A *Drosophila* mutant of LETM1, a candidate gene for seizures in Wolf-Hirschhorn syndrome

Angus G. McQuibban1,* Nicholas Joza2, Aram Megighian3, Michele Scorzeto3, Damiano Zanini3, Siegfried Reipert4, Constance Richter2, Rudolf J. Schweyen5,† and Karin Nowikovsky5,*

1Department of Biochemistry, University of Toronto, Canada M5S 1A8, 2Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr Bohrgasse 3, Vienna 1030, Austria, 3UPFNL, Department of Anatomy and Physiology, University of Padua, Italy, 4Department of Biochemistry and Cell Biology and 5Department of Microbiology, Immunobiology and Genetics, MFPL, University of Vienna, Dr Bohrgasse 9, Vienna 1030, Austria

Received September 29, 2009; Revised and Accepted December 17, 2009

Human Wolf-Hirschhorn syndrome (WHS) is a multigenic disorder resulting from a hemizygous deletion on chromosome 4. LETM1 is the best candidate gene for seizures, the strongest haploinsufficiency phenotype of WHS patients. Here, we identify the *Drosophila* gene CG4589 as the ortholog of LETM1 and name the gene *DmLETM1*. Using RNA interference approaches in both *Drosophila melanogaster* cultured cells and the adult fly, we have assayed the effects of down-regulating the LETM1 gene on mitochondrial function. We also show that *DmLETM1* complements growth and mitochondrial K⁺/H⁺ exchange (KHE) activity in yeast deficient for LETM1. Genetic studies allowing the conditional inactivation of LETM1 function in specific tissues demonstrate that the depletion of *DmLETM1* results in roughening of the adult eye, mitochondrial swelling and developmental lethality in third-instar larvae, possibly the result of deregulated mitophagy. Neuronal specific down-regulation of *DmLETM1* results in impairment of locomotor behavior in the fly and reduced synaptic neurotransmitter release. Taken together our results demonstrate the function of *DmLETM1* as a mitochondrial osmoregulator through its KHE activity and uncover a pathophysiological WHS phenotype in the model organism *D. melanogaster*.

INTRODUCTION

The human Wolf-Hirschhorn syndrome (WHS) is a contiguous gene disorder caused by a hemizygous deletion of a set of genes on chromosome 4 mostly restricted to band 4p16.3 (the WHSCR-1 and WHSCR-2 critical regions). WHS patients display pleiotropic phenotypes including short stature, severe growth and motor delay, mental retardation, seizures and major malformations including craniofacial, heart, renal and skeletal anomalies (1). The variation and severity of the symptoms in WHS patients is linked with the size of the chromosome deletion and it is now widely recognized that the various patient symptoms cannot result from the loss of a single pathogenic gene. However, patient studies have not yet revealed what gene contributes to any specific WHS symptoms. LETM1 has been identified as an excellent candidate gene for seizures in WHS. Recent studies showed that LETM1 is deleted in most WHS patients exhibiting seizures and preserved in those without seizures (2,3).

To understand the complexity of the human disease, animal models that can complement patient studies are needed. Studies in a mouse model have presented a genotype:phenotype correlation between FGFR3, MAEA and CTBP1 with skeletal malformations, hematopoietic dysgenesis and growth defects, respectively (4). However, one of the most severe pathogenic phenotypes of WHS is epilepsy. To date, no mouse has been generated with a gene deletion of LETM1 even though LETM1 is believed to be tightly linked to the pathogenesis of epilepsy in WHS.

Extensive research has led to functional characterization of the human LETM1 and the yeast homolog Mdm38. Originally, yeast Mdm38 was found in two different genetic screens, one for suppressor genes of the *mrs2* mutants lacking the mitochondrial Mg²⁺ transporter, and the other for genes required

*To whom correspondence should be addressed at: 1, King’s College Circle, Toronto, Canada M5S1A8 (G.A.M.)/Dr Bohrgasse 9, Wien 1030, Austria (K.N.). Tel: +1 4169786820(G.A.M.)/+43 1427774538 (K.N.), Email: angus.mcquibban@utoronto.ca (G.A.M.)/karin.nowikovsky@univie.ac.at (K.N.)
†Deceased on February 2009.

© The Author 2009. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
to maintain the mitochondrial distribution and morphology (MDM genes) (5,6). Absence of Mdm38p conferred a growth defect on non-fermentable substrates. Studies in yeast involving KOAc-induced swelling experiments on isolated mitochondria as well as K⁺/H⁺ exchange (KHE) assays using fluorescent indicators entrapped in sub-mitochondrial particles (SMPs) showed that mdm38 mutant mitochondria were refractory to KOAc-induced swelling, and that SMPs lacking Mdm38p were lacking coupled K⁺ and H⁺ fluxes across the membrane (6,7). Strong confirmation that MDM38 encodes an essential component of the mitochondrial KHE system came from the findings that the KHE ionophore nigericin restored yeast growth on respiring substrates to wild-type levels, as well as KHE activity of mutant SMPs. Furthermore, organelar morphological defects upon down-regulation of Mdm38/LETM1 were also reverted by nigericin (8,9).

In this study, we demonstrate that CG4589 is the fly ortholog of yeast and human LETM1 and name the gene DmLETM1. Specifically, we show that CG4589 encodes a mitochondrially localized protein of functional homology to yeast and human LETM1. Our studies reveal the effects of DmLETM1 down-regulation in Drosophila-cultured cells and the adult fly. Importantly, we uncover, for the first time, the impact of disrupting mitochondrial K⁺ homeostasis on cellular viability in a metazoan. We address the role of DmLETM1 in the development of Drosophila and in the adult fly and propose that this will serve as a useful model of WHS disease.

RESULTS

Drosophila CG4589 encodes a functional homolog of the yeast LETM1 protein and restores KHE activity in yeast

In Drosophila melanogaster, a TBLASTN (10,11) search of the expressed sequence tag (EST) database has revealed an open reading frame annotated as CG4589 with significant similarity to human LETM1 and yeast MDM38. CG4589 encodes a predicted protein of 1013 amino acids (AA) sharing AA sequence and protein architecture that is highly conserved in all LETM1 sequences in eukaryotic organisms: an N-terminal mitochondrial localization sequence, one putative trans-membrane domain, two C-terminal putative Ca²⁺ binding EF hands and three alpha-helical coiled–coiled domains (Fig. 1A). Blast alignments indicated that the protein encoded by CG4589 shares 39 and 42% sequence identity and 63 and 64% sequence similarity with yeast MDM38 and human LETM1, respectively (Fig. 1B) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?request IDs: Y962PPTU011 and Y918GF80013).

To test for functional homology, we expressed CG4589 in yeast and screened for rescue of the yeast MDM38 deletion phenotype. The mutant strain mdm38Δ demonstrates a mild growth phenotype on fermentable substrates but is unable to grow on a non-fermentable substrate as a result of pleiotropic mitochondrial defects caused by mitochondrial K⁺ overload (6,9). As seen in Figure 2A, overexpression of CG4589 in the mdm38Δ mutant strain significantly improved growth on a fermentable substrate, and more importantly, restored non-fermentable growth at 28°C or at 16 and 35°C, where the phenotype is even more pronounced. In addition to the in vivo complementation that suggests functional replacement of KHE activity, we took advantage of the fact that isolated mitochondria from mmd38Δ cells lack the mitochondrial KHE activity and thus are refractory to KOAc-induced swelling (Fig. 2B) (6). Importantly, the KHE activity in isolated mitochondria from mdm38Δ cells was restored to wild-type levels in the presence of CG4589 (Fig. 2B), indicating that this gene product restores KHE activity in isolated mitochondria. We have therefore named CG4589 DmLETM1.

DmLETM1 encodes a mitochondrial protein

To gain an initial understanding of the protein product of DmLETM1, we first analyzed its cellular localization by generating Drosophila S2 cells that express DmLETM1 fused to GFP. The distribution of DmLETM1-GFP in these cells was indistinguishable from that of mitochondria, as seen by the clear colocalization of the green fluorescence of the fusion protein and the red fluorescence of the mitochondrial network imaged by immuno-staining for the mitochondrial ATPase complex V-subunit α (Fig. 3).

DmLETM1 regulates mitochondrial morphology

Next, we treated Drosophila S2 cells with RNAi against DmLETM1. In contrast to control cells, DmLETM1KD cells displayed a fragmented mitochondrial network. Moreover, mitochondria appeared globular and swollen (Fig. 4Ab). We have previously found that nigericin, an antibiotic that catalyzes electroneutral KHE (12), restored aerobic growth of mmd38Δ yeast strains and reverted mitochondrial swelling in situ (9). This prompted us to investigate whether nigericin would rescue the RNAi knockdown phenotype in fly cells. Strikingly, the mitochondrial morphological changes were reverted upon the addition of nigericin, which compensates for the missing KHE (Fig. 4Ac–e). As studies performed in yeast revealed that upon down-regulation of MDM38 swollen mitochondria were degraded in the vacuoles in a process called mitophagy (9), we evaluated the organelar changes in control and DmLETM1KD S2 cells by counterstaining mitochondria with dsRed and lysosomal compartments with Lyso-Tracker Green. In order to allow accumulation of autophagosomes and retention of ingested material, control and DmLETM1KD cells were treated with a protease inhibitor. We observed little colocalization of mitochondria and lysosomal compartments in control cells, indicating low mitochondrial turnover (Fig. 4Ba–c). In contrast, DmLETM1KD cells revealed significant mitochondrial swelling and colocalization of mitochondria and lysosomal compartments indicating increased mitochondrial turnover or mitophagy (Fig. 4Bd–f). Colocalization of lysotracker and mitochondria were quantified in three different treatments (Fig. 4Bg). Further, electron microscopy confirmed that mitochondria of DmLETM1KD cells were also found within structures that appear as autophagosomes (Fig. 4Bh). Taken together, the data presented here on mitochondrial morphology, functional rescue with nigericin and induced mitophagy agree with previously reported yeast and human cell-culture studies (8,9,13,14).
Figure 1. CG4589 encodes a member of the LETM1 protein family. (A) Schematic architecture of the protein sequence of DmLETM1. The single predicted transmembrane domain is marked by a grey box. The C-terminal part of the sequence contains two putative EF-hand Ca^{2+}-binding sites (black boxes) three coiled–coiled domains marked by oval circles. (B) CG4589/DmLETM1 is the homolog of human and yeast LETM1. Clustal W alignment of Drosophila melanogaster (Dm. CG4589), Homo sapiens (Hs. LETM1) and Saccharomyces cerevisiae (Sc. Yol027c) AA sequences. AA identity and similarity are highlighted in black and grey, respectively. Black balk indicates the TM between the amino acids 237–253. The putative EF Ca^{2+} calcium binding hands are boxed. Putative leucine zipper motif is marked by asterisks.
Ubiquitous knockdown of DmLETM1 is lethal during Drosophila development

Having determined that DmLETM1 is crucial in maintaining mitochondrial morphology and KHE activity, we next investigated the role of mitochondrial K\(^{+}\) homeostasis in a multicellular organism by in vivo RNA interference.

To first determine the phenotype independently of a potential positional effect of the UAS-IR CG4589 insertion site, three independent insertion lines of the UAS-IR CG4589 transgene (T1: w[1118]; P[G2208]v6661/TM3, T2: w[1118]; P[G2208]v6662/TM3 and T3: w[1118] P6663/CyO) were evaluated. Homozygous T1, T2 and T3 UAS-IRCG4589 were created. Crosses of all three lines with tubulin-GAL4 (tub-GAL4) and daughterless-GAL4 (da-GAL4) driver line resulted in lethality at early larval stage upon DmLETM1 knockdown. Crosses with actin-GAL4 (act-GAL4) resulted likewise in early larval stage with the exception of T2 showing ~60% lethality at larval stage and 40% at pupal stage.

As presented here, one representative line of transgenic RNAi flies ubiquitously expressing a UAS-IR-CG4589 construct under the control of the da-GAL4 transgene completed embryogenesis and developed into early larvae at normal frequencies, reflecting most likely incomplete gene knockdown

Figure 2. DmLETM1 complements for the yeast *mdm38* deletion phenotypes. (A) Complementation of the growth phenotype. Serial dilution of yeast LETM1 mutant cells (*mdm38Δ*) transformed with the empty plasmid pVT103-U or pVT103-U-CG4589. Cells were grown for 3 days at 28°C, 4 days at 35°C or 7 days at 16°C on media with fermentable (YPD) or non-fermentable (YPG) substrate. (B) Restoration of the mitochondrial KHE activity. KHE activity was determined by measuring the KOAc induced swelling of isolated mitochondria using the light scattering technique. Mitochondria from W303 wild-type cells (WT, broken line) and *mdm38Δ* cells expressing the control vector pVT103-U (*mdm38Δ*, grey line) or pVT103-U-CG4589 (*mdm38Δ* + DmLETM1, black line) were prepared as described in Materials and Methods. Inhibition of the KHE activities by quinine (Q), a specific KHE inhibitor is also shown (triangles).
or a strong maternal effect (B. Dickson and G. Dietzl, personal communication). However, larval development was arrested at the third-instar stage and DmLETM1<sup>KD</sup> larvae exhibited pre-mature lethality, suggesting that DmLETM1 plays an important role during larval development. A time-course analysis of lethality revealed that at day 7 and 8 after egg laying there was 31 and 61% lethality, respectively, of the da-DmLETM1<sup>KD</sup> larvae (Fig. 5A). However by day 9, no surviving da-DmLETM1<sup>KD</sup> larvae remained. In addition, RNAi expressing 6 days old larvae showed dramatically reduced body size compared with same day aged wild-type (da-GAL4) (Fig. 5B). Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) confirmed that DmLETM1 was significantly down-regulated in larvae expressing the hairpin construct (Fig. 5C).

**Tissue-specific down-regulation of DmLETM1**

In order to examine the role of DmLETM1 in specific tissues of the fly and to further our understanding of the complex human WHS disease phenotypes, the DmLETM1 gene product was depleted from various tissues using the Gal4–UAS system (15). Homozygous UAS-IRCG4589 fly strains T1 and T2 were crossed with the mef2-GAL4 driver line that is expressed in muscle cells at all stages of development. The resulting larvae in both strains showed sluggish behavior with no climbing from the food onto the vial-walls and showed reduced body size. In addition, growth was arrested at the pupal stage. However, rare escapers (<10% of total pupae) developed into adult flies, suggesting that the knockdown was not complete, but allowing an assessment of the adult phenotype. These flies were extremely compromised, appearing very weak and smaller than control flies (Fig. 6A). In addition, they were unable to fly, suggesting that muscle function was severely impaired by the reduction of KHE activity in the mitochondria.

We also assessed the phenotypic effect of down-regulating DmLETM1 in the developing *Drosophila* eye. The eye of *Drosophila* is composed of about 800 individual ommatidia, consisting each of eight photoreceptor cells (R1–R8) and 12 accessory cells (16). The ommatidia are arranged in a hexagonal array. Examination of the external surface of the eye by light microscopy demonstrated severe disruption of the ommatidial array, including a severe roughening of the surface and a significant reduction of ommatidial numbers resulting in a small eye (Fig. 6B and C). In the absence of DmLETM1, eye facets occupied a strongly reduced surface area (by ~50%), which was concentrically surrounded by scar tissue. To directly assess whether DmLETM1 deletion caused anomalies of mitochondrial morphology and homeostasis, EM studies were performed at larval stages of the developing eye disc. Thin sectioning through ommatidia of both wild- and RNAi DmLETM1 mutants displayed structurally intact mitochondria (Fig. 6D and Supplementary Material). However, a significant number of individual ommatidia of DmLETM1-deficient eye discs showed dramatically swollen mitochondria with an electron transparent matrix and remnant cristae, a phenotype that was never seen in wild-type eyes (Fig. 6D and E). The mitochondria of such individual mutant ommatidia were affected collectively, as revealed by disorganized structures of the photoreceptors within the ommatidia (Supplementary Material). This mitochondrial phenotype was not accompanied by a massive increase in the classical hallmarks of cell death, such as condensed osmophilic nuclei (Supplementary Material, Fig. SE).

**Absence of DmLETM1 in the nervous system results in decreased locomotion and decreased neurotransmitter release in synapses**

To down-regulate DmLETM1 in neuronal tissues, UAS-IR-CG4589 T2 flies were crossed with elav-GAL4 [early expression in the central nervous system (CNS)] and s-nyb-GAL4 (late expression in neurons and synapses). The locomotor activity of adult flies of each genotype was monitored and alterations were quantified by video tracking adult fly movements (see Materials and Methods). The average speed during movement was slightly reduced in *n-syb* down-regulated DmLETM1 adult flies and significantly reduced in *elav* DmLETM1 down-regulated flies in comparison to the controls (Fig. 7A). The number of immobile episodes (pauses in locomotion of more than 2 s) was significantly increased in both DmLETM1 down-regulated lines when compared with the controls (Fig. 7C). Moreover, the average duration of immobile episodes was increased in both DmLETM1 down-regulated lines (Fig. 7B). As a consequence, the average total time spent being immobile by adult flies of both DmLETM1 down-regulated lines was notably increased in comparison to the controls (Fig. 7D). These data demonstrate...
the drastic effects of LETM1 down-regulation on locomotion, a phenotype also observed in WHS patients (17).

To better characterize the observed motor defects, both spontaneous and evoked synaptic activity of third-instar larva neuromuscular junction were recorded intracellularly (18). Third-instar larva neuromuscular junction represents a classical and well-established model for studying the general and evolutionarily maintained neurobiological mechanisms underlying synaptic function in invertebrates and vertebrates (19,20). Down-regulation of DmLETM1 by the n-syb-Gal4 driver, did not affect spontaneous neurotransmitter release (i.e. the spontaneous fusion of a neurotransmitter vesicle with the presynaptic membrane) (data not shown), but nerve-evoked neurotransmitter release was significantly

Figure 4. (A) RNAi down-regulation of DmLETM1 in S2 cells and rescue by treatment with nigericin. (a and b) Mito-Tracker red chloromethyl-X-rosamine staining of mitochondria in S2 wild-type cells treated with no RNAi (a) or RNAi directed against dMLETM1 (b) as visualized by fluorescence microscopy. Bars: 10 μm. (c) Drosophila S2 cells displaying normal morphology before RNAi treatment. (d) Swollen and fragmented mitochondrial morphology following DmLETM1 RNAi. (e) Mitochondrial morphology is restored by treatment with the ionophore nigericin following DmLETM1 RNAi. (c–e) Mito-Tracker red chloromethyl-X-rosamine and 4',6-diamidino-2-phenylindol staining of mitochondria and nuclei, respectively. Bars: 10 μm. (B) Mitochondrial turnover in DmLETM1<sup>kd</sup> S2 cells. (a) Lysosomal staining of control S2 cells stably expressing mito-dsRED (b), merged image is shown in (c). (d) Lysosomal staining of S2 cells treated with the RNAi against DmLETM1, (e) ds-RED signal, (f) the merged image. (g) Quantification of cells that showed mitochondrial and lysosomal colocalization in three separate experiments. Bars: 10 μm. (h) TEM analysis of S2 cells treated with the RNAi against DmLETM1 reveals mitochondria (M) inside an autophagosome (V). Bar = 100 nm.
reduced, as shown by stimulating the nerve with both single pulses (Fig. 8A) and with high frequency 10 Hz stimuli (Fig. 8B). This last condition is close to the physiological activity of larval motor neurons and leads to the rapid release of neurotransmitter vesicles (21). Tetanic 10 Hz stimulation was utilized, because it was demonstrated that mitochondrial calcium buffering is critical for the mobilization of reserve pool vesicles at D. melanogaster neuromuscular junction during prolonged high-frequency stimulation (22). The number of neurotransmitter molecules filling each vesicle was the same in control and LETM1 down-regulated flies as indicated by the overlapping of the amplitudes distributions of the spontaneous neurotransmitter release events (or minis) in both fly strains (Fig. 8C). These data show that DmLETM1 down-regulation does not interfere with the number of neurotransmitter molecules per vesicle but with the number of vesicles undergoing exocytosis upon arrival of nerve action potential. Thus, an alteration in the number or in the function of presynaptic mitochondria appears to affect synaptic function and plasticity. Taken together, the behavioral and electrophysiological data suggest a significant impairment in nervous system function, when DmLETM1 function is reduced specifically in neurons.

DISCUSSION

WHS is multigenic disorder resulting in a multifunctional pathology defined by the association of a typical facial appearance, severe growth delay, mental retardation and seizures. The human LETM1 gene is considered to be the major candidate gene for seizures in WHS, because it is deleted in all the typical WHS patients and preserved in the atypical WHS patients with no seizures (23). The protein encoded by LETM1 is a member of a novel and highly conserved protein family found in all sequenced eukaryotic genomes. The Saccharomyces cerevisiae ortholog of LETM1 encoded by the ORF YOL027c was named MDM38 or MKH1 and has been extensively studied at both the biochemical and functional level (6,24,25). These studies have demonstrated that LETM1 plays an essential role in mitochondrial K⁺ homeostasis by mediating the mitochondrial K⁺ efflux (KHE) that is of prime importance for organelle-volume control. Rehling and co-workers (24) presented data implicating the association of the Mdm38 Protein A-tagged protein with mitochondrial ribosomes and have suggested that membrane insertion of mitochondrially synthesized proteins is dependent on Mdm38p. However, we have directly demonstrated that defects observed in the biogenesis of cytochrome b, Atp6 and other respiratory chain proteins in mdm38 cells described by Frazier et al., clearly appeared as a secondary and late effect to shut-off of MDM38, while loss of mitochondrial KHE activity is an immediate response (9). Of note, the mitochondrial KHE function was still fully active in r8 mitochondria in the presence of Mdm38p (unpublished data). hLETM1 has also been reported to interact with the mitochondrial chaperone BCS1L. Even though BCS1L is required for the assembly of the respiratory chain, Tamai et al. (14) showed that mitochondrial swelling upon the depletion of LETM1 was not caused by defects of the respiratory chain. Recently, LETM1 has also been characterized in HeLa cells, demonstrating its impact on mitochondrial function (8,14); however studies in metazoan model organisms have yet to be reported. Indeed, in humans, the mechanisms through which LETM1 might cause the seizures disorder is poorly understood, underscoring the need for functional studies in a model organism.
Here, we have identified and characterized the fly ortholog of LETM1, CG4589 (DmLETM1). We showed that DmLETM1 localizes exclusively to mitochondria of Drosophila-cultured S2 cells and that knockdown of DmLETM1 in S2 cells induced mitochondrial fragmentation resulting in mitophagy, the clearance of damaged mitochondria by the autophagosomal machinery. Like in mammalian cells (8,13,14) and consistent with the yeast phenotype (6,9), mutant mitochondria appeared fragmented, swollen and significantly devoid of cristae structures. Importantly, the morphological changes were reverted

Figure 6. (A) Down-regulation of DmLETM1 in muscle cells results in severe growth retardation in adult flies. UAS-IRCG4589 flies were crossed to mef2-GAL4 flies. Same day aged wild-type (left) and DmLETM1KD flies (right). (B–E) Severe external damage and massive mitochondrial swelling in the adult eye in the absence of DmLETM1. Stereomicroscopic photographs of eyes from control (B) and UAS-IRCG4589 flies crossed ey-GAL4 flies (C). TEM analysis of eyes from control (D), bar: 500 nm and UAS-IRCG4589 flies crossed ey-GAL4 flies (E), bar: 1 μm.
upon the addition of nigericin, an antibiotic catalyzing electroneutral KHE and thus compensating for the missing KHE. Finally, physiological assays on KHE activity carried out in yeast strains lacking MDM38 confirmed the functional homology of DmLETM1. Overexpression of DmLETM1 in mdm38Δ mutants restored the mitochondrial KHE activity to wild-type level as demonstrated by KOAc-induced swelling experiments.

To date, there is no description in the literature that demonstrates the consequences of LETM1 down-regulation in a metazoan. In this paper, we demonstrate, for the first time, that DmLETM1 plays a vital role in Drosophila development by affecting both growth and survival through a mechanism that impinges upon mitochondrial morphology and function. Our data provide clear evidence that ubiquitous downregulation of DmLETM1 expression results in arrested larval growth and loss of viability. Finally, we analyzed the consequences of down-regulating DmLETM1 in a tissue-specific manner. Growth arrest following DmLETM1 down-regulation in muscle cells also results in a reduced body size. This is an interesting finding, because severe growth delay is one of the hallmarks of WHS (26), a strong indication that the fly could be a useful model to understand the complex WHS patient phenotypes. Down-regulation of DmLETM1 in the eye had drastic effects including swollen mitochondria, disorganization of the eye, loss of photoreceptor cells and reduced number of ommatidia. These data suggest a correlation between the regulation of mitochondria morphology and cell survival. Moreover, data provided here point to the role of DmLETM1 in the formation of normal ommatidia in the fly eye. Interestingly, patients with deletion on the short arm of chromosome 4 that includes LETM1 have various ocular defects (27). Depletion of DmLETM1 in the nervous system using pan-neuronal-specific drivers was not embryonic nor pupal lethal with flies reaching the adult stage. However, depletion induced with the n-syb- or elav-GAL4 driver severely affected the locomotory functions of the adult fly. Interfered flies were less responsive. The increased time

Figure 7. Adult fly locomotor activity. The locomotor movement of single flies placed in a uniformly illuminated arena was video-recorded for a 10 min period and video-tracked (see Materials and Methods). The considered parameters for the analysis were: mean (± standard error of means or SEM) speed during active movement (A); mean (± SEM) time duration of immobile episodes (fly immobile for more than 2 s; see Materials and Methods) (B); mean (± SEM) number of immobile episodes (C) and mean (± SEM) total time spent immobile during the observation time period (D). Asterisks indicate the statistical significance (P < 0.05) of the differences between control parental (T2) line and n-syb DmLETM1 or elav DmLETM1 (indicated by the lines above columns) assessed using the t-test for unpaired data. A total number of 24, 27 and 25 adult flies were analyzed for parental (T2) control, DmLETM1 n-syb and DmLETM1 elav down-regulated lines, respectively.
spent in immobile episodes and their increased number might indicate defects that could involve the brain structures contributing to the organization of fly locomotor activity (28). This behavioral neuronal phenotype was accompanied by a significant reduction of evoked neurotransmitter release in the neuromuscular junction of third-instar larvae. It is interesting to note that a role for presynaptic mitochondria in regulating synaptic activity was recently demonstrated (22). Impaired synaptic function (either ubiquitous or limited to specific neuronal circuits) can also lead to an ‘irritative’ neuronal lesion that might be responsible for epileptic attacks. However, this is still a matter of debate in WHS patients.

Neuronal and muscular degenerative diseases are often related to dysfunction of mitochondrial oxidative phosphorylation (29). The correlations between disease phenotype and mitochondrial loss-of-function are not always straightforward, because mitochondrial functions often overlap. Yet, mitochondrial and nuclear mutations in the respiratory complexes contribute to severe early encephalomyopathies (reviewed in 29). However, an important question in this study is whether the phenotype of DmLETM1 mutants might depend on mitochondrial dysfunction secondary to KHE inactivation or rather has unique features that reflect a primary disturbance in K\(^{+}\) homeostasis. The fact that nigericin rescued mitochondrial defects in cell culture suggests that our phenotype does not result solely from energy deprivation. As described for the hLETM1, gene silencing had no basal effect on uncoupled respiration or on levels and assembly of respiratory complexes and of the ATPase (8). Nevertheless, KHE inactivation causes matrix swelling and cytochrome c release and decreased respiratory rates might occur as a later response following LETM1 down-regulation resulting in a phenotype that mimics inactivation of respiratory chain complexes.

To date, several Drosophila models have been established to analyze the impact of certain respiratory mutations causing encephalomyopathies. Ubiquitous inactivation of Surf1 (a gene involved in the assembly of cytochrome c oxidase, COX), caused severe, global impairment of larval development and reduced spontaneous and visual-induced locomotion with marked underdevelopment of the CNS (30).

Figure 8. Neurotransmitter release at third-instar neuromuscular junction. (A) Amplitude of the evoked junctional potentials. Mean amplitude (± SEM) of excitatory junctional potentials (EJPs) evoked by 0.5 Hz segmental nerve stimulation in A3/A4 muscle fiber 6 or 7 of third-instar larvae of parental (T2) control (black) and DmLETM1 n-syb down-regulated (white) flies. \( * P < 0.001 \) (t-test for unpaired data). \( n = 675, 9 \) (control) and 900, 12 (DmLETM1 down-regulated) indicate number of analyzed EJPs and number of larvae, respectively. SEM is too small to be clearly visible in the graph; their values are 0.171 and 0.093 for control and DmLETM1 down-regulated line, respectively. (B) Ten Hertz stimulation. Relative (with respect to the last five single EJPs evoked by 0.5 Hz segmental nerve stimulation) mean (± SEM) EJPs amplitudes evoked by 10 Hz segmental nerve stimulation. Means are calculated for each of 150 consecutive evoked responses in nine parental (T2) control (black dots) and nine DmLETM1 n-syb down-regulated (grey dots) third-instar larvae. Each white or grey background area indicates a 50 responses period. Data from DmLETM1 down-regulated third-instar larvae are significantly different \( P < 0.001; \) two-way ANOVA) from controls. (C) Relative distribution of spontaneous junctional potential (mini) amplitudes. Percentage frequency distribution of minis amplitudes intracellularly recorded from A3/A4 muscle fiber six or seven of third-instar larvae from control parental T2 (black dots) and DmLETM1 n-syb down-regulated (grey dots) animals. Numbers of counted minis and number of larvae, respectively, are 879 and 5 in control and 894 and 5 in DmLETM1 down-regulated flies. Double Gaussian fit (continuous line for control parental T2 and dotted line for DmLETM1 n-syb down-regulated) is shown in both figure and figure inset. Double Gaussian fit shows a double peak distribution of minis amplitudes. The first higher peak shows that a high percentage of minis amplitudes are distributed around a mean amplitude which roughly corresponds to the mean quantum amplitude, i.e. the mean mini amplitude evoked by the fusion of a single neurotransmitter vesicle (37). The second peak is correspondent to a mean mini amplitude which is roughly twice the quantum amplitude (37). \( R^2 \) for each fit curve is indicated in the graph.
Importantly, impaired locomotor behavior in these larvae was not due to structural and/or functional abnormalities of the segmental muscle fibers or reduction in contractile efficiency and evoked junction potential were normal. Down-regulation of DmLetm1 in neuronal cells led to specific reduced evoked neurotransmitter release in the CNS. We examined the average number of type 1b-s synaptic boutons and found it unchanged in parental and down-regulated lines (data not shown), indicating that the electrophysiological findings reflect functional synapse impairment and not neuromuscular junction (NMJ) developmental alteration. In a Drosophila model, the hirundo gene encoding a structural COX subunit decreases COX activity and results in in vivo mitochondrial Ca\(^2\) fluxes. In this study, the authors concluded that the reported effects of DmLETM1 suppression on mitochondrial function, an issue of decreased KHE activity on mitochondrial function, are not general defects in mitochondrial respiration, motor dysfunction and premature death. Of note, the question was raised whether there was a direct link between mitochondrial encephalopathy and COX decrease, or if there was an intermediate causative step involving reactive species, or ion channels as proposed by Liu et al. (31).

Taken together, we would argue that the fly phenotypes we observe upon DmLETM1 down-regulation are the result of the loss of KHE activity and not general defects in mitochondrial respiration. Indeed, in a genome-wide RNAi screen analyzing the role of OXPHOS in obesity in Drosophila (Pospisil et al., in press), the lethality of numerous OXPHOS knockdown mutants was assessed using a range of tissue-specific GAL4 drivers including the muscle (C57-GAL4) and neuronal (n-syb-GAL4) compartments. Among the knockdown mutants analyzed (21 members of complex I of the respiratory chain, 12 members of the complex IV and 13 proteins of complex V, 12 members of the complex IV and 13 proteins of complex V, 12 members of the complex IV and 13 proteins of complex V, 12 members of the complex IV and 13 proteins of complex V, 12 members of the complex IV and 13 proteins of complex V), few candidates with a lethal phenotype when down-regulated were found, including COX decrease, or if there was an intermediate causative step involving reactive species, or ion channels as proposed by Liu et al. (31).

Future studies will have to further clarify the physiological role of LETM1 in neurons by disturbing mitochondrial cation homeostasis including K\(^+\) and Ca\(^{2+}\) and the mechanism underlying the decreased release of neurotransmitter vesicles.

Very recently, through a genome-wide siRNA Drosophila screen, Jiang et al. (32) found that DmLETM1 suppression strongly decreased mitochondrial Ca\(^{2+}\) uptake and the matching H\(^+\) fluxes. In this study, the authors concluded that Drosophila and human LETM1 is the mitochondrial Ca\(^{2+}/H^+\) antiporter rather than the KHE. This conclusion is surprising because the physiological role of the Ca\(^{2+}/H^+\) antiporter is to catalyze Ca\(^{2+}\) efflux and to protect cells from mitochondrial Ca\(^{2+}\) overload; and down-regulation of the Ca\(^{2+}/H^+\) exchanger should rather have resulted in an increased mitochondrial Ca\(^{2+}\) accumulation. Further, the reported inhibitory effect of Ruthenium Red is inconsistent with an effect of Ca\(^{2+}/H^+\) exchanger, which is insensitive to this inhibitor (33). We suspect that the reported effects of DmLETM1 suppression on mitochondrial Ca\(^{2+}\) transport may be a secondary consequence of decreased KHE activity on mitochondrial function, an issue that will require further investigation.

In sum, the ability to induce in an animal model like Drosophila the severe growth and motor defects that resembles that of WHS patients provides compelling evidence that the fly will be a useful model to further understand WHS.

### MATERIALS AND METHODS

#### Drosophila strains

Flies were maintained on standard medium at 25°C in a temperature-controlled incubator. The GAL4–UAS system (15) was used to direct expression of CG4589 RNAi to specific tissues. Silencing experiments in Drosophila were carried out with transgenic GAL4/UAS lines (6). The UAS-IR CG4589 line carries a transgene construct expressing under the control of a 10× UAS enhancer a 344 bp long inverted repeat which was amplified with forward primer 5′-CGCGAATTCCGACGAAAGGAGTGAGAAGAGA-3′ and reverse primer 5′-CGCTCTAGACGAGCTGCTT GCCGTTGAG-3′ cloned into pMF3 yielding pGD264.

Three UAS-IR CG4589 transgene insertion lines (T1: w[1118]; P(GD2208)v6661/TM3, T2: w[1118]; P(GD2208) v6662/TM3 and T3: w[1118] Pt6663/CyO) were used in this study. Homozygous T1, T2 and T3 UAS-IRCG4589 flies were created and crossed to act-GAL4, tub-GAL4 and da-GAL4 driver lines for ubiquitous down-regulation of CG4589, to eyless-GAL4 (ey-GAL4) for eye-specific knockdown, to mef2-GAL4 for down-regulation in muscles and to elav-GAL4 and s-syb-GAL4 in neuronal tissues. Crosses with ey-GAL4 were viable except for T1 (lethal between pupa stage and fly eclosion). Crosses with s-syb or elav were all viable. All experiments shown were done with T2 if not otherwise stated.

#### Cloning DmLETM1

To express DmLETM1 in fly cell lines, the entire ORF of CG4589 was amplified from a D. melanogaster cDNA with the primers 5′-GGGACAAGTTTTGTCACAAAAAAGCGG CTTAATGAAACGCCCTGTGCTACACAGGA-3′ and 5′-GGGACCACACTTTGTACAAGAAACGTGGTAGAGCA GTTTCTTGGCGTGATCGC-3′ and cloned into the gateway system according to the manufacturer’s instructions, generating GFP-tagged UAS-CG4589.

#### Time course analysis of lethality

Parental UAS-IRCG4589 males were allowed to deposit eggs on apple juice medium over several hours. The embryos hatched and about larvae were transferred to separate dishes of power food medium. At the time points indicated, the dishes were rinsed and the number of surviving larvae was counted; n = 25 larvae per time point. Standard conditions were used (25°C). da-GAL4 served as a control.

#### RT–PCR

For RT–PCR, total RNA was extracted from larvae using TRI Reagent (Sigma), treated with DNase I, and reverse transcribed using RTG You-Prime-First-Strand Beads (GE Healthcare). Semi-quantitative PCR was performed in a reaction using combined CG4589 primers 5′-TACCGGAAAGATCT GCTTCGACC-3′ and 5′-GAGGGTCTTCTCTGCTGATCGTGTC G-3′, and G3PDH primers 5′-CCACTGCGAGAGGTGTC AACTA-3′ and 5′-GCTCAGGGTGATTGCCTATGCA-3′.
**RNAi in cell culture**

RNAi in *Drosophila* S2 Schneider cells was conducted as previously described (34). Briefly, double-stranded RNA 400 nucleotides in length were amplified and purified using the RiboMax procedure (Promega, USA). Purified RNA was added to cell cultures and incubated for 3 days. 5 mM glutaraldehyde (Sigma, USA) was added for 30 min before processing cells for imaging by confocal microscopy. Lysosomal compartments were visualized by adding Lyso-Tracker prior to microscopy according to the manufacturer’s instructions (Invitrogen, USA) and the protease inhibitor E64 was added to cultures 16 h prior to imaging.

**Locomotion assay**

The locomotor activity of adult flies was analyzed by recording with a video tracking system, for a 10 min period, the movements of single adult flies inside an arena.

Parental and DmLETM1 down-regulated adult flies (maintained at constant 22°C temperature and 60% humidity and light/dark 12 h cycles), 3–5 days after pupal eclosion, were anaesthetized under CO₂ and single adult flies were collected and placed into small transparent vials (4.5 cm in length and 1.5 cm in diameter) with no food and water *ad libitum* through a piece of wet paper. Each vial contained a single fly. Flies were then left to rest overnight (at least 10 h) in order to have a complete recover from CO₂ anesthesia.

Five minutes before the experiment, the wet paper was removed from each vial. The vial was then placed inside a box where a series of ultrabright white leads generated a uniform illumination. After having closed the box, the movement of the fly inside the vial (the arena) was videorecorded using a Sony digital videocamera. Video signals were online fed to a PC through a video-interface. A specific software (AnyMaze, Stoelting, USA) was utilized to track the path covered by the moving animal during the recording time period of 10 min and to calculate its total length. Everytime the animal stopped to walk for more than 2 s, the pause was considered an immobile episode. During the immobile episode, the animal body did not change its coordinates inside the arena, even if it was moving its legs or wings. The software automatically analyzed the number of immobile episodes and their total time duration (sum of the time duration of each immobile episode). An average of each immobile episode time duration was calculated from these two parameters. The software calculated also the time spent during active movement together with the average speed during active movement. A total number of 24 control (parental T2) flies, 25 *n-syb* GAL4 and 27 *elav*-GAL4 DmLETM1 down-regulated flies, respectively, were analyzed.

The tests were performed at the same time interval of the day for all strains.

Statistical significance between control and DmLETM1 down-regulated flies was analyzed using the *t*-test for unpaired data.

**Electrophysiology**

Experiments were performed at 20–22°C on third-instar larval body wall preparations dissected in Ca²⁺-free HL3 saline (35) and pinned on the silicone-coated surface (Sylgard 184, Dow Corning, USA) of a 35 mm Petri dish (36). After dissection, Ca²⁺-free HL3 saline was replaced with Ca²⁺-free 1 mM HL3. Before starting electrophysiological recordings, third-instar larval body walls were left to incubate in the new Ca²⁺-free 1 mM HL3 solution for at least 15 min. Electrophysiological recordings were done on fibers six or seven of abdominal segment 3–4 using intracellular glass microelectrodes (1.2 mm o.d.; 0.69 mm i.d.; 10–12 MΩ resistance; Science Products, Germany) filled with a 1:2 solution of KCl 3 M and CH₃CO₂K 3 M. Fibers with a membrane resting potential lower than −60 mV were discarded. In each fiber, both KOAc-induced swelling of isolated mitochondria

The light scattering method was used to measure the mitochondrial KHE activity: KOAc-induced swelling experiments were performed in hypotonic potassium acetate buffer and recorded with a spectrophotometer Hitachi U-2000 as described by Simon and Bergeman (4). Briefly, isolated mitochondria were resuspended in 0.6 m sorbitol buffer, pH 7.4, to a final concentration of 10 mg of protein per mL; and 100 µl of this suspension was incubated 5 min with 5 µM antimycin A (Calbiochem, USA), washed, treated with 0.5 µM A23178 (Sigma) and EDTA prior resuspension in KOAc swelling buffer (55 mM KOAc, 5 mM TES, 0.1 mM EGTA and 0.1 mM EDTA) and immediately recorded at A₅₄₀.

KOAc-induced swelling of isolated mitochondria

The null mutant *mdm38Δ* was constructed from the *S. cerevisiae* yeast strain W303 (ATCC no. 201239) as previously described (4). Transformation of this strain was performed according to Dimmer et al. (8) using the yeast expression vector pVT103-U (9) empty or pVT103-U encoding CG4589-HIS from the *ADH* promoter. To clone CG4589 into the vector pVT103-U, CG4589 was amplified with the primers 5’-AACTGGATCCCATACGTGTCGTTATCG-3’ and 5’-CATGCTCGAGTCCGTTGTTGCTGG-3’ using CG4589-HIS in frame with 6× H1S in frame with CG4589. Expression of CG4589 in yeast mitochondria was verified by western blotting.

Complementation experiments were performed using *mdm38Δ* transformed with pVT103-U with or without sCG4589. Serial dilution of transformants grown to OD₆₀₀ = 1 were spotted onto fermentable YPD, SD-ura and non-fermentable (YPG) plates (10) and incubated as indicated.

**Electrophysiology**

Experiments were performed at 20–22°C on third-instar larval body wall preparations dissected in Ca²⁺-free HL3 saline (35) and pinned on the silicone-coated surface (Sylgard 184, Dow Corning, USA) of a 35 mm Petri dish (36). After dissection, Ca²⁺-free HL3 saline was replaced with Ca²⁺-free 1 mM HL3. Before starting electrophysiological recordings, third-instar larval body walls were left to incubate in the new Ca²⁺-free 1 mM HL3 solution for at least 15 min. Electrophysiological recordings were done on fibers six or seven of abdominal segment 3–4 using intracellular glass microelectrodes (1.2 mm o.d.; 0.69 mm i.d.; 10–12 MΩ resistance; Science Products, Germany) filled with a 1:2 solution of KCl 3 M and CH₃CO₂K 3 M. Fibers with a membrane resting potential lower than −60 mV were discarded. In each fiber, both KOAc-induced swelling of isolated mitochondria

The light scattering method was used to measure the mitochondrial KHE activity: KOAc-induced swelling experiments were performed in hypotonic potassium acetate buffer and recorded with a spectrophotometer Hitachi U-2000 as described by Simon and Bergeman (4). Briefly, isolated mitochondria were resuspended in 0.6 m sorbitol buffer, pH 7.4, to a final concentration of 10 mg of protein per mL; and 100 µl of this suspension was incubated 5 min with 5 µM antimycin A (Calbiochem, USA), washed, treated with 0.5 µM A23178 (Sigma) and EDTA prior resuspension in KOAc swelling buffer (55 mM KOAc, 5 mM TES, 0.1 mM EGTA and 0.1 mM EDTA) and immediately recorded at A₅₄₀.

**Electrophysiology**

Experiments were performed at 20–22°C on third-instar larval body wall preparations dissected in Ca²⁺-free HL3 saline (35) and pinned on the silicone-coated surface (Sylgard 184, Dow Corning, USA) of a 35 mm Petri dish (36). After dissection, Ca²⁺-free HL3 saline was replaced with Ca²⁺-free 1 mM HL3. Before starting electrophysiological recordings, third-instar larval body walls were left to incubate in the new Ca²⁺-free 1 mM HL3 solution for at least 15 min. Electrophysiological recordings were done on fibers six or seven of abdominal segment 3–4 using intracellular glass microelectrodes (1.2 mm o.d.; 0.69 mm i.d.; 10–12 MΩ resistance; Science Products, Germany) filled with a 1:2 solution of KCl 3 M and CH₃CO₂K 3 M. Fibers with a membrane resting potential lower than −60 mV were discarded. In each fiber, both KOAc-induced swelling of isolated mitochondria

The light scattering method was used to measure the mitochondrial KHE activity: KOAc-induced swelling experiments were performed in hypotonic potassium acetate buffer and recorded with a spectrophotometer Hitachi U-2000 as described by Simon and Bergeman (4). Briefly, isolated mitochondria were resuspended in 0.6 m sorbitol buffer, pH 7.4, to a final concentration of 10 mg of protein per mL; and 100 µl of this suspension was incubated 5 min with 5 µM antimycin A (Calbiochem, USA), washed, treated with 0.5 µM A23178 (Sigma) and EDTA prior resuspension in KOAc swelling buffer (55 mM KOAc, 5 mM TES, 0.1 mM EGTA and 0.1 mM EDTA) and immediately recorded at A₅₄₀.
spontaneous and evoked neurotransmitter release were recorded. No more than one fiber for each larval body wall was utilized for electrophysiological recording.

**Spontaneous neurotransmitter release.** Spontaneous neurotransmitter release was analyzed by intracellularly recording miniature end-plate potentials (meps or minis) in current-clamp conditions. Tetrodotoxin 4 μM was added to the bath in order to prevent action potential neurotransmitter release. Signals were amplified by a voltage-clamp amplifier (SEC-10; NPI, Germany) in current-clamp mode. Resting membrane potential was clamped at −70 mV. Minis were recorded for 240 s.

**Evoked neurotransmitter release.** Excitatory junctional potentials were intracellularly recorded under current-clamp condition. Resting membrane potential was clamped at −70 mV. Single segmental nerve to abdominal segment A3 or A4 was stimulated (stimulus duration 0.1 ms, 1.5 threshold voltage) using a suction microelectrode, filled with extracellular bathing solution and connected to a stimulator (S88, Grass, USA) via a stimulus isolation unit (SIU5, Grass, USA) in a capacitative coupling mode. Signals were amplified in current-clamp mode by voltage-clamp amplifier (SEC-10, NPI, Germany). For all experiments, the subsequent stimulation protocol was utilized: 75 stimuli at 0.5 Hz (a stimulation frequency which does not fatigue D. melanogaster third-instar larva neuromuscular junction) were followed by a 15 s 10 Hz tetanic stimulation.

**Data storage and analysis.** Amplified signals were digitized using a digital A/C interface (National Instruments, USA) and then fed to a PC for both on-line visualization and off-line analysis using an appropriate software (WinEDR, Starthclyde University; Pclamp 6.04, Axon, USA). Stored data, were off-line analyzed using an appropriate software (pClamp 6.04 Axon, USA) Graphs, curve fits and statistical analysis were made using a specific software (Prism, GraphPad, USA).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at *HMG* online.

**ACKNOWLEDGEMENTS**

We thank Georg Dietzl for helpful experimental advice. We are indebted to Barry Dickson for generously sharing the Drosophila lines w[1118]; P[G2D2208]v6661/TM3, w[1118]; P[G2D2208]v6662/TM3, w[1118] and P[G2D2208]v6663/CyO from the transgenic RNAi library and the GAL4 driver lines used in this study We thank Mauro A. Zordan for the possibility to utilize the videotracking system in our locomotor behavior experiments and for his helpful technical and critical suggestions, and Paolo Bernardi for critical reading of the manuscript. We are indebted to Andrew Pospisilik for sharing his results with us before publication.

**Conflict of Interest statement.** None declared.

**FUNDING**

This study was supported by the Austrian Science Fund (FWF) to R.J.S., a Canadian Institutes of Health Research (CIHR) operating grant to G.A.M. G.A.M. is a New Investigator of the CIHR and the Parkinson Society of Canada. M.S. was supported by a university of Padova grant (assegno di ricerca di Ateneo CPDR078721/07).

**REFERENCES**


H+ antiporter. Science, 326, 144–147.