Functional characterization of drim2, the *Drosophila melanogaster* homolog of the yeast mitochondrial deoxynucleotide transporter

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Running title: *The Drosophila deoxynucleotide carrier dRIM2*

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**Background:** Carrier-mediated influx of cytosolic deoxynucleotides is a major source of precursors for mitochondrial DNA synthesis.

**Results:** dRIM2 is required to maintain normal deoxynucleotide pools in *Drosophila* mitochondria and its knockout is lethal at the larval stage.

**Conclusion:** dRIM2 is a deoxynucleotide carrier and is essential to maintain mitochondrial function.

**Significance:** Our data provide the first animal model of RIM2 deficiency.

**ABSTRACT**

The *CG18317* gene (drim2) is the *D. melanogaster* homolog of the *S. cerevisiae* Rim2 gene, which encodes a pyrimidine (deoxy)nucleotide carrier. Here we tested if the drim2 gene also encodes for a deoxynucleotide transporter in the fruit fly. The protein was localized to mitochondria. *Drosophila* S2R⁺ cells, silenced for drim2 expression, contained markedly reduced pools of both purine and pyrimidine dNTPs in mitochondria, whereas cytosolic pools were unaffected. In vivo drim2 homozygous knockout was lethal at the larval stage, preceded by (i) impaired locomotor behavior, (ii) decreased rates of oxygen consumption and (iii) depletion of mtDNA. We conclude that the *Drosophila* mitochondrial carrier dRIM2 transports all DNA precursors and is essential to maintain mitochondrial function.

Deoxy- and ribonucleoside triphosphates (dNTPs and rNTPs) are essential for the replication and transcription of the mitochondrial genome. An appropriate supply of these precursors is thus necessary for the maintenance of functional mitochondria throughout the life of cells and organisms (1, 2). The main site of deoxy- and ribonucleotide synthesis is the cytoplasm where they are produced by two *de novo* pathways interconnected through the ribonucleoside diphosphates, which are both the immediate precursors of rNTPs and the substrates for ribonucleotide reductase, the key enzyme in the *de novo* synthesis of dNTPs (3). *De novo* synthesis of thymidylate, the first committed step of thymidine deoxynucleotide *de novo* synthesis, occurs in the nucleus and in the mitochondrial matrix (4, 5). In most but not all organisms, NTPs and dNTPs are also synthesized by salvage of (deoxy)nucleosides by dedicated nucleoside- and nucleotide- kinases. Mammals contain two parallel deoxynucleoside salvage pathways, located in the cytosol and in mitochondria, respectively. The rate-limiting enzymes are two cytosolic and two mitochondrial (mt) deoxynucleoside kinases whose combined substrate specificities permit the salvage of all deoxynucleosides in each of the two subcellular...
compartment (6). Ribonucleotide salvage consists primarily in the recycling of ribonucleosides and free purine bases and occurs in the cytoplasm. The nuclear envelope is freely permeable to nucleotides and the precursors made in the cytoplasm are therefore easily available for nuclear DNA replication and transcription. The mt inner membrane is instead impermeable to nucleotides, and cytosolic nucleotides need membrane carriers to reach the mt matrix where mtDNA transactions take place. At present only a few mt nucleotide carriers are known (7). In yeast they include the three isoforms of the ATP/ADP exchanger, a GTP/GDP carrier (Ggc1p) (8) and a (deoxy)nucleotide carrier (Rim2p) (9). Two human genes, SLC25A33 and SLC25A36 (7, 10), were suggested to code for nucleotide carriers on the basis of their homology to the S. cerevisiae RIM2. The prediction has been confirmed only in the case of SLC25A33 (11, 12). The product of SLC25A33 is a protein 33% identical to Rim2p. It has been named PNC1 (11), to highlight its function as a Pyrimidine Nucleotide Carrier, demonstrated by transport studies with the recombinant protein reconstituted in liposomes (11) and later by isotope-flow experiments in intact cells (12). The transport properties of yeast Rim2p and human PNC1 are very similar. When reconstituted into liposomes both proteins exchange all pyrimidine ribo- and deoxyribo-nucleotides and show some activity with guanine nucleotides but not with adenine nucleotides. Thus they appear to be responsible for the import of most nucleic acid precursors into mitochondria. The characterization of Rim2p activity led to the proposal that the carrier imports nucleoside triphosphates in exchange with monophosphates (9).

Rim2p deletion had been found to cause total loss of mtDNA in yeast long before the transport activity of the protein had been identified biochemically (13). So far there are no data on the in vivo effects of PNC1 loss of function in humans or mice. The existing information comes from experiments of PNC1 silencing by siRNA in cultured human cells. Knockdown (KD) of PNC1 led to depletion of mtDNA, reduced transcription of mt genes and impairment of oxidative phosphorylation (11, 14). In situ analysis of nucleotide flow in cells with downregulation of PNC1 revealed a slower mitochondrial uptake of uridine triphosphate and a slower release of thymidine nucleotides to the cytoplasm (12). The same study investigated also the function of the SLC25A36 gene product. Downregulation of the protein, which is 60% identical to PNC1, had no effect on mitochondrial pools. Therefore, the activity of SLC25A36 remains unknown (12). The genome of Drosophila melanogaster contains only one gene with significant similarity to yeast Rim2 and the two human genes. This gene, denominated CG183173 and here indicated as rim2, maps on chromosome 2 (position 22B1), spans 7,503 base pairs and produces three different transcripts, all containing the typical features of mitochondrial carriers (15). There is currently no information concerning the functions of rim2. On account of its homology to Rim2p and PNC1, it may be involved in the mitochondrial transport of nucleotides. We considered it could be a useful model to investigate how the deletion of a nucleotide carrier affects mitochondrial function in a multicellular animal.

We first silenced rim2 expression in the Drosophila S2R+ cell line (16) and found depletion of all mitochondrial dNTP pools, suggesting that the protein is involved in the transport of all four DNA precursors. We then produced rim2 knockout (KO) flies and found that the homozygous loss of rim2 is lethal, blocking larval development at the 3rd instar. We analyzed different phenotypic aspects of the rim2-/- larvae detecting profound alterations of mitochondrial structure and function, and impairment of larval locomotion that could be related to depletion of mtDNA. Our data suggest that rim2 codes for a nucleotide transporter essential for the maintenance of functional mitochondria in Drosophila.

EXPERIMENTAL PROCEDURES

Cell cultures – The Drosophila S2R+ cell line derived from a primary culture of late stage (20-24 h old) D. melanogaster embryos (16). It was obtained from Drosophila Genomics Resource Center (DGRC) (http://dgrc.cgb.indiana.edu/). S2R+ cells grow at 25°C without CO2 in Schneider’s medium (Life Technologies) with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) as a loose, semi-adherent monolayer, showing a doubling time of about 48 h.

dsRNA production and RNAi procedures – dsRNAi synthesis was performed employing the T7 Megascript kit (Life Technologies) (17, 18). The oligonucleotides primers used to synthesize
dsRNA starting from cDNA were drim2_T7 forward (F) and reverse (R) (primer sequences are reported in Table 1). These primers give two complementary 700 bp RNA products that anneal as temperature decrease, forming a final 700 bp dsRNA. About 2x10^6 cells suspended in 1 ml of serum-free medium were mixed with 2 μg/ml dsRNA, plated in a 24 wells plate and incubated at room temperature (RT) for 1 h. Subsequently, one volume of complete medium 2X was added and cells were grown in the presence of dsRNA for 2 days at 25°C.

**dNTP pool extraction and analysis** – At the end of the treatment with dsRNA, about 10x10^6 S2R* cells were centrifuged in 15 ml tubes for 10 min at 400×g and the pellet washed twice with ice-cold PBS. The cells were then resuspended in 200 μl of extraction buffer (0.21 M mannitol, 0.07 M sucrose, 0.2 M EGTA, 10 mM TrisHCl pH 7.5, 0.5% BSA) and a suspension of glass beads (0.1 mm diameter) corresponding to about ½ the volume of the cellular pellet was added. The cell-bead suspension was introduced in a Bullet Blender Storm homogenizer (Next Advance) and shaken for 2 min at speed 8, then 400 μl of extraction buffer were added and the glass beads were removed by a short centrifugation. Mitochondrial and cytosolic nucleotide pools were isolated from the whole cell homogenate by differential centrifugation and methanol extraction as described (19). All manipulations took place in a cold room. The pellet remaining after mitochondrial pool extraction was dissolved in 1 ml of 0.3 M NaOH. The A_{260} nm of the NaOH fraction was used to normalize the number of cells from which the pools of the different samples were extracted (20). The sizes of the dNTP pools were determined with a DNA polymerase-based assay (21) with the modifications reported in (22). Two different aliquots of each pool extract were analyzed and pool sizes were expressed as pmol dNTPs/million cells, with cell numbers calculated as indicated above.

**Immunolocalization of DRIM2** – HA-tagged drim2 cDNA was cloned in a pACT vector under the control of Actin 5c promoter (23). 500,000 cells were seeded on round coverslips and grown for 16 h, then they were transfected with 20μl CellFectin II Reagent and 2.5μg vector and incubated for 8 h. The medium was removed and replaced with complete Schneider medium (containing 10% heat-inactivated FBS). After 48h from transfection cells were washed once with 1x PBS and incubated with 100 nM MitoTracker Red CMXROS and 1μg/ml Cyclosporin H in Schneider’s Medium for 20 min (23). Then the cells were washed in 1X PBS and fixed in 4% paraformaldehyde for 20min. After a second wash in PBS cells were permeabilized for 5 min with 50 mM NH4Cl in PBS + 0.1% Triton, then blocked for 1h in 3% goat serum in PBS, washed again and incubated with 1:100 monoclonal mouse α-HA antibody (Sigma-Aldrich) at 4°C O.N. Cells were washed again with PBS then incubated with 1:500 FITC-conjugated α-mouse IgG (Sigma-Aldrich) with 2% goat serum for 45min. After a final wash in PBS the slides were mounted with Vectashield mounting medium. Images were taken with a Leica SP5 confocal microscope at 63X magnification.

**Fly stocks and breeding conditions** – Flies were raised on standard cornmeal medium and were maintained at 23° C, 70% relative humidity, on a 12h light: 12h dark cycle. The UAS fly strains (Trasformant ID #44203 and #44202) used to perform post-transcriptional silencing were from the VDRC (Vienna Drosophila RNAi Center). Other *D. melanogaster* strains were obtained from the Bloomington Stock Center.

**Egg-to-adult viability** – For each of the transgenic lines around 300 fertilized eggs were collected on standard yeast–glucose–agar medium in a Petri dish (60×15 mm). The fertilized eggs were incubated at 23°C, and for each experimental condition, the number of individuals reaching the third instar larva, pupa, or adult, and the relative percentages were calculated (24).

**Genomic DNA extraction** – Single individuals (flies or larvae) were homogenized in separate vials in 50 μl of extraction buffer containing 10mM Tris-HCl (pH 8.2), 1mM EDTA and 25 mM NaCl. Proteinase K was added to a final concentration of 200 μg/ml and the homogenate incubated for 45 min at 37° C followed by heat inactivation of the enzyme at 95° C for 5 min. Genomic DNA used to quantify the mtDNA copy number was extracted starting from 10 larvae using the phenol/chloroform precipitation.

**Primers** – All oligonucleotides used in this work were reported in the Table 1. They were designed using the on-line tool Primer-BLAST (25).

**Generation of knockout strain** – We obtained from Exelixis Drosophila Stock Center available stocks bearing PiggyBac insertions (PBac(RB) CG18317^e01575^ and PBac(RB) CG18317^e00041^) at the boundaries of the *drim2* locus. In order to obtain the gene deletion we exploited the specific recombination between the FRT element within the PBac elements catalyzed by FLP recombinase

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RNA isolation and qRT-PCR experiments – Total RNA was extracted from approximately 10 larvae or 2X10⁶ cells using Trizol (Life Technologies) and further purified by precipitation with LiCl 8M. RNA samples were checked for integrity by capillary electrophoresis (RNA 6000 Nano LabChip, Agilent Technologies). For each sample, 1 μg of RNA was used for first-strand cDNA synthesis, employing 10 mM deoxynucleotides, 10 μM oligo-dT and SuperScript II (Life Technologies). qRT-PCRs were performed in triplicate in a 7500 Real-Time PCR System (Life Technologies) using SYBER Green chemistry (Promega). The ΔΔCt method implemented in the 7500 Real Time PCR System software was used to calculate the relative expression ratio (28). The drim2 oligonucleotides primer used were drim2 F and R. Specific primers were designed for CoxI (CoxI F and R). The 16S primers used were 16S F and R (29). Rp49 was used as endogenous control and the oligonucleotides employed were Rp49 F and R.

Body wall preparations – A small portion of the tip was cut from third instar larvae, internal organs were removed by gently squeezing from end to end, and the preparation was turned inside-out by rolling the cuticula along a holding tweezer.

Mitochondrial pattern in muscle fibers – Body wall preparations of larvae were stained with 500 nM MitoTracker Red CMXRos (Life Technologies) for 45 min. Tissues were then washed in 1X PBS and fixed in 4% PFA for 20 minutes. After a brief wash they were mounted in 80% glycerol. Scans of muscle 6 were taken with a Leica SP5 Confocal Microscope at 63X magnification.

Measurements of oxygen consumption – Oxygen measurements were made using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Measurements were performed both in whole tissues of Drosophila larvae (body wall preparations) and in S2R+ Drosophila cells, using different Seahorse technologies. The instrument was maintained on a temperature of 25°C. In the case of tissues, each dissected larva was placed into a well of an islet capture 24-well microplate. Islet capture screens were used to keep the larvae in place. Basal oxygen consumption rates (OCR), reported in the unit of picomoles per minute, was measured several times before injecting the first drug to be tested. Chemicals were sequentially added in each well as described in figure legends. Cells were seeded onto XF-24 well-plates at 20,000 cells/well and cultured for 48 h. The following day the culture medium was replaced with serum-free Schneider medium (Life Technologies). Basal OCR were measured three times, loaded compounds were then sequentially injected.

Analysis of larval locomotor behavior – The locomotor activity of a single third stage larva inside an arena was recorded for a 120 seconds period using a video tracking system. The arena consisted in a Petri dish (5 cm in diameter) covered by a thin layer of agar gel 1%. The Petri dish was placed inside a box, the internal walls of which were painted black, containing a ring of ultrabright white leads to generate a uniform illumination. After closing the box, the movement of the larva inside the arena was video recorded using a Canon digital video camera (10 frames per second). A specific software (AnyMaze) was utilized to track the path covered by the moving animal during the recording time period of 120”.

The software calculated the total length of the path, the average speed and the maximum speed of the larva. A total number of 50 larvae were analyzed for each genotype. The tests were performed at the same time of the day for all strains.

Electron Microscopy – Third stage larvae were dissected in Ca²⁺-free haemolymph-like saline-3 (HL3) and transferred into a fixation solution containing 3% glutaraldehyde, 2% paraformaldehyde, 100 mM sucrose, and 2 mM EGTA, in 0.1 mM sodium phosphate buffer, pH 7.2. Samples were then post-fixed for 2 h with cold 1% OsO₄ in 0.1 mM sodium cacodylate buffer, pH 7.2, rinsed 3-4 times (5 min each) in 0.1 mM sodium phosphate buffer pH 7.2. Subsequently larvae were dehydrated through an ethanol gradient, followed by a propylene oxide-resin gradient. Finally samples were embedded in Epon resin (Sigma-Åldrich) and polymerized at 60°C for 3 days. The analysis was performed on 60 nm ultrathin sections of larval body-wall muscles, stained for 20 min with uranyl acetate, and examined with a Philips CM10 electron microscope (FEI Company).
analyses the shortest and the longest diameter of mitochondria, were measured using ImageJ software (http://rsb.info.nih.gov/ij/). In addition we measured the area and the density of mitochondria in the same larvae. In particular, we first measured the area occupied by all cell profiles present on each section, for each genotype considered. Over this area we then measured the total number and the area occupied by mitochondria. The data were collected considering the total area occupied by cell profiles on sections cut at 2 different levels (about 50 µm apart) of the same block, and placed on different grid. The analysis were performed using “Netherlennder” system (30).

**Determination of mtDNA levels** – Total DNA from larvae was extracted using phenol/chloroform precipitation. The amount of mtDNA was assessed by the ratio of mtDNA to nuclear DNA (nDNA) copy number determined by quantitative real time amplification of the 16S gene and the nuclear Rpl32 gene. Primers used in this work (16S F and R; Rpl32 F and R) were those previously reported by (29). We generated two gene-specific calibration curves with six 10-fold serial dilutions (100–1,000,000 copies) of plasmids containing the cloned target sequences (Life Technologies). Concentration of plasmid stock solutions was assessed with a NanoDrop ND-1000 spectrophotometer (Nanodrop), and the plasmid copy number of dilutions was calculated using Avogadro’s number. Reactions were performed in triplicate using the SYBR Green chemistry Real Time PCR System instrument (Life Technologies). Data were normalized to the ratio (arbitrary set to 100%) (31, 32).

**DNA microarray design** – Probes were designed using the Agilent eArray Custom Microarray Design Service (https://earray.chem.agilent.com/earray/index.jsp), which applies proprietary prediction algorithms to design 60 mer oligo-probes. Microarrays were synthesized in situ using the Agilent ink-jet technology with 8 x 60 K format. A total of 32,162 probes representing D. melanogaster transcripts were successfully obtained. A custom microarray platform, named “Drosophila 1.0” (eArray Design ID: 035757), showed 30,814 duplicate probes and 1,348 single probes. Each array included default positive (1,011 probes) and negative (308 probes) controls. Probe sequences and other details on the microarray platform can be found in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number: GPL17290.

**Microarray labeling and hybridization** – Gene expression profiling was carried out on drim2+/− and drim2−/− Drosophila larvae using the Drosophila 1.0 custom platform (Agilent Technologies). Total RNA was obtained from the whole body of 3rd instar larvae for each genotype. Four and three biological replicates were analyzed for drim2+/− and drim2−/− samples respectively for a total of 7 microarray experiments. 800 ng of total RNA was labeled with “Agilent One-Color Microarray-Based Gene Expression protocol” according to the manufacturer’s instructions. The synthesized cDNA was transcribed into cRNA and labeled with Cy3-dCTP. Labeled cRNA was purified with RNeasy Mini columns (Qiagen). The quality of each cRNA sample was verified by total yield and specificity calculated with NanoDrop ND-1000 spectrophotometer measurements. 1.65 µg of labeled cRNA was used in each reaction and hybridization was carried out at 65°C for 17 h in a hybridization oven rotator (Agilent). The arrays were washed using Agilent Gene expression washing buffers and Stabilization and Drying Solution, as suggested by the supplier. Slides were scanned on an Agilent microarray scanner (model G2565CA) and Agilent Feature Extraction software version 10.5.1.1 was used for image analysis. Gene expression data are available in the GEO database with the accession number: GSE48012.

**Statistical analysis of gene expression data** – Inter-array normalization of expression levels was performed with quantile method (33) to correct possible experimental distortions. A normalization function was applied to the expression data of all the experiments and the values of within-arrays replicate spots were then averaged. Feature Extraction Software which provided spot quality measures was used to evaluate the quality and reliability of the hybridization. In particular, the flag “glsFound” (set to 1 if the spot had an intensity value significantly different from the local background and to 0 when otherwise) was used to filter out unreliable probes: the flag equal to 0 was to be noted as “not available (NA).” Probes with a high proportion of NA values were removed from the dataset in order to carry out a more solid, unbiased, statistical analysis. Forty percent of NA was used as the threshold in the filtering process, and a total of 25,350 Drosophila transcripts were...
obtained. Principal Component Analysis (PCA),
cluster analysis, and profile similarity searches
were performed with Multi Experiment Viewer
version 4.8.1 (tMev) of the TM4 Microarray
Software Suite. The identification of differentially
expressed mRNAs was performed with two class
Significance Analysis of Microarray (SAM)
program with default settings (34). The
normalized expression values of the biological
replicates for each genotype were log2
transformed and mediated. Gene Ontology (GO)
analysis of differentially expressed genes was
performed using the DAVID tool (35).

RESULTS

The Rim2p protein was previously characterized
in Saccharomyces cerevisiae as a mitochondrial
pyrimidine nucleotide transporter (9). Yeast
Rim2p is a member of the mitochondrial carrier
protein family distinguished by some typical
features: the amino acid sequences include three
repeats, each containing two putative trans-
membrane sequences (TMSs) and the signature
motif [PX(DE)XX(KR)]. Therefore the main
membrane sequences (TMSs) and the signature
sequences and calculated the levels of homology
of yeast, human and fruit
A
mitochondria (Fig. 2
clearly indicated that dRIM2 localizes to
-repeats, each containing two putative trans-
features: the amino acid sequences include three
repeats, each containing two putative trans-
membrane sequences (TMSs) and the signature
motif [PX(DE)XX(KR)]. Therefore the main
structure fold is a six α-helical bundle (11). To
evaluate the degree of conservation of the Rim2p
across species, we performed an amino acid
alignment of yeast, human and fruit-fly Rim2p
sequences and calculated the levels of homology
(Fig. 1). The drim2 gene of D. melanogaster
codes for three different transcripts, named A, C
and D. The corresponding protein isoforms
showed comparable degrees of similarity to the
yeast sequence (= 40%). Interestingly, all
Drosophila isoforms are closer to human PNC-1
(52-54 % identity) than to yeast Rim2p.

To characterize the subcellular localization of
dRIM2 we transfected Drosophila S2R+ cells with
a pACT vector expressing HA-tagged dRIM2
cDNA under the control of the Actin 5c promoter.
Mitotracker staining and immunodetection of HA-
dRIM2 with an α-HA monoclonal antibody
clearly indicated that dRIM2 localizes to
mitochondria (Fig. 2A).

Next, we downregulated the expression of
derm2 in S2R+ cells and tested the effects on the size of the mitochondrial
deoxynucleoside triphosphate (dNTPs) pools. In
cells incubated for 72 h with dsRNA, real-time
RT-PCR analysis showed that mRNA was
decreased by 80%. If dRIM2 is a carrier for
pyrimidine nucleotides, its downregulation should
affect the dTTP and dCTP pools more than the
purine deoxynucleotides dATP and dGTP. To test
this prediction, we isolated the mitochondrial and
cytosolic dNTP pools from control and silenced
S2R+ cells by adapting a procedure previously
devised for the quantification of mammalian
mitochondrial dNTPs (19). No data are available
on dNTP pool sizes in Drosophila cells and we
therefore wished to establish the relative
abundance of the four dNTPs and the ratios
between cytosolic and mitochondrial pools.
In mammals dTTP is generally the largest dNTP
pool, and dGTP the smallest, with dCTP and
dATP occupying intermediate positions. Pool
sizes in Drosophila are reported in Fig. 2B and C.
As in mammalian cells (22), mitochondrial pool
sizes corresponded to about 3-10% of the
cytosolic pools. The dATP pool was the largest in
both cytosol and mitochondria, followed by
dTTP, dGTP and dCTP in the cytosol and dTTP,
dCTP and dGTP in mitochondria. In both
compartments the dCTP pool was particularly
small, and comparable in size to the dGTP pool.
Since the sizes of dNTP pools are strongly
influenced by the position of the cell in the cell-
cycle (36) we took care of comparing the
concentrations of dNTPs in cultures of drim2-
silenced and control S2R+ cells with similar
frequencies of S-phase cells. We observed no
difference in the proportion of S-phase cells
between silenced and control cultures, with values
of about 20-25% depending on the experiment.
Therefore, we feel confident that the differences
in dNTP pools we measured in the two sets of
cultures were not caused by differences in cell-
cycle distribution. However, whereas the
cytosolic pools were virtually identical in control
and silenced cultures (with the exception of the
dATP pool that was higher in the latter), all
mitochondrial dNTPs were significantly lower in the
silenced cells, with levels ranging between
30% (dCTP) and 60% (dATP) of the controls.
Thus, downregulation of drim2 reduced the
mitochondrial concentrations of both pyrimidine
and purine dNTPs, suggesting that the protein is a
general transporter for all four DNA precursors.
We cannot extend this conclusion to RNA
precursors, as we did not measure the
mitochondrial ribonucleotide pools.

Next we attempted to knockdown the drim2 gene
in vivo by using GAL4/UAS-driven RNAi in
living flies (18, 37). Despite high levels of
silencing (about 80%) (Fig. 3A), drim2 KD
individuals did reach the adult stage and lived
longer than the controls (Fig. 3B). No effects on
egg to adult viability were observed. These results
suggest that even a low residual level of drim2
mRNA is sufficient to maintain the wild-type (wild-type) phenotype. Thus we generated a *Drosophila* *drim2* KO using the technique described in (26) that exploits the specific recombination between FRT sites in the presence of flippase. Heterozygous KO flies (*drim2*+/−) were balanced with a strain expressing GFP (w; L2, Pin1,CyO-GFP), allowing discrimination between homozygous GFP-negative *drim2*−/−, and heterozygous GFP-positive *drim2*−/+ larvae. Real time-PCR showed that *drim2*−/+ larvae had about 50% *drim2* mRNA levels compared to a wild-type control (w1118), whereas *drim2*− flies were null, as expected (Fig. 4A). The *drim2*−/+ third-instar larvae were visibly smaller than their heterozygous counterparts (Fig. 4B). Nevertheless, KO larvae did present mouth hooks, the distinctive character of the third larval stage indicating that their smaller size was not due to a developmental delay (Fig. 4C). The KO heterozygous *drim2*−/+ larvae developed into normal adults with no developmental defects. On the contrary, none of *drim2*− larvae reached adulthood. Although *drim2*− individuals survived through larval development, most of them died at the third larval instar and the survivors failed to progress beyond the pupal stage (Fig. 4D). To further characterize the phenotype, we measured locomotor activities (total distance travelled, overall average speed and inactivity) with the Any Maze software. Wild-type and *drim2*−/+ larvae behaved similarly, whereas *drim2*−/+ showed marked locomotor defects (Fig. 4E, F, G).

Confocal images of body wall preparations stained with Mitotracker Red showed a normal mitochondrial pattern along the z-lines in w1118; however, the *drim2*−/+ individuals showed spatially disorganized mitochondria that failed to line up along the z-lines (Fig. 5A). This abnormal pattern was confirmed by transmission electron microscopy (EM) (Fig. 5B). EM carried out in *drim2*−/+ and *drim2*−− larvae (first panel, Fig. 6A) revealed alterations of mitochondrial number and shape compared to w1118 (Fig. 6A). The *drim2*−/+ mitochondria appeared more elongated, and considerably bigger than those of w1118 larvae (Fig. 6A). Morphometric analysis indicated that, on average, the major mitochondrial diameter in *drim2*−/+ larvae was significantly increased and the minor diameter reduced relative to w1118 mitochondria (Fig. 6B, C). In *drim2*−− larvae, mitochondria had a rounder shape (Fig. 6A); the averaged major diameter was unchanged relative to the wild-type t whereas the minor diameter was longer than that of mitochondria of both w1118 and *drim2*−/+ (Fig. 6B, C). Accordingly, estimates of the mitochondrial area indicated that *drim2*−/+ organelles were larger than those from w1118 and *drim2*−− flies (Fig. 6D). Furthermore, mitochondrial density, i.e. the number of mitochondria per surface unit, was reduced in *drim2*−/+ individuals compared to wild-type controls and significantly higher in *drim2*−− larvae (Fig. 6E).

As Rim2 deletion in yeast and PNC1 downregulation in mammalian cells decreases mtDNA content (13, 14), we measured the mtDNA copy number in KO larvae. Unexpectedly, whilst mtDNA was almost 40% depleted in *drim2*−/+ larvae, *drim2*−− individuals had levels of mtDNA close to wild-type (Fig. 7A), possibly a consequence of the higher mitochondrial density (Fig. 6E) (38, 39).

A reduced mtDNA copy number might lead to reduced expression of the mtDNA-encoded subunits of the respiratory chain. We measured the levels of mitochondrial transcripts for *Cyclooxygenase-1* (COX1) and RNA (16S) relative to the housekeeping gene *Rpl32* by real time-PCR (40) (Fig. 7B). Mitochondrial transcription was lower in both *drim2*−/+ and *drim2*−− larvae. In order to establish whether defects in *drim2* function also affect mitochondrial respiration we measured oxygen consumption rates of muscle body-wall preparations of *Drosophila* larvae with the Seahorse technology (41). Controls and *drim2*−− larvae maintained a steady respiratory rate which was inhibited by rotenone and antimycin A, demonstrating its mitochondrial origin. On the contrary *drim2*−/+ larvae showed severe impairment of oxygen utilization that was insensitive to respiratory inhibitors (Fig. 8A). The same measurements were performed also in S2R+ cells silenced for *drim2*. The rate of oxygen consumption was significantly decreased in cells silenced for 96 h, and the cells responded less than the controls to uncoupler (FCCP) and to (oligomycin or rotenone plus antimycin A) (Fig. 8B). No significant differences of respiratory profile were detected between cells undergoing a mock interfering treatment for almost 48 h and the controls. The basal respiration of the two cultures was similar, both being of mitochondrial origin since they were stimulated by FCCP and inhibited by oligomycin or rotenone plus antimycin A (Fig. 8B).

In order to define the gene expression pattern specifically associated with *drim2* KO, we performed protein-coding microarray analyses...
Mitochondria contain multiple copies of a small circular DNA coding for essential polypeptide components of the respiratory chain and F-ATP synthase complexes embedded in the inner mitochondrial membrane. Replication and transcription of mtDNA occur during the whole life of the cell, even after it has reached terminal differentiation. Both processes are particularly active during cell proliferation and early development, when mitochondrial biogenesis is induced (42) and thus the request for dNTPs and rNTPs is particularly high. In yeast and mammals the cytoplasm is the main site for nucleotide production, but some synthesis occurs also in mitochondria and in the nucleus (4, 5). Due to the impermeability of the mitochondrial inner membrane to nucleotides, these are taken up into mitochondria by a partly unknown repertoire of membrane carriers. The nucleotide transporter we have studied here is the Drosophila ortholog of yeast Rim2p and human PNC1, two homologous pyrimidine nucleotide transporters (9, 11, 12).

In yeast, the genetic inactivation of Rim2 causes loss of mtDNA and a petite phenotype (13). In cultured human cells, downregulation of PNC1 by siRNA can lead to decreased mtDNA copy number (14). Thus in both yeast and humans the dependence of mitochondria on nucleotides made in the cytosol has been demonstrated. The study of nucleotide, and especially deoxynucleotide, metabolism has been relatively neglected in Drosophila. Most attention has been dedicated to a distinctive multisubstrate deoxynucleoside kinase responsible for the salvage of all deoxynucleosides (43), as opposed to the four separate deoxynucleoside kinases existing in mammals (6). Given the presence in the genome of the key enzymes for dNTP and rNTP de novo synthesis, we assumed that the general picture defined in mammalian cells also applies to Drosophila and that also in this species mitochondria obtain nucleic acid precursors from extra-mitochondrial sources. Thus, after confirming that dRIM2 is localized to mitochondria (Fig. 2A), we hypothesized that its deletion may impact the mtDNA content and impair mitochondrial transcription, with negative consequences for oxidative phosphorylation and energy-dependent processes.

We studied the effects of dRIM2 ablation on the dNTP pools required for the maintenance of mtDNA. No information was available on the composition of dNTP pools of Drosophila cells and we first analyzed the total pools of control S2R’ cells. They contained relatively more dTTP and dATP than dCTP and dGTP, a pool composition different from that commonly observed in mammalian cells where dTTP and dCTP frequently are the most abundant dNTPs. When we separated mitochondrial and cytosolic pools we found that, in Drosophila, as in human cells, the dNTP pools of mitochondria amount to no more than 10% of the total dNTPs. In drim2-silenced cells both mitochondrial pyrimidine and purine dNTP pools were reduced. Although these data only give a static picture of the mitochondrial pools and the actual nucleotide transport was not directly measured, the general decrease of all four dNTPs suggests that dRIM2 acts as a general deoxynucleotide transporter. Cytosolic pools were unaffected, underscoring a specific function of the carrier in the import of nucleotides from the site of their synthesis in the cytoplasm into the mitochondrial matrix where they are consumed for mtDNA synthesis. The possible lack of intramitochondrial dNTP synthesis in Drosophila suggested by the genomic data is supported by the appearance of cell toxicity when silencing was prolonged beyond 3 days. Although the treatment did not completely remove drim2 mRNA, the downregulation was sufficient to impair cell viability in vitro. This was not the case when we downregulated the protein in vivo using the GAL4/UAS system to ubiquitously activate drim2-dsRNAi. A 30% residual level of gene activity was sufficient for the flies to develop normally into adults (Fig. 3).
A very different picture appeared after *in vivo* KO of *drim2*. Homozygous *drim2*+/− larvae exhibited a lethal phenotype and died during the third larval stage without reaching adulthood. The homozygous KO produced an overall impairment of larval development associated with reduced dimensions compared to wild-type larvae (Fig. 4B-D). It is known that most *Drosophila* mutants for genes involved in mitochondrial and nucleotide metabolism either do not undergo metamorphosis, arresting their development at larval stage or manifest developmental defects. This phenomenon is probably related to the particularly high energetic burden of metamorphosis. Larval lethality is frequently associated with neuromuscular and behavioral defects, functions that in *Drosophila* are particularly sensitive to OXPHOS-dependent energy drop (44-46). Here, *drim2*+/− larvae showed severe defects in their locomotor activity (Fig. 4E-G).

From the morphological point of view, mitochondria of both *drim2*+/− KO and heterozygous *drim2*+/− larvae displayed evident anomalies (Fig. 6). EM analysis in *drim2*+/− larvae revealed a higher mitochondrial density (Fig. 6E), possibly a physiological response compensating for the progressive loss of mitochondrial function. However, expression profiling did not demonstrate activation of mitochondrial biogenesis. Genes such as *PGC-1α*, *NRF-1*, *NRF-2* and *TFAM* were not up-regulated in *drim2*+/− larvae. An alternative explanation for the increased number of mitochondria may be reduced mitochondrial turnover rather than increased biogenesis.

Interestingly, among the genes involved in nucleotide metabolism that are differentially expressed in homozygous KO larvae only nucleoside diphosphate kinase (NDK) was clearly upregulated, while all others were downregulated. Since NDK catalyzes the final step in the synthesis of dNTPs and rNTPs, this result suggests a homeostatic response to reduced availability of nucleoside triphosphates in mitochondria. The downregulated genes encode both for synthetic and catabolic enzymes and participate in the regulation of different pool components in combination with other enzymes. Changes detected in individual members of a complex enzyme network are difficult to interpret and may reflect general unspecific stress rather than a concerted metabolic adjustment related to the lack of mitochondrial nucleotides.

Despite the severity of the KO phenotype, direct quantitation of mtDNA in the *drim2*+/− larvae did not show the expected depletion (Fig. 7A) possibly due to their observed higher mitochondrial density (Fig. 6E). Moreover *drim2*+/− larvae show a decrease of mitochondrial transcripts (Fig. 7B). These results indicate that *drim2* and *PNC1* are necessary for mtDNA transcription and replication in flies and humans, respectively.

Mitochondrial disorders are characterized by impaired oxygen consumption, and homozygous KO larvae and *drim2*-silenced cells are no exception (Fig. 8), as expected on the basis of the reduced mtDNA copy number and transcription. Yeast Rim2 was identified in screens for suppressors of high iron toxicity in strains deleted for the two yeast mitochondrial iron transporters *Mrs3* and *Mrs4* or for the vacuolar iron transporter *CCC1* (47, 48). Thus the protein was proposed to play a dual role as pyrimidine nucleotide and iron transporter. However, recent data show that in wild-type yeast deletion of RIM2 alone is irrelevant for mitochondrial iron supply (49). The *Drosophila* genome contains *mfrn*, the gene for mitoferrin that is the homologue of the yeast iron carriers *Mrs* 3 and 4. Therefore, we assume that the effects of *drim2* KO detected here depend primarily on the lack of nucleotide transport into mitochondria.

Our findings strongly suggest that the *D. melanogaster* CG18317 (*drim2*) gene is essential to maintain mitochondrial function by providing deoxynucleotides for mtDNA transactions. *dRIM2* is the first (deoxy)nucleotide carrier characterized in *Drosophila* and our KO larvae are the first animal model of RIM2 deficiency. The data presented here may offer a key to understand the functional role of RM2 in a multicellular animal, and further support a general function in deoxynucleotide transport in mitochondria.
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FOOTNOTES

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TABLE AND FIGURE LEGENDS

TABLE 1. Sequences of oligonucleotides used in this study. Primer sequences are indicated as 5'-3' direction.

FIGURE 1. Sequence alignment of RIM2 proteins from different species. Sequences included in the alignment, performed with Multiple Sequence Alignment T-Coffee, are those of Saccharomyces cerevisiae, Homo sapiens (SLC25A33) and Drosophila melanogaster (dRIM2 isoforms A, C and D). The alignment highlights identical residues (•) and similar ones (• and :), the positions of the six transmembrane α-helices (red lines) and the three signature motifs (blue boxes).

FIGURE 2. Mitochondrial localization of dRIM2 and effects of drim2 silencing on the deoxynucleotides pool contents of S2R+. A, Drosophila cells were transiently transfected with pACT-drim2 HA tagged, incubated with Mitotracker dye (red) and then immunolabeled with the anti-HA antibody (green). Overlay of images confirms the mitochondrial localization of dRIM2. Pyrimidine and purine dNTP pool sizes in cytosol (B) and mitochondria (C) of drim2 silenced S2R+ cells and in controls. Pool sizes are expressed as pmol dNTP per million cells. Values are means ± standard deviation (s.d.) from four experiments for control and five experiments for RNAi (Student’s t-test • p< 0.05, ---p< 0.005).

FIGURE 3. Developmental effects of drim2 KD in vivo. All experiments were carried out in w1118 (closed column) as well as in specific controls w; UAS-drim2 (grey column) and in KD w; UAS-drim2/Act5C-Gal4 (open column) third-stage larvae. A, drim2 mRNA levels in each strain measured by qRT-PCR. B, Relative percentage of egg to adult viability calculated at three developmental stages, i.e., third-stage larvae, pupae and adults. Data plotted are means ± s.d. (Student’s t-test ---p< 0.005).

FIGURE 4. Morphological, developmental and behavioral effects of drim2 KO in vivo. All experiments were carried out in parallel in w1118 (closed column), drim2+/+ (grey column) and drim2−/− (open column) third-stage larvae. A, drim2 mRNA levels, expressed as relative quantity of template in the sample, were determined by qRT-PCR. B, Third-stage KO larvae. Heterozygous drim2+/− larva (right) and homozygous drim2−/− larva (left). Notice the smaller size of the latter. C, Both hetero- and homozygous KO larvae present mouth hooks, the distinctive characters of the third larval stage. D, Relative percentage of egg to adult viability calculated at three developmental stages, i.e., third-stage larvae, pupae and adults. E-G, Larval locomotor activity characterized by the three parameters, each calculated in a total recording time period of 120 seconds: (E) total distance travelled (i.e. as millimeters covered), (F) overall average speed (millimeters over 120 seconds) and (G) inactivity (seconds over 120 seconds). Data plotted are means ± s.d. (Student’s t-test ---p< 0.005).

FIGURE 5. Mitochondria disposition around z-lines. Confocal images (A) and electromicroscopy (B) of body wall preparations of w1118 and drim2−/− larvae. Arrows indicate the z-lines.

FIGURE 6. Electron microscopic analysis on third-stage larval body-wall sections. Characterization was carried out on w1118, drim2+/+ and drim2−/− larvae. A, Cross section ultrastructure of larval muscles, illustrating the distribution and morphology of mitochondria. (B and C) Morphometric analyses of mitochondrial dimensions in terms of minor (B) and major diameter (C), both expressed in nanometers and presented as box plots. D, Mean ± standard error of the total area occupied by mitochondria (expressed in square microns) over the tissue profile area measured (w1118: 447 µm²; drim2+/+; 231 µm²; drim2−/−: 256 µm²). E, Mitochondria density ± standard error was plotted as the number of mitochondria per square micrometer of the tissue profile area (w1118: 447 µm²; drim2+/+; 231 µm²; drim2−/−: 256 µm²). (Student’s t-test • p< 0.05, ** p< 0.01, ***p< 0.005).

FIGURE 7. Mitochondrial DNA content and analysis of mitochondrial transcription in larvae. All experiments were carried out on w1118, drim2+/+ and drim2−/− larvae. A, mtDNA content was measured by quantitative real-time-PCR. Data are ratios of mtDNA to genomic DNA relative to the control ratio in w1118 larvae. Values are expressed as a mean of three independent experiments and error bars represent the s.d. of
the mtDNA/nDNA ratio among the replicates. B, Analysis of mitochondria transcription. Transcripts of the mitochondrial genes Cyclooxygenase-1 (COX1) and (16S) were measured by real time-PCR and related to the level of transcripts of the nuclear gene Rpl32. For each conditions data are presented as the mean of relative quantity of template in the sample ± s.d. from three individual experiments. (Student’s t-test - p< 0.05, ** p< 0.01, *** p< 0.005).

FIGURE 8. Oxygen consumption rates in larvae and in S2R+ cells. A, Oxygen consumption was measured in w1118 (black vehicles), drim2+/+ (grey vehicles) and drim2−/− (empty vehicles) third- stage larvae. The addition of 5 μM rotenone plus 5 μM antimycin A is indicated by the arrow. B, Respiratory profile of control and drim2-silenced S2R+cells. We analyzed untreated cells (black vehicles), cells silenced for 48 hours with dsRNA (grey vehicles) and cells silenced for 96 hours (empty vehicles). Ten mM glucose, 1 μM FCCP and 5 μM rotenone plus 5 μM antimycin A were added at the times marked by arrows.

FIGURE 9. Altered gene pathways in drim2 KO Drosophila. Heat map representing a selection of deregulated transcripts, provided by DAVID tool, in drim2−/− vs. drim2+/+ involved in purine (22 transcripts) and pyrimidine (13 transcripts) metabolism (A and B respectively), as well in Oxidative phosphorylation (10 transcripts) and Glycolysis/gluconeogenesis (6 transcripts) (C and D respectively). A color-coded scale for the normalized expression values is used: yellow and blue represent high and low expression levels in drim2−/− vs respect to drim2+/+, respectively. The expression level of each transcript was calculated as the Log2 (drim2−/− / drim2+/+) and the complete list of differentially expressed genes identified by SAM algorithm is provided in the Supplementary Information (Table S1).
| Primer name | Sequence 5’-3’ |
|-------------|----------------|
| dmm2_T7 F   | taatagcctactctagggagatgtacctgtgtttgccaaagtsaa |
| dmm2_T7 R   | taatagcctactctagggagatgtacctgtgtttgccaaagtsaa |
| dmm2KOSATTY F | atctgactccttgaatctg |
| dmm2KOSATTY R | atctgactccttgaatctg |
| dmm2KOSATT G | gagaccaacagggacctatg |
| dmm2KOSATT Y | ctctgactccttgaatctg |
| dmm2 F      | tggctacgccg gastraeaa |
| dmm2 R      | tggctacgccg gastraeaa |
| Corl F      | tpcctcttgattacggtcaccagpa |
| Corl R      | tpcctcttgattacggtcaccagpa |
| 165 F       | tggctacgccg gastraeaa |
| 165 R       | tggctacgccg gastraeaa |
| Rsp9 F      | gagaccaacctctggttaaac |
| Rsp9 R      | gagaccaacctctggttaaac |
| Rsp32 F     | aggccoaagatgagtaagaa |
| Rsp32 R     | tggctacgccg gastraeaa |
FIGURE 1
FIGURE 2

A. Mito tracker, FITC α-HA, overlay

B. Cytosolic pool

C. Mitochondrial pool

- dTTP
- dCTP
- dGTP
- dATP

Not treated

drim2 RNAi

pmol/10^6 cells
FIGURE 3

A

Relative mRNA levels

w118  w. UAS-dim2  w. UAS-dim2/Ad5C-Gal4

B

Relative % survival

larvae  pupae  adults

w118  w. UAS-dim2  w. UAS-dim2/Ad5C-Gal4  w118  w. UAS-dim2  w. UAS-dim2/Ad5C-Gal4
FIGURE 4

A

B

C

drim2

D

E

F

G

Distance travelled

Average speed

Inactivity

mm

m/m/120°

sec/120°

FIGURE 5

w1118

drim2

A

B

10 μm

1,8 μm

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FIGURE 6
FIGURE 7

Mitochondrial transcription

B

Relative mRNA levels

- COX1
- 16S

FIGURE 8

A

B

Glu Oligo FCCP R+A

R+A

CCR (pmol/min)

Time (min)

0 40 60 120

0 100 200 300 400

0 300 600 900 1200 1500 1800

0 30 60 90 120

0 100 200 300 400

0 300 600 900 1200 1500 1800
FIGURE 9

**A**

- CG11883: cytosolic/mitochondrial 5'-nucleotidase
- Prot2
- CG32549: putative purine 5'-nucleotidase
- Ady43A: adenosine kinase
- CG16758: purine-nucleoside phosphorylase
- CG11811: guanylate kinase
- CG2590: adenylosuccinate lyase
- Act13ECG9210: Adenylyl cyclase 35C
- Gycbeta100B: Guanylcylic cyclase beta-subunit at 100B
- Aprt: adenine phosphoribosyltransferase
- Rpl11T28: IMP dehydrogenase
- ade5: RNA polymerase III 128kD subunit
- if23Tcg: phosphoribosylaminomimidazole corboxylase
- f23626: DNA-directed RNA polymerases I and II subunit RPAC2
- Rspl8: AMP deaminase
- Pde8: Phosphodiesterase 8
- mae4: DNA polymerase epsilon subunit 2
- Gyccalpha99B: Guanylcylic cyclase alpha-subunit at 99B
- CG21793: adenosinephosphatidase
- Pde6: Phosphodiesterase 6
- Veil: ecto-5'-nucleotidase
- nmdyn-D7: nucleoside-diphosphate kinase

**B**

- CG11883: cytosolic/mitochondrial 5'-nucleotidase
- CRMP: Collapsin Response Mediator Protein (dihydropyrimidinase)
- CG32549: putative purine 5'-nucleotidase
- CG16758: purine-nucleoside phosphorylase
- Rpl11T28: RNA polymerase III 128kD subunit
- CG6364: DNA-directed RNA polymerases I and III subunit RPAC2
- h2k11Tcg: uridine kinase
- if23626: CTP synthase
- Mes4: DNA polymerase epsilon subunit 2
- Veil: ecto-5'-nucleotidase
- su(r) D: dihydrorpyrimidine dehydrogenase (NADP+)
- nmdyn-D7: nucleoside-diphosphate kinase

**C**

- ND75: NADH-ubiquinone oxidoreductase 75 kDa subunit (NUAM)
- ND3: mitochondrial NADH-ubiquinone oxidoreductase chain 3
- CG9140: NADH-UBIQUINONE OXIDOREDUCTASE 51 KDA SUBUNIT (NUBM)
- CG4169: Ubiquinol-cytochrome C reductase complex core protein 2 (UCR2)
- CG4759: Cytochrome c1, heme protein, mitochondrial (CY1)
- RFeS6: Ubiquinol-cytochrome C reductase iron-sulfur subunit (UCRI)
- Ctype: Cytochrome c oxidase polypeptide Vb (COXIV)
- CG7596: Cytochrome c oxidase polypeptide 30 (COX30)
- CG9603: Cytochrome c oxidase polypeptide VIa (COXK, COXJ)
- CG7712: NADH-ubiquinone oxidoreductase B14 subunit (NBDM)

**D**

- CG10924: phosphoenolpyruvate carboxykinase (GTP)
- AcCoAS: Acetyl Coenzyme A synthase
- CG31075: aldehyde dehydrogenase (NAD+)
- CG2767: alcohol dehydrogenase (NADP+)
- CG10467: aldose 1-epimerase
- CG9900: glucose-6-phosphate 1-epimerase

**FIGURE 9**
Functional characterization of drim2, the Drosophila melanogaster homolog of the yeast mitochondrial deoxynucleotide transporter
Caterina Da-Re, Elisa Franzolin, Alberto Biscontin, Antonia Piazzesi, Beniamina Pacchioni, Maria Cristina Gagliani, Gabriella Mazzotta, Carlo Tacchetti, Mauro A. Zordan, Massimo Zeviani, Paolo Bernardi, Vera Bianchi, Cristiano De Pitta and Rodolfo Costa

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