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Identification of novel substrates of Malin E3 ubiquitin ligase in Lafora disease

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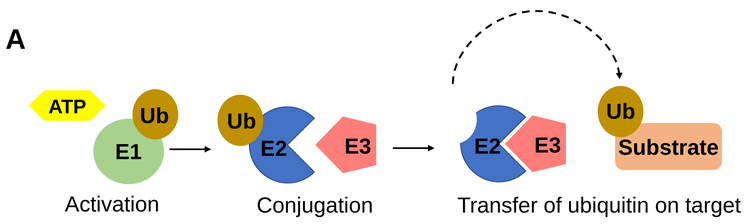
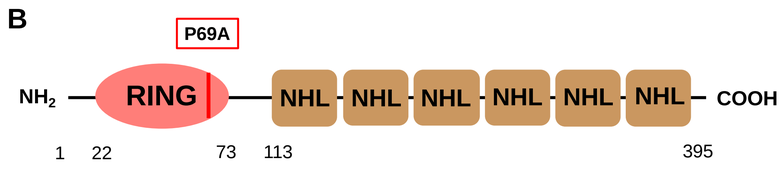
Lafora disease (LD) is a progressive neurological disorder characterized by epileptic seizures, myoclonus, cerebellar symptoms and psychic deterioration. There is no cure and patients are treated in a palliative way with anti-epileptic drugs, towards which, after some time, they become resistant. At the basis of the disease, there is a malfunction of two proteins, laforin and malin, encoded respectively by two genes*:* *EPM2A* and *EPM2B*. The two proteins form a complex and its incorrect functionality generates an error in the metabolism of glycogen leading to the accumulation of polyglucosan inclusions in patients. The polyglucosans, have an anomalous structure that prevents its normal degradation leading to the formation of Lafora bodies. Studies conducted on brain samples of LD mouse models show a greater accumulation of polyglucosans at the level of astrocytes compared to neurons. Considering the role of malin, known to be an E3 ubiquitin ligase, involved in the ubiquitination of specific substrates, we performed a proteomic analysis of the enriched ubiquitinated fraction of proteins, in HEK293T cells expressing either wild type or an inactive form of malin carrying the pathogenic P69A mutation (the most prevalent mutation of *EPM2B* gene). In comparison to cells expressing the nonfunctional malin-P69A, a list of more ubiquitinated putative substrates in cells expressing wild type malin was obtained. The aim of this study is to validate these candidates as substrates of malin and then focus on the consequences of ubiquitination on their physiological function. This information will allow the identification of putative therapeutic targets and develop new treatments that could ameliorate the pathology present in Lafora disease.

Ubiquitination process



Structure of Malin

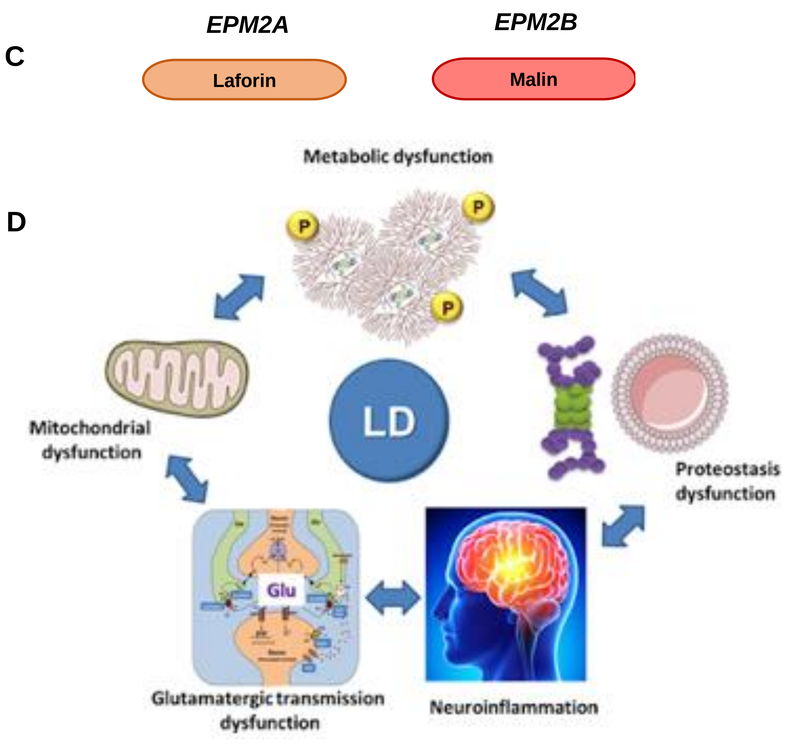


The family of E3 ubiquitin ligases are involved in the last stage of the ubiquitination process (A). The function of the E3 ubiquitin ligases is to transfer a protein, ubiquitin, to a target protein. The type of chain (linear or branched), the length (poly or mono) and the lysine residue of the binding ubiquitin determine the fate of the target protein. Malin belongs to this group of enzymes and, in particular, to the subgroup of RING E3 ubiquitin ligases. RING E3s directly transfer the ubiquitin to the substrate acting as a scaffold to allow the E2 conjugating enzyme to orient itself in a way to facilitate the transfer of ubiquitin. Malin (B), in addition to the RING domain, is characterized by 6 repeating NHL regions that are important for the association with substrate proteins (targets).

Targets of malin for ubiquitination

Protein	Outcome	Reference (PMID)
AGL	Regulation by ubiquitination	17908927
AMPK subunits alpha and beta	K63 linked polyubiquitination	20534808
BECLIN, VPS34, VPS15, ATG14L, UVRAG	Impairment of the maturation of autophagosomes	31758957
DISHEVELLED2	K48 and K63 ubiquitination and degradation	22223637
EAAT2/GLT-1	Localization of GLT-1 at the plasma membrane	33368637
GL	Ubiquitination and inhibition of glycogen accumulation	18070875
GLYCOGEN SYNTHASE	Ubiquitination and proteasome-dependent degradation	17952067
LAFORIN	Polyubiquitination and degradation	15930137
p62	Autophagy receptor that targets substrates for autophagy degradation	26546463
PYRUVATE KINASE M1,M2	Ubiquitination and nuclear translocation only of PKM2	26493215
R5/PTG	Ubiquitination and inhibition of glycogen accumulation	18029386; 17952067; 18070875
R6	Ubiquitination and inhibition of glycogen accumulation	18070875; 23624058

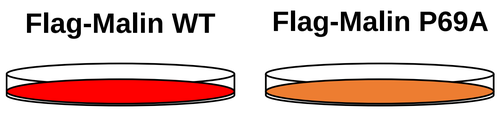
The table shows the substrates that are ubiquitinated by malin. For some of these substrates the type of polyubiquitin link that is formed is reported. Proteins with K48-linked poly-ubiquitin chains are directed to proteasomal degradation, while K63-linked poly-ubiquitin chains are involved in the regulation of processes such as DNA repair, protein trafficking, or RNA translation. For some substrates the effect of ubiquitination is described. For example, malin ubiquitinates GLT1 and determines its localization at the level of the plasma membrane.



Lafora disease is the consequence of mutations that can occur at the level of one of the two genes (C) that encode for laforin or malin proteins which together form a functional complex. Whether there is a laforin or malin defect, the phenotype that manifests in patients is the same.
An overview (D) of the dysfunctions that distinguish the disease is reported.*

Proteomic approach

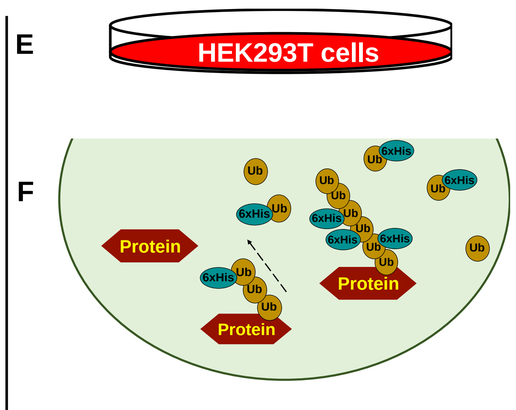
Strategy: exploit *EPM2B* P69A, the most recurrent mutation in patients



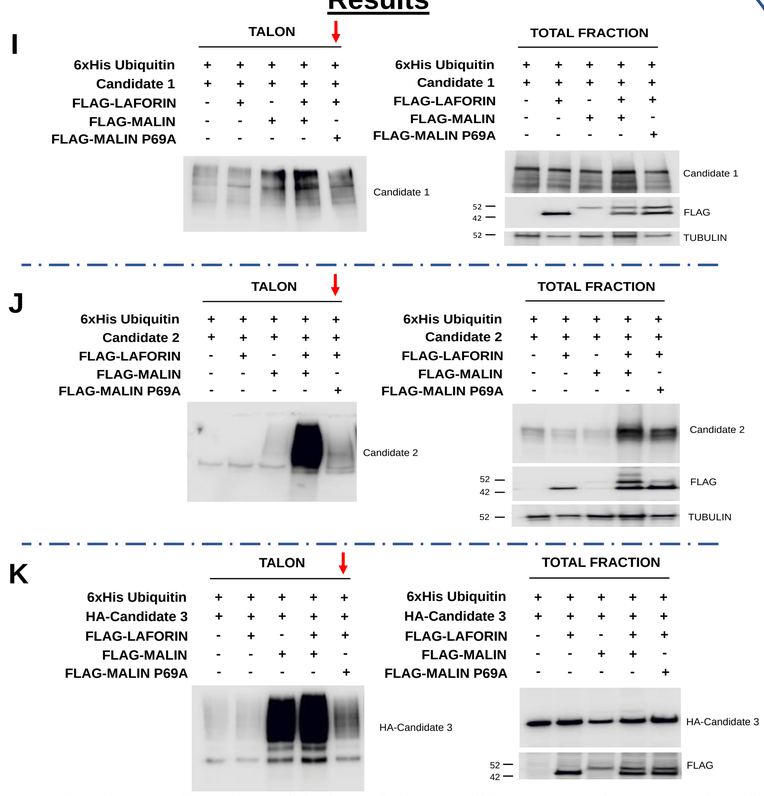
A proteomic approach was used to search for new Malin substrates. The HEK293T cell line was transfected with Malin wt and the mutated form of Malin P69A, the most recurrent mutation in patient. To follow ubiquitination, cells were also transfected with ubiquitin. By comparing the mutated versus the WT, two protein profiles were obtained and possible candidates were extrapolated from the analysis. In particular, the chosen protein candidates were the ones that were more ubiquitinated in cells transfected with Malin wt vs the ones transfected with the mutant P69A.

Immobilized metal affinity chromatography (IMAC) for validation of substrate candidates **

A metal affinity chromatography is used to validate the substrates obtained from the proteomic approach. Cells are transfected with ubiquitin tagged with 6xHis (E). Substrates that will be ubiquitinated by malin E3 ubiquitin ligase will contain ubiquitins tagged with 6xHis as well as endogenous ubiquitins (F). Cells are lysed under denaturing conditions and a TALON Metal Affinity Resin complexed with cobalt ions is used (G). The resin is highly selective for his-tagged proteins and this will allow us to purify the candidate substrate of interest (H).



Results



The results (I-K) outline the validation of the candidates as substrates of malin. The HEK293T cells were all transfected with ubiquitin and the candidate of interest and, alternatively, with the proteins of the laforin-malin complex and the non-functional complex using the malin P69A mutant. The ubiquitination of the candidate substrate does not occur when there is a defect at the level of malin therefore, its E3 ubiquitin ligase function is impaired (see red arrow).

Conclusions:

The validation of these candidates as substrates of E3 ubiquitin ligase opens up new horizons for understanding better Lafora disease. Certainly, the next step is to study in which physiological mechanisms these substrates are involved and what are the effects that may arise following their lack of ubiquitination by malin. This line of study, on one hand, could help to delineate better already known dysfunctions (see above, D) or, on the other hand, new unknown dysfunctions could be discovered. Overall, the discovery of these new candidates, following this approach, could help develop new innovative therapies to fight a disease that still has no cure to date.

- García-Gimeno, M.A.; Knecht, E.; Sanz, P. Lafora Disease: A Ubiquitination-Related Pathology. *Cells* **2018**, *7*, 87. https://doi.org/10.3390/cells7080087

** https://www.takarabio.com/products/protein-research/purification-products/his-tagged-protein-purification/bulk-resins-and-gravity-columns/cobalt-resin

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