Modelling Mucopolysaccharidosis type II in *Drosophila*: new CRISPR/Cas9-mediated knockout lines

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Introduction

Mucopolysaccharidosis type II (MPS II) is a rare lysosomal storage disorder due to the deficit of the lysosomal hydrolase iduronate 2-sulfatase (IDS), leading to undegraded glycosaminoglycans storage. In humans, MPS II presents with important clinical features, including organomegaly, cardiopathies and bone deformities. The severe form presents a heavy neurological involvement and occurs in about 2/3 of the cases. The applied therapy is the enzyme replacement, but it doesn’t have any effects on the brain disease. Moreover, the pathogenic mechanisms underlying the disease are mostly not understood [D’Avanzo et al 2020].

Thanks to its simplicity, *Drosophila melanogaster* is a very suitable model for the study of disease pathogenesis. It has been used for genetic studies for more than a century. Short generation time, the ease of maintenance, and the genetic and molecular tools available to study flies make *Drosophila* a wonderful genetic system to do gene manipulation and pathway analysis [Ugur et al 2016].

The *Ids* gene also has a single homologue/ortholog in *D. melanogaster* (*Ids/CG12014*), which is highly expressed in the central nervous system. The amino acid sequence of human IDS shares 47% identity with the one of the fruit fly [Holmes et al 2017]. Therefore, we set out to develop a fruit fly model for MPS II with the main objective of having a simpler model for basic and pharmacological studies.
Model generation

To create a knockout fruit fly model for MPS II, we used the CRISPR/Cas9 non-homologous end-joining (NHEJ) strategy to generate INDEL in first and second exon of the homologous ids gene (Ids(CG12014) in Drosophila.

We followed the protocol described by Port and Bullock [Port and Bullock 2016]. Briefly, we used the online “flyCRISPR Optimal Target Finder tool” (http://targetfinder.flycrispr.neuro.brown.edu/) to design and selected two optimal gRNA for ids gene. The properly designed oligos were cloned into the pCFD4 plasmid (Addgene #49411). DH5a competent E. coli cells were then transformed and inserted verified by single colony sequencing. The final plasmid was then amplified and sent to Best Gene Inc. (Chino Hills, CA, USA) for injection services in yw;nos-Cas9(II-attP40) Drosophila embryos. Hatched flies were crossed as reported in Figure 1 to obtain the final CRISPR mutant stock for the ids gene.

The resulting flies supposed to be homozygous for ids-/-, due to absence of markers, were tested for the ids/- mutation via High Resolution Melting Analysis (HRMA), a powerful technique used for the detection of mutations and polymorphisms based on the thermal denaturation properties of double-stranded DNA samples. Positive samples were confirmed by Sanger sequencing.

For all analyses we selected 3 out of 12 Ids-KO fruit fly models obtained by CRISPR/Cas9 (Ids2.3, Ids2.9, Ids2.10).

Real-time qPCR

The expression level of the ids gene (CG12014) was analysed by real-time qPCR. RNA was extracted by third instar larvae by using the TRizol Reagent (Invitrogen), reverse transcribed using the LunaScript RT SuperMix (NEB) and detected by qPCR using the Luna® Universal qPCR Master Mix (NEB). To assess whether the KO of the ids gene could cause alterations of the endolysosomal pathway, the following genes were also analyzed: Lamp1, Atg8a, Rab11. Ceramide Synthase (both C-terminal and Homeo Domain, Schlank C-ter and Schlank HD) and lipase 3 (lip3) were also analyzed to evaluate a possible imbalance of the lipid pathway.

Ids enzymatic activity

The IDS enzymatic activity was assessed by performing a fluorometric assay employing the substrate 4-Methylumbelliferyl a-L-idopyranosiduronic acid 2-sulphate disodium salt (Carbosynth) as previously reported [Voynyi et al. 2001].

Eclosion and puation rate

To assess survival rate into developmental stages, L1-L2 larvae were collected on apple juice agar plates (20-25 larvae per plate for a total of 100 larvae per genotype) and supplied with yeast paste. The number of emerging pupae and adults (alive 24 h after hatching) was counted. Data are expressed as percent of eclosion (number of adult flies eclosed/number of pupae; number of pupae/number of larvae).
Hatching rate
Females and males were mated inside cages covered with apple juice agar plates supplemented with yeast. After 24 h the plate with new embryos were removed, and after a further 48 h at 25 °C the hatched larvae are counted over the total fertilized embryos.

Lifespan
Lifespan were determined by collecting newly eclosed animals and placing them at low density (<20 animals per vial) at 25°C in a constant 12 h light/dark cycle in a humidity-controlled incubator. The aging animals were transferred to new vials at least three times per week, with deaths scored. The lifespan plots were generated by calculating the percentage of survivorship every second day and plotting viability as a function of time (days).

TMRE staining
TMRE is a cell-permeant, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria. For the staining, muscles were dissected and incubated with TMRE. After washing, they were mounted and immediately imaged by confocal microscopy.

Behavioral test
To assess possible locomotor and neurological defects, crawling and climbing assay were performed. In the crawling assay, a total of 50 L3 larvae (divided in 10 groups) were video-recorded for 30 seconds while moving on an agarose plate. Video were analyzed using ImageJ (Rasband, W.S., ImageJ, NIH, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) for the total distance traveled and the average speed. Ids2-3, Ids2-9 and Ids2-10 Ids-KO third instar larvae were compared to w- controls. In the climbing assay, or negative geotaxis assay, the natural tendency of flies to move against gravity when agitated is utilized to study genes or conditions that may hinder locomotor capacities. About 100 adults were analyzed at 1, 15 and 30 days of life. Only Ids2-3 flies were analyzed due to the pupal stage lethality of Ids2-9 and Ids2-10.

Results
The method proved to be extremely efficient as we obtained 12 potential MPS II knockout-lines (Ids-KO). We analyzed 3 KO-lines (Ids2-3, Ids2-9, Ids2-10) resulted in a strong downregulation of the Ids gene of about 85-90% in third instar larvae, well reflected also in the absence of enzymatic activity (Fig. 2). Moreover, mutations in Ids2-9 and Ids2-10 models were lethal at pupal stage (Fig. 3a), whereas the pupation rate were not affected (Fig. 3b). The hatching rate was significantly reduced in all the three Ids-KO models, with a strong embryonal lethality (Fig. 3c,d).

Due to the pupal stage lethality of Ids2-9 and Ids2-10, only Ids2-3 adults were analyzed for lifespan. Survival probability resulted almost comparable with w- flies till about 58 days, when Ids-KO flies all die in bulk, unlike controls (Fig. 3e).

Figure 2
a) Ids gene expression measured by real-time qPCR on third instar larvae (n=25). Ids-KO data were normalized to w1118 wild-type control; b) Ids enzymatic activity levels measured on third instar larvae(n=15 flies), mean ± SD. ***p<0.001.
Locomotor ability was strongly reduced in all three KO-lines in third instar larvae, and starting at 15 days, also in adults for Ids^{2,3}. In the crawling assay, both the total distance travelled by third instar larvae (Fig. 4a) and the mean speed (Fig. 4b) were strongly reduced in all the Ids-KO models compared to w- controls. Climbing ability was also compromised for Ids^{2,3} adults at 15 and 30 days in both the parameters tested: % of flies able to climb (Fig. 4d) and distance travelled (Fig. 4c). These parameters also suggest a neurological impairment of these models.
The RT qPCR (Fig. 5) showed increased expression in the Ids-KO models (Ids2.3, Ids2.9) of genes involved in the endo-lysosomal and autophagic pathways (Lamp1, Atg8a, Rab11). The lipid pathway seemed to be involved too, since Ceramide synthase (Schlank) was overexpressed, whereas Lipase 3 (Lip3) was downregulated, suggesting a block of lipid catabolism.

The TMRE assay (Fig. 6) showed an increased labeling in the muscles of the Ids-KO models compared to the w-controls, indicating a hyperpolarization of the mitochondria in MPS II and a probable increased activation and imbalance of these organelles.

Conclusions

In conclusion, we obtained and characterized for the first time three Drosophila KO-lines, showing first preliminary data on the alteration of the endo-lysosomal, autophagic and lipid pathways, and alteration at the level of mitochondria. Therefore, these MPS II models will be very useful tools for studying the pathogenesis of the disease and new therapeutic targets and drugs.

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References

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