glycogen phosphatase, glycogen in both laforin and malin ko mice is hyperphosphorylated, and this hyperphosphorylation correlates with polyglucosan accumulation and LB formation<sup>3, 18, 19, 21, 24</sup>. As for malin, it is a confirmed ubiquitin E3 ligase whose substrates include GS, hyperactivity of which is a long-established cause of polyglucosan formation in phosphofructokinase deficiency and in various natural or engineered models of polyglucosan diseases<sup>1</sup>. We anticipate that future experiments will mitigate the recent observations weakening these gene function-based hypotheses, and will, in ways we cannot presently see, bring them together to explain the toxic glycogen structural transformation underlying LD.

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