The mitochondrial aspartate/glutamate carrier (AGC or Aralar1) isoforms in *D. melanogaster*: biochemical characterization, gene structure, and evolutionary analysis

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Abstract

Background

In man two mitochondrial aspartate/glutamate carrier (AGC) isoforms, known as aralar and citrin, are required to accomplish several metabolic pathways. In order to fill the existing gap of knowledge in *Drosophila melanogaster*, we have studied *aralar1* gene, orthologue of human AGC-encoding genes in this organism.

Methods

The blastp algorithm and the ‘‘reciprocal best hit” approach have been used to identify the human orthologue of AGCs in Drosophilidae and non-Drosophilidae. Aralar1 proteins have been overexpressed in *Escherichia coli* and functionally reconstituted in liposomes for transport assays.

Results

The transcriptional organization of *aralar1* comprises six isoforms, three constitutively expressed (*aralar1-RA, RD and RF*), and the remaining three distributed during the development or in different tissues (*aralar1-RB, RC and RE*). Aralar1-PA and Aralar1-PE, representative of all isoforms, have been biochemically characterized. Recombinant Aralar1-PA and Aralar1-PE proteins share similar efficiency to exchange glutamate against aspartate, and same substrate affinities than the human isoforms. Interestingly, although Aralar1-PA and Aralar1-PE diverge only in their EF-hand 8, they greatly differ in their specific activities and substrate specificity.

Conclusions

The tight regulation of aralar1 transcripts expression and the high request of aspartate and glutamate during early embryogenesis suggest a crucial role of Aralar1 in this Drosophila developmental stage. Furthermore, biochemical characterization and calcium sensitivity have identified Aralar1-PA and Aralar1-PE as the human aralar and citrin counterparts, respectively.

General Significance

The functional characterization of the fruit fly mitochondrial AGC transporter represents a crucial step toward a complete understanding of the metabolic events acting during early embryogenesis.

Keywords: *Drosophila melanogaster*; *CG2139*; aspartate/glutamate carrier (AGC); aralar; citrin; phylogenetic footprint;

Abbreviations: AGC, aspartate/glutamate carrier; MAS, malate-aspartate shuttle; MCF, mitochondrial carrier family; NRG, nuclear respiratory gene; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TSS, transcriptional start site.

1.Introduction

The mitochondrial carrier family (MCF)represents one of the largest families of transporters widespread across all species. MCF members share a common structural organization, consisting of three tandemly repeated sequences of approximately 100 amino acids in length, and mediate the transport of metabolites across the inner mitochondrial membrane [1-3]. The aspartate/glutamate carrier (AGC) catalyzes an electrogenic exchange of cytosolic glutamate plus a proton for mitochondrial aspartate [4], and it is one of the most important family member, since it is a key component of the malate-aspartate shuttle (MAS),which transfers the reducing equivalents of NADH from cytosol to mitochondria in order to regenerate NAD^+ necessary for glycolysis. Furthermore, AGC plays an essential role in many other metabolic processes including the synthesis of urea and nitrogen-containing metabolites [5], gluconeogenesis from lactate [5], calcium-mediated regulation of mitochondrial respiration [4, 6-9], insulin secretion from islet cells [10], zygote development [11, 12], N-acetylaspartate (a myelin precursor) synthesis [13-16] and glial synthesis of glutamate and glutamine[17]. Increasing evidences support the hypothesis that MAS activity is crucial for breast cancer cell proliferation [18]. In this regard, it might divert energetic cell metabolism towards glycolysis, promoting cancer cell growth [19]. Furthermore, AGC is involved in taurine metabolism, since it can efficiently transport also cysteinesulfinate [4, 20], an intermediate of cysteine degradation that serves as a precursor of taurine [21].

In *Homo sapiens,* two isoforms named AGC1 and AGC2 (also known as aralar and citrin) and encoded by the *SLC25A12* and *SLC25A13* genes, respectively, have been identified and characterized [4, 22]. They belong to a subfamily of calcium-binding mitochondrial carrier sharing a characteristic bipartite structure, which consists of a C-terminal domain responsible for transport activity, and an N-terminal domain containing 8 EF-hands that regulates transport activity [4, 23].

Aralar is mainly expressed in the heart, retina, skeletal muscle and brain [24, 25], whereas citrin is widely expressed in the liver, epithelial cells and breast [4]. Aralar is involved in retinal visual function [26, 27], in myelination process [13] and in glutamate-induced excitotoxicity [28, 29]. Mutations in the *SLC25A12* gene have been found in autism spectrum disorders [25, 30-32], as well as in a rare human disease (OMIM number 612949) implying developmental delay, epilepsy and hypotonia [15, 33]. *SLC25A13* mutations are responsible for citrin deficiency that causes adult-onset type II citrullinemia (CTLN2, OMIM number 603471), neonatal intrahepatic cholestasis (NICCD, OMIM number 605814) [29, 34-39], as well as failure to thrive and dyslipidemia caused by citrin deficiency (FTTDCD) [40].

In *D. melanogaster,* a single gene annotated in FlyBase as *CG2139* or *aralar1*encodes a ortholog of the human AGC isoforms [41].A*ralar1* gene is involved in high energy demanding processes, together with many other genes, such as those responsible for embryonic epithelial repair [42]. Previous studies indicated that *aralar1* locus is involved in the determination of bristle number in *D. melanogaster*, which are structures that may also have a neurosensory function [43], as well as this locus may influence wing size phenotype [44].

Differently from other fruit fly mitochondrial transporters, such as adenine nucleotide translocase [45], uncoupling protein [46, 47] dicarboxylate [48], thiamine pyrophosphate [49, 50], and glutamate carriers [51], a genomic analysis of this insect has highlighted the presence of a single gene (*CG2139*) encoding various *aralar1* spliced isoforms. The presence of so many alternatively spliced isoforms in mitochondrial carriers [47, 52, 53] and more in general in this organism is not surprising, since alternative splicing is a peculiar mechanism very often used by *D. melanogaster* to tightly control tissue- and stage-specific protein isoforms with different functions in development, as highlighted by Venables et co-workers *"Drosophila uses every alternative splicing strategy imaginable with an elegance and complexity that often eclipses mammals"* [54].

Despite the high metabolic relevance of this transporter, very few data are available on its biological role in fruit fly cellular processes, and no data at all are available on its transport properties in Drosophila.

In the present study, we have described (i) the expression profile of *D. melanogasteraralar1*gene in different developmental stages and tissues; (ii) the evolutionary changes that have shaped *aralar1* gene structure in arthropods; (iii) the functional characterization of recombinant Aralar1-PA and Aralar1-PE and their identification as the fruit fly mitochondrial aspartate/glutamate carriers.

2. Material and methods

2.1. Blast search of AGC orthologs in *D. melanogaster* and other species

The *D. melanogaster* ortholog of human AGCs was identified using the blastp algorithm (http://flybase.org/blast/). A BLAST search strategy, using *D. melanogaster* CDS and/or peptides as queries against genomic and ESTs databases, was also adopted to identify putative genes encoding AGC in the genome of other Drosophilidae and several non-Drosophilidae species [55]. A complete list of the species investigated in this study and the accession number in which *aralar1* genes have been identified is shown in Supplementary Table 1. These searches were performed using organism-specific (www.flybase.org,www.vectorbase.org and http://hymenopteragenome.org) and generalist genomic repositories (NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ‘‘reciprocal best

hit" approach was used to identify *aralar1* genes in different species. In this approach, a common evolutionary origin is supposed when in the compared genomes two gene sequences represent each other the best BLAST hit[56].For each genomic sequence identified by using the above-mentioned criteria, exon/intron boundaries were carefully annotated after prediction *in silico* carried out with the aid of ESTs and cDNA sequences, if available, or predicted by the Genscan tool [57], or inferred by sequence similarity with transcript sequences of closely related species. Multiple alignments of amino acids, as well as coding and non-coding DNA sequences, were obtained using the MultAlin 5.4.1 software available at the MultAlin server (http://multalin.toulouse.inra.fr/multalin/) [58, 59]. The matrix-scan pattern matching tool of the Regulatory Sequence Analysis Tools from the RSAT server (http://rsat.ulb.ac.be/) was used, in combination with multiple alignments to identify Nuclear Respiratory Gene (NRG) elements [60] in the non-coding sequences of Drosophilidae and non-Drosophilidae *aralar1* genes.

2.2. Reverse-transcription analyses of *aralar1* transcriptional isoforms

Oregon-R flies were raised on standard culture medium at 24 °C. Total RNA for gene expression analysis was achieved from 0.5- to 1-g samples of Oregon-R individuals at different developmental stages (embryo, larvae, pupae and adult flies). Except for ovaries and testes, male and female tissues equally contributed to each dissection. Total RNA from all the developmental stages and tissues was extractedby employing Trizol. Purified RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and diluted to 1 mg/ml for reverse transcription. RNA (1 µg) was reverse transcribed by employing the Quantitect reverse transcription kit (QIAGEN) [61, 62] following the manifacturer's protocol. Amplification was carried out using the Platinum Taq DNA Polymerase (Thermo Fisher).

Primers used for amplification were:

aralar_RA_F ATTAGACGCGGGAATTGCTC
aralar_RA_R CTCCTCGTGAAAGTCGTGCAG
aralar_RB_F ATAGTGCGAACGTGCCTGA
aralar_RB_R ATGGCGAGATGAACCCAGTG
aralar_RC_F CCGATGCCAAGAATCTCCGT
aralar_RC_R TCCTCGTGAAAGTCGTGCAG
aralar_RE_F CAAATCACGCCGCTGGAGAT
aralar_RE_F GGCCCCTTTATCAAGCGCTA

2.3. Construction of the expression plasmids encoding *D. melanogaster*Aralar1 isoforms and mutants in *E. coli* and *S. cerevisiae*

The two protein isoforms Aralar1-PA and Aralar1-PE were considered for the functional characterization of D. melanogaster *CG2139* gene. Aralar1-RA and Aralar1-RE were amplified by polymerase chain reaction (PCR) from the cDNA of the fruit fly clones LD35441 and GH21613, respectively. Both clones were provided by Drosophila Genomics Resource Center (Indiana University 1001 East Third St., Bloomington IN, 47405-7107). The oligonucleotide primers (sense and antisense) used in PCR reactions carried suitable restriction sites at their 5′ ends for the further cloning of the amplified inserts in the pET-21b/V5-His *E. coli* expression vector [51]. The absence of a stop codon in the reverse primer sequence led to the expression of Aralar1 proteins containing V5/His-tag at their C-termini [63].

The WT Aralar1-RE cDNA was employed as a template to generate mutants named Δ5 (deletion from residue 301 to 305), Δ8 (deletion from residue 301 to 308), Δ12 (deletion from residue 299 to 310), and 5Ala (replacement of residues $K_{301}RRRK_{305}$ by consecutive alanines). All of the mutations were introduced by the overlap extension PCR method [64, 65], using oligonucleotides with suitable mutations in their sequences. Primers used for amplification were:

aralar_PE_FTAGGAATTCACCAATGCACATCCCGTTTCC and

aralar_PE_R CGAAAGCTTGGATCCCGTGGCCGTCG for all mutants.

The following specific primers were used to generate the above-mentioned mutants:

aralar_PE_ Δ5 _F CCATCAAGCAGGCTGGTGGATACCTCCGAGTAGCCGCA

aralar_ PE _ Δ5 _R TGCGGCTACTCGGAGGTATCCACCAGCCTGCTTGATGG

aralar_ PE _ Δ8 _F CCATCAAGCAGGCTGGTGGAGTAGCCGCATCGACTATAGTGAC

aralar_ PE _ Δ8 _R GTCACTATAGTCGATGCGGCTACTCCACCAGCCTGCTTGATGG

aralar_ PE _ Δ12 _F GCGCCGTCCATCAAGCAGGCCGCATCGACTATAGTGACCTGAGCAA

aralar_ PE _ Δ12 _R TTGCTCAGGTCACTATAGTCGATGCGGCCTGCTTGATGGACGGCGC

aralar_ PE _5Ala_F
CCATCAAGCAGGCTGGTGGGCGGCTGCCGCAGCGATACCTCCGAGTAGCCGCA

aralar_RE_5Ala_R
TGCGGCTACTCGGAGGTATCGCTGCGGCAGCCGCCCACCAGCCTGCTTGATGG

The aralar1-RA and aralar1-RE ORFs were recovered from pET-21b/V5-His clones by a EcoRI/AgeI digestion and cloned in the modified yeast expression vector pYES2/ V5-His [49], in which the inducible GAL1 promoter had been replaced with the constitutive TDH3 promoter. The

frameshift of transcripts was avoided cloning a KpnI/EcoRI fragment carrying the ATG start codon at '5-end. Fragments used were: GGTACCAGGATCCGATCTTGAACTGACTGAGATGGCGAATTC and GGTACCAGGATCCGATCTTGAACTGACTGAGATGGCGAATTC for aralar1-RA and for aralar1-RE, respectively.

2.4. Bacterial expression and purification of the recombinant proteins

Aralar1 proteins were overexpressed at high levels in *E. coli* BL21(DE3) [49].Their identities were assessed by MALDI-TOFMS of trypsin digests of the corresponding bands excised from a Coomassie blue-stained polyacrylamide gel [66, 67]. Inclusion bodies were purified using sucrose density gradient centrifugation, next they were washed at 4ºC, firstly with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8), then twice with a buffer containing Triton X-114 (2%, w/v) and 10 mM HEPES (pH 8), and finally with 10 mM PIPES pH 6.5. Aralar1-PA and Aralar1-PE were solubilized in 2% sarkosyl (w/v), and a small residue was removed by centrifugation (258000 g, 30 min). Solubilized proteins were diluted 10-fold with 10 mM PIPES pH 6.5 and reconstituted into liposomes [68].

2.5. Reconstitution into liposomes and transport assays

Reconstitution mixture (700 µl) contained 0.5-1 µg of solubilized proteins, Triton X-114 (1,3 % w/v), L-α-phosphatidylcholine from egg yolk (1,3 % w/v), as sonicated liposomes, 10 mM glutamate (except where otherwise indicated), and 20 mM PIPES at pH 6.5 [69]. These components were carefully blended, and the blend was recycled 13 times through the same Amberlite column (Bio-Rad) [69]. External substrate was eliminated from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with 50 mM NaCl and 10 mM PIPES at pH 6.5. Transport at 25ºC was started by adding L-[^{14}C]glutamate (Scopus Research BV, Wageningen, Netherlands) at the indicated concentrations to substrate-loaded proteoliposomes (exchange reaction), or to empty proteoliposomes (uniport reaction). In both cases, transport was terminated by adding 30 mM pyridoxal phosphate (PLP). In control samples, inhibitor was added together with the external radioactive substrate based on the inhibitor stop method [70, 71]. Finally, external substrate was removed and radioactivity intoproteoliposomes was measured. The experimental values were adjusted by subtracting control values. The initial transport rate was measured from the radioactivity taken up by proteoliposomes after 1 min (in the initial linear range of substrate uptake). The free Ca^{2+} concentrations were determined fluorimetrically with fura-2-acetoxymethyl ester (Fura-2 AM) (Thermo Fischer Scientific). Fura-2 AM is a membrane-permeable, non-invasive

derivative of the ratiometric calcium indicator fura-2. The excitation wavelengths used are 340 nm and 380 nm for Ca^{2+}-bound and Ca^{2+}-free fura-2 AM, respectively. In both states, the emission maximum is about 510 nm. The ratios 510 nm/340 nm and 510 nm/380 nm are directly related to the amount of Ca^{2+} [72]. Calcium dependence on transport activity was determined using de-ionized ultrapure water. In experiments for evaluating the influence of the membrane potential on the activity of the recombinant proteins, valinomycin was added to proteoliposomes in order to provide de-energized conditions. K^+ diffusion potentials were generated using valinomycin and K^+ gradients. In these experiments, substrate and buffer were neutralized with NaOH [4].

2.6. Yeast strains, growth conditions and functional complementation of yeast AGC1Δ by ARALAR1-PA and ARALAR1-PE

W303 (wild-type) (*MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}*) yeast strain was provided by the EUROFAN resource center EUROSCARF (Frankfurt, Germany). The yeast AGC1gene deletion (*agc*1Δ) and the cloning of endogenous yeast deleted gene AGC1 were achieved as described before [73].

The recombinant aralar1-RA-pYES2 and aralar1-RE-pYES2 plasmids were introduced into the agc1Δ yeast strain using the lithium acetate method [74], and transformants were selected on minimal medium lacking uracil, supplemented with 2% glucose.

Functional complementation was achieved growing cells on liquid complete medium (1% Bacto yeast extract, 2% Bacto Peptone, pH 5.0) supplemented with 2% glucose (YPD) or 0.5 mM oleic acid dissolved in 10% Tween40 (YPO) as carbon sources. Growths were started from medium log precultures grown on complete medium YPD and diluted with YPO to an optical density of 0.01 at 600 nm.

Simultaneously,washed cells weredilutedand spotted on complete solid medium YPO, then plates were incubated for 72 h at 30 °C. Four-fold serial dilutions of both the transformed strains, wild-type and AGC1Δ cells were analyzed.

2.7. Other methods

Proteins were resolved by SDS-PAGE and stained with Coomassie blue dye [75]. The amounts of recombinant pure Aralar1-PA and Aralar1-PE incorporated into liposomes were measured as previously described [76] and proved to be approximately 20% of the protein amount added to the reconstitution mixture. Moreover, recombinant proteins were resolved by SDS-PAGE analysis [77, 78], then transferred to a nitrocellulose membrane and analyzed by Western blotting employinga mouse anti-V5 monoclonal antibody (Sigma-Aldrich) [79, 80]. A multiple sequence alignment

(MSA) of the fruit fly Aralar1 proteins, human aralar and citrin isoforms was obtained by using ClustalW to insert gaps in the MSA [81]. All data were analyzed using the statistical software GraphPad Prism Software (7.0 version). Pairwise comparisons between means of different groups were performed using a Student's t-test (two tailed, unpaired). Multiple comparisons were performed using a univariate ANOVA. Values at $P \leq 0.05$ were considered statistically significant, while values at $P \leq 0.01$ and $P < 0.001$ were considered very significant [82, 83]. Orientation of functional Aralar-1 in the membrane of reconstituted proteoliposomes was investigated by ELISA assay performed as previously described [84]. ELISA tests were carried out on intact and permeabilized proteoliposomes by using either the rabbit polyclonal antiserum generated against 35-177 amino acids of Drosophila Aralar-1 proteins as described in [85] or the anti-V5 antibody directed against the V5 epitope placed at their C-termini (Sigma-Aldrich).

3. Results

3.1. Identification of genes encoding the aspartate/glutamate carrier in Drosophilaspecies

A single gene, annotated in FlyBaseas *CG2139* or *aralar1*, encodes a *D. melanogaster* ortholog of the human AGC isoforms (also known as aralar and citrin) [4], with no additional hits obtained throughout database mining. A*ralar1* locus spans roughly 10 Kbp on the third chromosome (3R:30,445,635..30,455,867 [-]) in the 99F4-99F5 cytogenetic band of polytene chromosomes of *D. melanogaster*. The transcriptional organization of *aralar1* reported in FlyBase (Figure 1A) comprises sixtranscriptional isoforms. The utilization of alternative transcriptional start sites, alternative splicing and alternative UTRs, produces five different transcriptional isoforms (namely RA, RB, RC, RD, RE), which in turn can be translated into four different proteins (PA/PD, PB, PC, PE). An additional translational isoform is reported in FlyBase, Aralar1-PF, which is translated from the *aralar1-RF* transcript that is structurally identical to *aralar1-RA* isoform by mean of a read-through translational mechanism [86]. In this regard, *aralar1-RA* transcript is translated into a 682 amino acids long protein (Aralar1-PA), whereas *aralar1-RF* transcriptional isoform is translated into a polypeptide having 12 additional aminoacids at its C-terminus due to translation progression beyond the canonical stop codon. Three different promoters contribute to the transcription of *aralar1* locus. *Aralar1-RA* (identical to *aralar1-RF*) and *aralar1-RD* are transcribed from the same downstream promoter, with *aralar1-RD* transcript possessing a shorter 3'UTR if compared to that of*aralar1-RA/RF* isoforms. *Aralar1-RB* arises from the central promoter,whereas *aralar1-RC* and *aralar1-RE* are originated from the upstream promoter. The two latter isoforms differ by a short, 36 bp long exon, which is specifically incorporated into the *aralar1-RE* transcript. Given the availability of a wide range of sequenced Arthropoda genomes, we have surveyed *aralar1* gene structure in Arthropoda.

Orthologous genes in Drosophilidae have been retrieved using a BLAST strategy, and locus structure has been inferred with the aid of ESTs and cDNA sequences annotated in FlyBaseand NCBI databases.Sequences with significant similarity to *D. melanogasteraralar1* gene have been detected in the genome of 22 additional Drosophila species (Supplementary Table 1), and their organization has been compared. Similarly to *D. melanogaster* annotation, in all the investigated Drosophilaspecies three transcriptional start sitescan be inferred from ESTs and cDNAs comparison and from inter-species similarity. In order to give a better snapshot of *aralar1* locus, its organization has been divided into three schemes highlighting the exon/intron organization relative to the three TSSs (Transcriptional Start Site) (Supplementary Tables 2-5). The comparison clearly suggests that the exon-intron structure of the locus is highly conserved in the 23 analyzed species. As can be observed, exon length is poorly variable. The first exon of all transcriptional isoforms is the most variable in length. Indeed, they encode the 5' UTRs and the leader peptides that are expected to be divergent in sequence, even if closely related species are compared. The length of the coding region of the last exon, encoding the protein C-terminus, also presents variable length and sequence in the analyzed Drosophila species. Internal coding exons are instead extremely conserved in length. As expected, introns are the most plastic regions of eukaryotic genes, and they are longer in species distantly related to *D. melanogaster*. It is worth to note that although the presence of the*aralar1-RE* specific exon is not supported by ESTs or cDNAs in the vast majority of the Drosophilidae species (not shown), it can be inferred by sequence similarity, suggesting that it could be also a functional exon in other species. In support of this hypothesis, the *aralar1-RE*specific exon, which can be 33-39 bp long depending upon the species, is flanked by a non-canonical donor splice site (GC)conserved in all the Drosophila species, suggesting the usage of an atypical splice site in the common ancestor of Drosophila and Sophophora genera (Supplementary Figure 1).

3.2. Conservation of regulatory elements

Inter-species DNA sequence comparison is an excellent tool for the identification of cis-regulatory DNA sequences interested in eukaryotic gene expression regulation, especially in non-coding DNA sequence. In the noncoding sequences of Drosophilidae *aralar1* genes we have searched for conserved motifs by using phylogenetic footprinting and DNA pattern discovery softwares. The nuclear respiratory gene (NRG) element has been previouslyreported as a palindromic 8-bp motif (TTAYRTAA) that is shared by all nuclear OXPHOS genes [87], as well as by many other nuclear genes involved in biogenesis and function of mitochondria in insects [60]. In *D. melanogaster*, NRG elements are usually located within an intron, in close proximity to the transcription start site (TSS), but it can be found in different gene locations, such as upstream the TSS. A NRG element

(TTATATAA) associated with high weight and low P value in the RSAT output (8,2 and $6.0e^{-05}$ respectively) is located in the first intron, 518 bp downstream of the transcription start site related to *D. melanogaster aralar1-RB* transcriptional isoform. This 8-bp motif is extremely conserved and located in the first intron in all the Drosophilidae *aralar1* orthologs with high frequency (Figure 1B). An additional NRG element, associated with a slightly lower score and a moderately higher P-value in the RSAT output (6,7 and $1,5e^{-04}$ respectively), is located downstream within the same intron, 686 bp upstream the TSS related to the RA/RD/RF transcriptional isoforms (Figure 1C).

The functional importance of the NRG element in *aralar1* genes is suggested by its presence in the non-coding regions of *aralar1* genes belonging to the 32 non-Drosophilidae Arthropoda species investigated in this work (Supplementary Table 1). Notwithstanding, the very long divergence times (divergence between Drosophilaand Parasteatoda/Limulus genera is estimated approximately 600 MYA, from Treebase http://www.timetree.org) [88], single or multiple NRG elements have been detected in *aralar1* genes of the majority of Arthropoda species, and their intragenic localization is often strictly conserved (not shown). However, standing to the availability of transcriptional data in the vast majority of the studied cases, we have reconstructed the complete exon/intron structure of *aralar1* related to the shortest transcriptional isoform, which we have arbitrarily assumed to be homologous to *D. melanogaster* RA-RD-RF transcripts. In many of these species, the NRG elements have been found in the first intron related to the shortest transcriptional isoform, suggesting that their positions could have been remodeled during insects' evolution. Also the length of the first intron, with respect to the most proximal TSS, is on average longer in non-Drosophilidae insects (mean length=15149 bp, 29 species) than in *D. melanogaster* homologs (mean length=245 bp, 23 species),supporting the hypothesis of a functional role of the NRG elements, although in a different position.

3.3. All eukaryotic *aralar1* genes have a common evolutionary origin.

Sequences homologous to *D. melanogaster aralar1* gene have been found in the genomes of 23 Drosophilidae species. They share not only significant sequence similarity with *D. melanogaster* genes, but also a strikingly conserved exon/intron organization (Figure 2 and Supplementary Tables 2-5). As a first step towards understanding the evolutionary history of *aralar1* genes, we have compared the Drosophilidae gene organization with their counterparts in an informative range of 32 Arthropod species consisting of 30 insects species (namely 7 Diptera, two Lepidoptera 1 Coleoptera, 18 Hymenoptera and 2 Hemiptera), an Arachnida (*Parasteatoda tepidariorum*) and a Xiphosurida (*Limulus polyphemus*).

Similarly to what found in Drosophilidae, a single *aralar1* gene has been detected in the genome of all these species. The complete list of the identified *aralar1* genes and the accession numbers in which they have been found in the respective genomes is shown in Supplementary Table 1. Variations in the exon/intron organization of the investigated genes are reported in Supplementary Table 6. Comparison of all the investigated genes, in particular those in insects and Arachnida (*Parasteatoda tepidariorum*), as well as in Xiphosura (*Limulus polyphemus*), suggests the presence of a common ancestor before Arthropods' divergence. Intron loss seemingly occurred in Diptera-Lepidoptera-Coleoptera, while intron gain suggests a lineage-specific process.

The existence of an internal exon, specific for a transcriptional isoform homologous to *D. melanogasteraralar1-RE,* can be inferred in a subset of the analyzed speciesincluding Hymenoptera (14 out of 18 species) and Diptera (6 out of 7 non-Drosophilid species), whereas we have not detected it in Coleoptera (one species) and Lepidoptera (two species), as well as in the evolutionary distant Hemiptera species.

In humans, the nearly identical exon/intron organization of *ARALAR* (*SLC25A12*) and *CITRIN* (*SLC25A13*) genes (19 and 21 coding exons, respectively)is consistent with a duplication of an ancestral gene, which was probably present in the genome of the Vertebrate's ancestor, due to the existence of two paralogue genes in the genome of extant vertebrate species so far sequenced (not shown). Alternatively spliced transcript variants have been reported for the human *ARALAR* gene (as provided by ac.nos NM_003705, NR_047549, XM_011512070). While the NM_003705 is translated into the Aralar1 protein (NP_003696), the functional roles of the predicted (XM_011512070) and the putatively non-coding (NR_047549) transcriptional isoformsare currently unknown. Comparisons of exon phase, exon length, and intron positions, suggest that Arthropod and human genes have a unique intron-rich ancestor predating the divergence of such lineages. Since position conservation in humansand in numerous Arthropod lineages can be supposedin order to identify retained ancestral introns,we have deducedthat such common ancestor gene containedat least 15 introns.

3.4. Expression pattern of *Drosophila melanogasteraralar1* gene

The developmental expression pattern of *aralar1* in *D. melanogaster* is widely described in FlyBase, which reports the modENCODE mRNA-Seq temporal expression data (mRNA-Seq_U, [89]. Relative data are summarized in Figure 3. In order to integrate these data with the expression pattern of *aralar1* transcriptional isoforms, we have carried out a Reverse Transcriptase-PCR analysis (RT-PCR) on total RNA samples isolated from distinct Drosophila developmental stages (Figure 4A) or from dissected adult tissues (Figure 4B).

With this aim, we have devised a set of primers specific to easily discriminate the five reported isoforms. Unfortunately, we were not able to find oligos suitable for performing quantitative analysis, so we could only conduct semiquantitative analyses based on RT-PCR. However, RA/RF and RD, cannot be distinguished, due to their nearly identical sequence, as described above. We will refer to *aralar1-RA* to indicate the RA/RD/RF isoforms through this paragraph.

The obtained results have revealed a constitutive expression of *aralar1-RA* transcript throughout development (Figure 4A). By contrast, the expression of the other isoforms is patchy distributed during the development or in different tissues. *Aralar1-RB* isoforms detected in early embryo development, during all the pupal stages and in adults, being only limited to females. Finally, *aralar1-RC*is detected in embryos (at least until 12-15 hours after egg laying), in all the pupal stages and in both sexes during the adult stage, while *aralar1-RE* is expressed in the early-embryo development and in both sexes during the adult stage. Consistent with the expression pattern detected in whole adult males and females, we have found that *aralar1-RA*, *aralar1-RC* and *aralar1-RE* are ubiquitously expressed in all the main fruit fly body regions (namely head, thorax and abdomen), whereas *aralar1-RB* expression is limited to the abdomens of females, due to its ovary-specific expression (Figure 4B).

3.5. Bacterial expression and functional characterisation of recombinant Aralar1 proteins

A ClustalW alignment of Drosophila Aralar1 with the human AGC isoforms has revealed that all the proteins conserve the same domain structure organization consisting of an N-terminal domain with 8 EF-hands, a carrier domain and a C-terminal domain with unknown function (Figure 5) [23]. Five out of the six Drosophila Aralar1 protein isoforms do not present any difference in their N-terminal EF-hands and carrier domains (Figure 5). Aralar1-PE diverges from the other isoforms because of an insertion of 12 additional amino acid residues in the loop of its eighth EF-hand (EF-hand 8) [23]. In order to check if this peculiarity may affect its transport activity, we have chosen Aralar1-PE to be biochemically characterized together with Aralar1-PA that also shows a shorter N-terminus similar to that found in Aralar1-PD and Aralar1-PF (Figure 5).

Aralar1-PA and Aralar1-PE have been overexpressed at high levels in *E. coli* BL21(DE3) (Figure 6A, lane 3 for Aralar1-PA, lane 4 for Aralar1-PE). They accumulate as inclusion bodies and have been purified by centrifugation on sucrose gradient and washing (Figure 6A, lane 5 for Aralar1-PA, lane 6 for Aralar1-PE), with a yield of 50 – 60 mg/ml bacterial culture. These proteins have been not revealed in bacterial cells harvested just before the induction of expression (Figure 6A, lane 1 for Aralar1-PA, lane 2 for Aralar1-PE), or in cells harvested after induction but do not carrying the coding sequence for Aralar1-PA and Aralar1-PE in the expression vector (data not shown). Their

identities have been assessed by western blot analysis employing a mouse anti-V5 monoclonal antibody (Figure 6A, lanes 1-6).

Aralar1-PA and Aralar1-PE have been functionally reconstituted into liposomes and the orientation of their N- and C-terminal regions in the proteoliposomes was performed by ELISA assay, indicating that both termini of Aralar1 proteins reconstituted into the proteoliposomal membrane protrude toward the outside, which in intact proteoliposomes is the only side of the membrane accessible to antibodies (Supplementary Figure 2). Aralar1-PA and Aralar1-PE transport activity have been tested in homo-exchange experiments (same substrate inside and outside). Using external and internal substrate concentrations of 1 and 10 mM, respectively, both proteins efficiently catalyze [^{14}C]glutamate/glutamate and [^{14}C]aspartate/aspartate exchange reactions. A very active uptake of [^{14}C]glutamate and [^{14}C]aspartate has also been observed when proteoliposomes had been preloaded with 10 mM aspartate or glutamate, respectively (hetero-exchange reactions). Both proteins do not catalyze any homo-exchange of phosphate, ADP, ATP, malonate, malate, oxoglutarate, ketoisocaproate, citrate, carnitine, ornithine, lysine, arginine, glutathione, choline, proline, and threonine (data not shown). No [^{14}C]glutamate/aspartate exchange has been observed using boiled Aralar1-PA and Aralar1-PE for incorporation into liposomes or reconstituting sarcosyl-solubilized bacterial material deriving either from cells not carryingany expression vector for Aralar1-PA and Aralar1-PEor from cells collected just before the induction of expression.

We have displayed kinetics of Aralar1-PA and Aralar1-PE uptake in Figure 6B. Each protein reconstituted into proteoliposomes contains 10 mM internal glutamate, and uptake has been measured in the presence of external 0.5 mM [^{14}C]glutamate (exchange). Exchange reactions follow first-order kinetics,and isotopic equilibrium is approached exponentially, maximum uptake is reached after 90 min (rate constants 0.066 and 0.080 min^{-1} and initial rates of 20.78 and 59.51 nmol/min x mg protein for Aralar1-PA and Aralar1-PE, respectively). Conversely, no [^{14}C]glutamate uptake is observed in the absence of an internal substrate (uniport), highlighting that both proteins are unable to catalyze a unidirectional transport of glutamate (Figure 6B). After 50 min incubation, when radioactive uptake by proteoliposomes has approached equilibrium, the addition of 20 mM unlabeled aspartate leads to an extensive efflux of radiolabeled glutamate from glutamate-loaded proteoliposomes (Figure 6B). A similar efflux of labeled substrate is observed by adding 20 mM unlabeled glutamate (not shown). This efflux further confirms the strict exchange mechanism catalyzed by Aralar1-PA and Aralar1-PE. The unidirectional transporthas been further investigated in backward experiment, as it provides a more convenient assay [53], i.e., by measuring the efflux of [^{14}C]glutamate from prelabeled active proteoliposomes; in the absence of an external substrate no efflux of [^{14}C]glutamate has been observed, even after 60 minutes of

incubation,but an extensive efflux has occurred upon the addition of 10 mM external glutamate or aspartate (data not shown).

Substrate specificity of recombinant Aralar1-PA and Aralar1-PE has been examined in detail by measuring the uptake of [^{14}C]glutamate into proteoliposomes preloaded with different possible substrates. Table I reports the results of our explorative statistical analysis. Both proteins efficiently exchange external [^{14}C]glutamate for internal L-glutamate or L-aspartate. Aralar1-PA also catalyzes a lower but significant uptake of [^{14}C]glutamate in the presence of internal L-cysteinesulfinate, whereas no transport activity has been found with L-α-aminoadipate, L-glutamine and L-asparagine (Table I). Differently from Aralar1-PA and yeast AGC [90], but similarly to the human AGC isoforms [4], Aralar1-PE exchanges external [^{14}C]glutamate for internal cysteinesulfinate at the same rate as aspartate, and it shows a significantly high [^{14}C]glutamate uptake in the presence of internal L-α-aminoadipate and L-glutamine. Both isoforms are virtually unable to exchange [^{14}C]glutamate for internal D-aspartate and D-glutamate, indicating a high degree of stereo specificity.

The [^{14}C]glutamate/glutamate exchange reactions catalized by Aralar1-PA and Aralar1-PE are hampered by many well-known inhibitors of several MCF members, being completely inhibited by 50 μM *p*-chloromercuribenzoate [4] and 1 mM pyrocarbonate [90] (100 and 96% inhibition, respectively for both proteins) and, to a slightly lesser extent by 50 μM mersalyl [76] and 0.1% tannic acid [53] (80 and 85% inhibition, respectively for both proteins), and also by 1 mM *N*-ethylmaleimide [53] (40 and 50% inhibitionfor Aralar1-PA and Aralar1-PE, respectively). Moreover, 10 mM bathophenanthroline or 0.1 mM bromocresol purple [53] are unable to exert a significant inhibition. Additionally, no inhibition has been observed with 10 μM bongkrekic acid [68]and 2.5 mM 1,2,3-benzenetricarboxylate [81], which are specific inhibitors of the mitochondrial ADP/ATP and citrate carrier, respectively.

Considering that the AGC-catalyzed aspartate/glutamate antiport reaction is electrogenic [4], the effect of membrane potential has been examined on the aspartate/glutamate exchange reaction mediated by our Aralar1 carriers. A K^+diffusion potential has been created across the membrane of proteoliposomes using valinomycin/KCl (calculated value ∼100 mV, positive inside). In such conditions, the rates of the [^{14}C]aspartate$_{out}$/glutamate$_{in}$exchanges of Aralar1-PA and Aralar1-PE are stimulated (Table II). In the absence of a membrane potential or when homo-exchanges have been measured, no effect has been observed. As a consequence, Aralar1-PA and Aralar1-PE are able to catalyze an electrogenic exchange of aspartate for glutamate.

We have determined the kinetic constants of the recombinant purified Aralar1-PA and Aralar1-PEby measuring the initial transport rate at various external [^{14}C]glutamate or [^{14}C]aspartate

concentrations, in the presence of a constant internal saturating concentration (10 mM) of unlabeled substrates. In five independent experiments carried out at the same internal and external pH (6.5), the half-saturation constants (K_m) of reconstitutedAralar1-PA and Aralar1-PE for glutamate are 0.26 ± 0.03 and 0.29 ± 0.07 mM, respectively, and for aspartate are 47 ± 2.3 and 51 ± 2.6 μM, respectively.Interestingly, the two isoforms significantly differ in their V_{max} values, which are 32.25 ± 1.69 and 94.29 ± 5.40 nmol/min/mg protein, for Aralar1-PA and Aralar1-PE, respectively for both substrates. The activity has been normalized by determining the amount of recombinant proteins recovered into proteoliposomes after reconstitution. We have further analyzed whether the differences in transport rates (Table I) and Vmax values found between the two isoforms could arise from the 12 additional amino acid residues present in the loop of the helix-loop-helix structure of the eighth EF-hand (EF-hand 8) of Aralar1-PE. First of all, we have checked, in de-ionized ultrapure water, the effect of Ca^{2+} or EGTA (a divalent ion chelator) on Aralar1-PA and Aralar1-PE transport activity (Figure 6C). The activities of both isoforms are increased of about 35% by the addition of 1 mM $CaCl_2$ and diminished by the addition of 0.5 mM EGTA. The inhibitory effect of EGTA is abolished in the presence of 1 mM $CaCl_2$ (Figure 6C). These results indicate that both isoforms are highly sensitive to calcium, since low calcium contamination in the reconstitution mixture is able to activate transport activity of both isoforms, whereas its removal by a Ca^{2+} chelator exerts an inhibitory effect. The Ca^{2+}-dependence of the transport activities of Aralar1-PA and Aralar1-PE has been further studied by measuring their transport rates as a function of the free Ca^{2+} concentration (Figure 6D). Both isoforms are highly Ca^{2+}-sensitive, since Aralar1-PA and Aralar1-PE have a half-maximal activation of about 0.34 and 0.15 μM, respectively.

3.6. Influence of the eighth EF-hand on the activity of ARALAR1 PE isoform

Once established that in our recostituted system calcium is able to regulate transport activity of both isoforms, we have checked if the shortening of the insertion consisting of 12 additional residues in Aralar1-PE or the replacement of some residues might affect its transport activity. In particular, the five positive charged residues (KRRRK from 301 to 305) have been replaced by consecutive alanine residues (5Ala mutant) or deleted (Δ5 mutant). Two further deletions have also been investigated, the first encompassing residues 301-308 (Δ8 mutant), and the second including residues 299-310 (Δ12mutant); in this latter mutant the insertion characterizing Aralar1-PE has been completely removed. Substrate specificity of recombinant Aralar1-PE mutants has been examined in detail by measuring the uptake of [14C]glutamate into proteoliposomes preloaded with different possible substrates. As reported in figure 7, the substitution of the five charged residues with Ala deeply increases transport activity of all the tested substrates, whereas its deletion

decreases tranport activity proportionally with the size of the deletion gradually leading Aralar1-PE to have the same substrate specificity of Aralar1-PA. The kinetic constants of the recombinant Aralar1-PE mutants are reported in Table III.The half-saturation constants (K_m) of reconstituted mutants do not vary with respect to the wild type, whereas as regards transport acitivity a significant change has been observed. In particular, 5Ala mutant shows a transport activity three-fold higher than that of the wild-type protein, whereas deletions negatively affect transport activity, which decreases proportionally with the size of the deletion. Interestingly, Δ12 mutant exhibits the same transport activity as Aralar1-PA.

3.7. Aralar1-PA and Aralar1-PE function as an AGC transporter in *S. cerevisiae*

The yeast *AGC1* null mutant does not grow on oleate even in rich medium [90]. This phenotype is explained since MAS is required for reduction of the cytosolic NADH derived from peroxisomal oleate oxidation [90]. Thus, the expression of a mitochondrial carrier protein able to exchange aspartate with glutamate should abolish the growth defect of the *agc1*Δ knockout. Aralar1-PA and Aralar1-PE expressed in *AGC1*null cells have fully restored growth of the *agc1*Δ strain on oleate, similarly to the yeast Agc1p (Figure 8), indicating that both Drosophila proteins function as AGC transporters. By contrast,in *agc1*Δ cells transformed with the empty vectorno growth rescue has been observed.

Discussion

By allowing the mitochondrial re-oxidation of the cytosolic NADH, the MAS plays a central role in the redox homeostasis and in different metabolisms such as glucose homeostasis and nitrogen metabolism. The MAS requires the concerted functioning of six proteins, two located in the cytosol, the malate dehydrogenase 1 (MDH1) and the aspartate aminotransferase 1 (GOT1), two located in the mitochondrial matrix, the malate dehydrogenase 2 (MDH2) and the aspartate aminotransferase 2 (GOT2), and the last two located in the inner mitochondrial membrane, the aspartate/glutamate and the oxoglutarate/malate (OGC) carriers, which connect MAS cytosolic reactions to the mitochondrial ones. Despite the central role in energetic metabolism, very little is known about MAS components in the fruit fly, and up to date no functional data are available on AGC and OGC. In mammals, there are two genes encoding AGC, *ARALAR*, also known as AGC1, more widely expressed but absent in the liver, and *CITRIN* also known as AGC2, expressed in fewer tissues but present in the liver [24]. By searching the FlyBase database (http://flybase.org/) for human AGC homologs, we have found 6 alternatively spliced-isoforms annotated as *aralar1* and none as citrin.

The expression profile of *aralar1* transcriptional variants studied in this work suggests that three isoforms (namely *aralar1-RA/RF* and *aralar1-RD*) are constitutively transcribed throughout the development and in adult tissues. Due to their overlapping sequences, it is not possible to discriminate the expression profile of these transcriptional isoforms, which are transcribed from the innermost promoter. The slight differences observed in their organization suggest that they might have a different stability (i.e. half-life of their mRNA molecules) due to the length of the 3' UTR (*aralar1-RD* and *aralar1-RA* isoforms). Similarly, the stability of Aralar1-PF protein, encoded by *aralar1*-RF transcript (identical to *aralar1-RA* transcript), could be affected by the presence of the additional 12 amino acids introduced upon the translational read-through process [86].

Conversely, *aralar1-RB*, *aralar1-RC* and *aralar1-RE* transcriptional isoforms have a more complex expression pattern, as described in the Results section. While *aralar1-RB* is undetectable by RT-PCR in adult males, it can be specifically detected in adult ovary, suggesting its role in oogenesis. We do not know whether *aralar1-RB* detected during the early embryo development is the result of a maternal contribution or of the active transcription in embryos, as instead appears evident for *aralar1-RC* and *aralar1-RE*, which are undetectable in female germline and are present in early embryo developmental stages. Furthermore, while *aralar1-RC* expression persists in the whole adult tissues with the exclusion of the germline tissues, *aralar1-RE* expression is confined in the early stages of embryo development and in somatic adult tissues. The expression of the more active *aralar1-RE* in the early embryogenesis might be related to the very active aspartate and glutamate metabolism occurring in this stage [91]. *Aralar1-RB* and *aralar1-RC*, together with the constitutively expressed isoforms, seem to be also important during metamorphosis. It is noteworthy that all the *aralar1* transcriptional isoforms contribute to the strong *aralar1* expression observed in adult fat bodies (mainly located in the abdomen), as can be inferred by the combination of our results (see Figure 4) and the modENCODE Tissue Expression Data. Fat bodies are important energy storages and utilization organs in Drosophila, as well as in other insects [92], and they play a fundamental role in the post-metamorphic energy metabolism and egg development. Taken together, these results suggest that an isoform-specific expression pattern becomes defined after the initial embryo development, when all the isoforms are transcribed, and its establishment appears to be important for certain developmental stages or in specific adult tissues.

It can be speculated that the observed transcriptional pattern could be related to the presence of two evolutionary conserved DNA motifs, located between the TSSs of *aralar1-RB* and *aralar1-RA*/*RF*/*RD* transcripts. Although we have not performed functional studies aimed to demonstrate their involvement in transcriptional regulation, it could be hypothesized that NRG elements might influence *aralar1* gene expression for two reasons. Firstly, its evolutionary conservation in 23

Drosophila species belonging to two distinct sub-genera indicates a constrained role. Indeed, intron sequences are less conserved, even in related species, and only functionally constrained motifs are maintained. The divergence between *D. melanogaster* and D. *grimshawi* is thought to have occurred 50Mya (Russo et al. 2003), and the conservation of such putative *cis*-acting sequence suggests a functional role. The NRG element can also be detected in non-Drosophilidae insects (Supplementary Table 6), thus enforcing its putative functional role, although its position related to the expressed transcriptional isoforms in these organisms is not easy to be determined.

Secondly, previous studies reported that NRG elements are conserved in a subset of Drosophila genes encoding mitochondrial proteins, while they have been lost in duplicated genes, which in turn have acquired testis-specific transcription patterns. Since *alarar1* gene lacks paralogues in *D. melanogaster* (and in all the analyzed Drosophila species), the presence of two NRG elements within it might have acquired a different role, such as the establishment of a regulated expression pattern of all, or at least some, of the transcriptional *aralar1*isoforms. In this view, the two NRG elements detected could act either cooperatively or independently, as positive or negative regulators. Whether or not the two NRG elements are functional to *aralar1* expression cannot be determined without the aid of mutant or transgenic strains carrying *aralar1* genes containing disrupted NRG elements, which necessarily calls for future analyses.

In order to verify if this complex tissue- and developmental stage-specific transcriptional regulation is associated to the expression of functionally different isoforms, the transport properties of Aralar1-PA and Aralar1-PE, representing the two main encoded products of all *aralar1* transcripts, have been determined. The two recombinant proteins, once reconstituted into liposomes, catalyze a very efficient aspartate/glutamate electrogenic exchange reaction and are able to restore the yeast defect of the *AGC1* null gene (Figure 8), confirming their identity as the *D. melanogaster* mitochondrial AGC. Similarly to the human isoforms [4], fruit fly Aralar1 isoforms do not show any significant difference in their substrate affinity, moreover, as the human isoforms, the Km value for aspartate was significantly lower than that for glutamate. Interestingly, although Aralar1-PA and Aralar1-PE differ only in their N-terminal halves, i.e., region known to interact with calcium [23], they showed a significant different specific activity, being Aralar1-PE more active than Aralar1-PA. A similar situation had been previously found in *H. sapiens*, where citrin resulted more active than aralar [4].

Although fruit fly and human AGC isoforms showed this overlapping behavior, it should be emphasized that the different specific activities found in the latter are mainly due to their different carrier domains [4], whereas all *D. melanogaster* AGC isoforms share the same carrier domain and differ in their N-terminal domains. In fact, the recombinant carrier domain reconstituted into liposomes, shows the same kinetic properties of Aralar1-PA (data not shown).

Interestingly, this N-terminal domain was also able to alter substrate specificity of the fruit fly AGC isoforms; since Aralar1-PE, similarly to human citrin [4], is able to exchange glutamate against cysteinesulfinate at the same rate as that of aspartate, while Aralar1-PA, similarly to human aralar, shows a glutamate/cysteinesulfinate exchange rate about one half lower than that of glutamate/aspartate. Furthermore, Aralar1-PE, differently from Aralar1-PA, significantly exchanges glutamate for L-α-aminoadipate and L-glutamine, suggesting a wider range of substrate specificity if compared to the latter.

A ClustalW alignment of Drosophila Aralar1 with human AGC isoforms has clarified that all proteins conserved the same structural organization including an N-terminal domain having 8 EF-hands with different functions (i.e. EF-hands 1-3 constitute a calcium-responsive unit, whereas EF-hands 4-8 are part of a static unit), a carrier domain and a C-terminal domain with unknown role (Figure 5) [23].Furthermore, calcium (EF-hands 1-3) and substrate binding sites are almost completely conserved between man and Drosophila, with a single conservative variation present in the calcium-binding site, where a threonine was replaced by a serine in the fruit fly isoforms (Figure 5). The most variable regions in the fruit fly Aralar1 isoforms are located in the N-terminus and into the loop of the helix-loop-helix structure of EF-hand 8 (Figure 5, green shaded) [23], where Aralar1-PE has an extra highly positive charged amino acid sequence. In the light of this observation, it is evident that N-terminus variations could play a fundamental role on the functional differences found between Aralar1-PA and Aralar1-PE. Site-direct mutagenesis experiments conducted on this additional region in EF-hand 8 have showed that the substitution of the charged residues by alanine can significantly affect substrate specificity and increase initial transport rate, whereas the progressive removal of these extra amino acids reduce initial transport rate and change substrate specificity until making Aralar1-PE similar to Aralar1-PA. Furthermore, this data are in agreement with the role proposed for the human AGC isoforms, in which the EF-hands 1–3 form a calcium-responsive mobile unit, EF-hands 4–8 form a static unit while the N-terminus region, external to the first EF-hand motif, seems not to be involved in any crucial regulatory function [23]. Finally, we have demonstrated that both proteins are Ca^{2+}-dependent, and similarly to citrin, Aralar1-PE shows a higher sensitivity to calcium being S0.5 about 2 times lower than that of Aralar1-PA. Hence, biochemical characterization and calcium sensitivity have identified Aralar1-PA and Aralar1-PE as the human aralar and citrin counterparts, respectively (Figure 6 C-D).

The functional characterization of the fruit fly mitochondrial AGC transporter represents a crucial step toward a complete understanding of the metabolic events acting during early embryogenesis, and it might strengthen the use of *D. melanogaster* as a model for genetics and developmental biology. Nevertheless, further studies of gain and loss of function are required to better understand

how the complex transcriptional and expression patterns of Aralar1 regulates specific metabolic pathways in the different developmental stages of the fruit fly.

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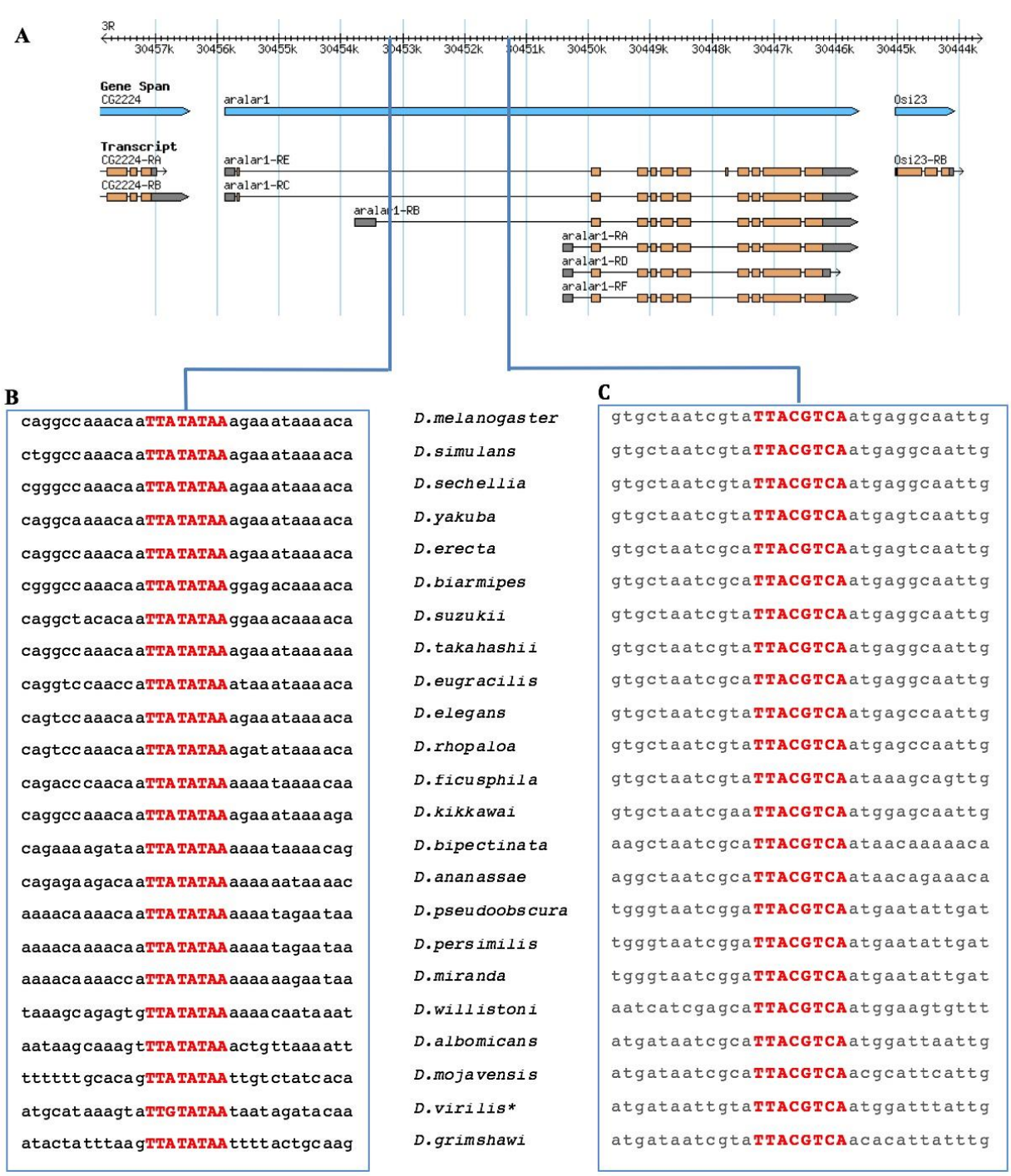

Figure 1 Chromosomal organization of the *aralar1* gene in *D. melanogaster*. A) Overall organization of *aralar1* locus and its map position in *D. melanogaster*. B) and C) multiple sequence alignments encompassing the two conserved NRG elements in the *aralar1* gene of 23 Drosophila species. * variant NRG motif in homologous position.

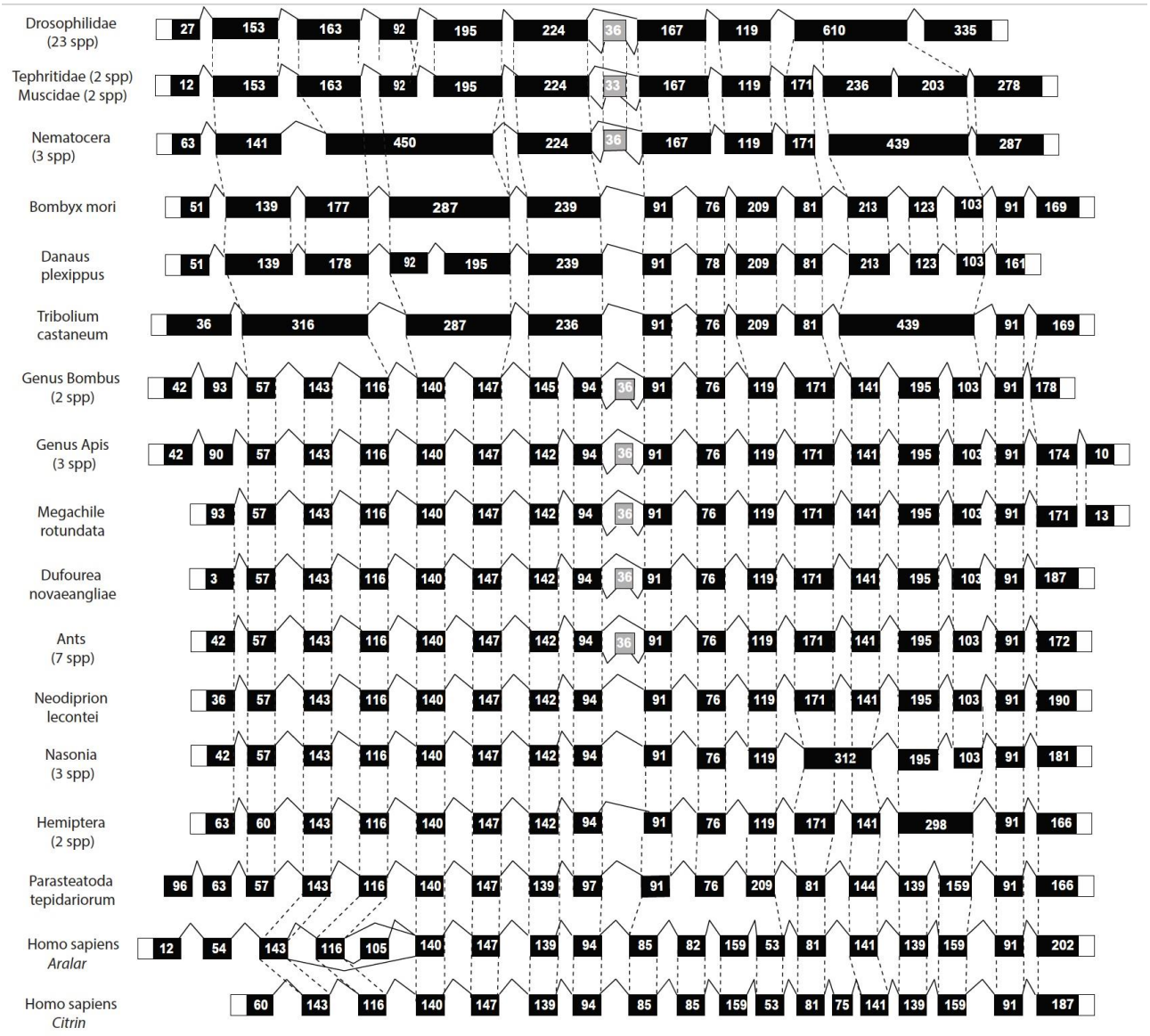

Figure 2. Eukaryotic AGC-encoding genes share an ancient intron-rich ancestor. The exon/intron structure of orthologous AGC-encoding genes of representative eukaryotic species is compared considering intron position, exon phase and length. Dashed lines indicate conservation of intron position. Translated AGC1-encoding regions are indicated in black, UTRs as white boxes. Internal *aralar1-RE* specific exons are indicated as gray boxes. Boxes are not in scale.

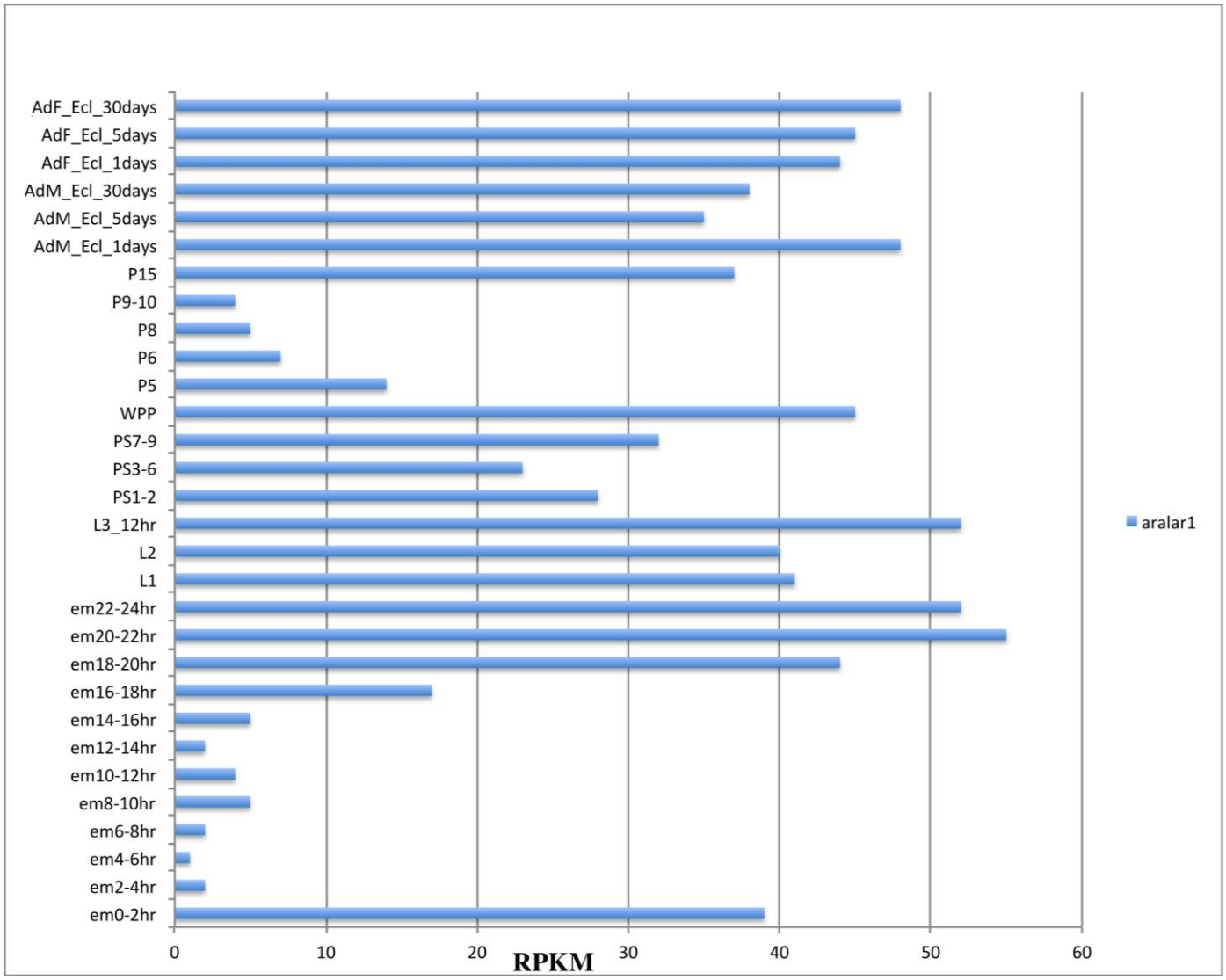

Figure 3. modEncode expression of *aralar1* through the development. Cumulative expression of all *aralar1* isoforms through the development is shown. Data were collected from modENCODE. Abbreviations legend. em: embryo stages; L: larval stages: P:pupae stages; AdM: adult males, various ages; AdF: adult females, various ages; RPKM: reads per kilobase million.

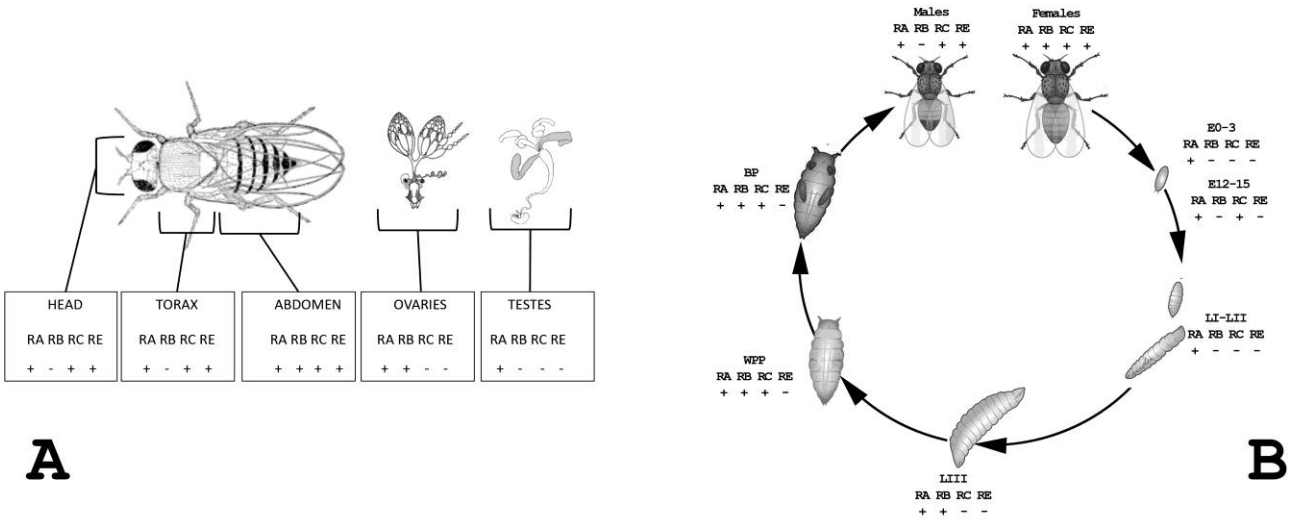

Figure 4. Transcriptional analysis of *aralar1* in Drosophila adult tissues.

The presence/absence of the indicated*aralar1* isoforms revealed by the detection of RT-PCR fragment in each analyzed RNA sample is indicated with "+" or "–", respectively.

Panel A. The impact of the four transcriptional isoforms of *aralar1* analyzed in adult heads, thoraces and abdomens, and in the reproductive organs of males and females of *D. melanogaster*.

Panel B. The presence/absence of four transcriptional isoforms of *aralar1* was analyzed by RT-PCR through development in the indicated stages. RNA samples were collected from embryos at two stages of embryonic development (0–3, 12–15 after egg lying), mixed first and second instar larvae (LI-LII), third instar larvae (LIII), white prepupae (WP), black pupae (BP), and adults (females and males).

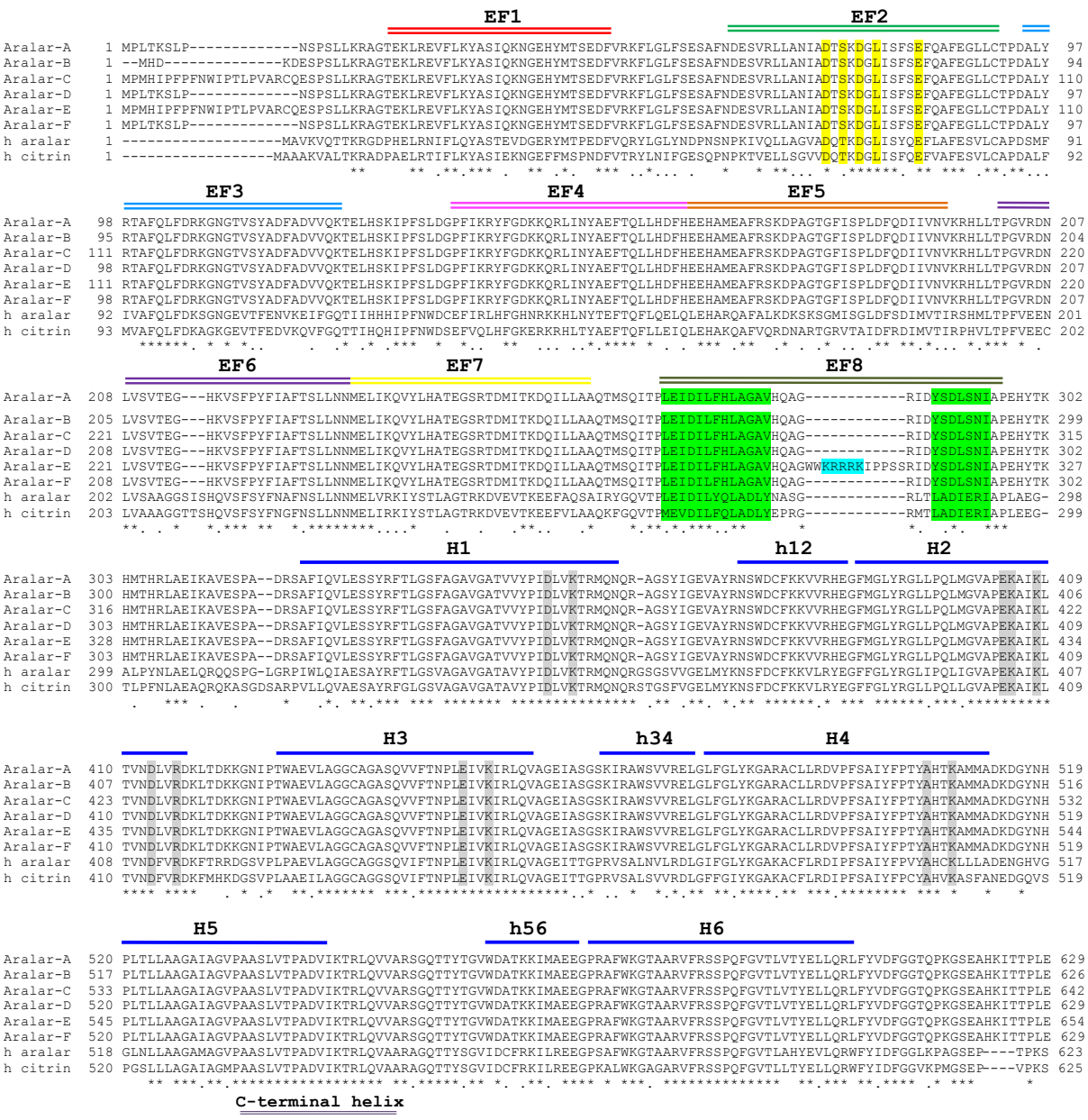

Figure 5. Alignment of Drosophila Aralar1-PA-PF and human aralar and citrin. Sequence conservation is indicated with an asterisk for identical residues, a dot for conserved substitutions, and a gap for non-conserved residues. The EF-hands, the six transmembrane helices H1-H6, the three small helices parallel to the membrane plane h12-h56, and the C-terminal α-helix are indicated by coloured bars and labels. The two α-helices of EF-hand 8 and the positive residues in

the loop of Aralar-1-PE are shaded in green and cyan, respectively. Residues that are involved in the coordination of calcium and the contact points of the substrate-binding site are shaded in yellow and gray, respectively. The alignment was obtained by using ClustalW.

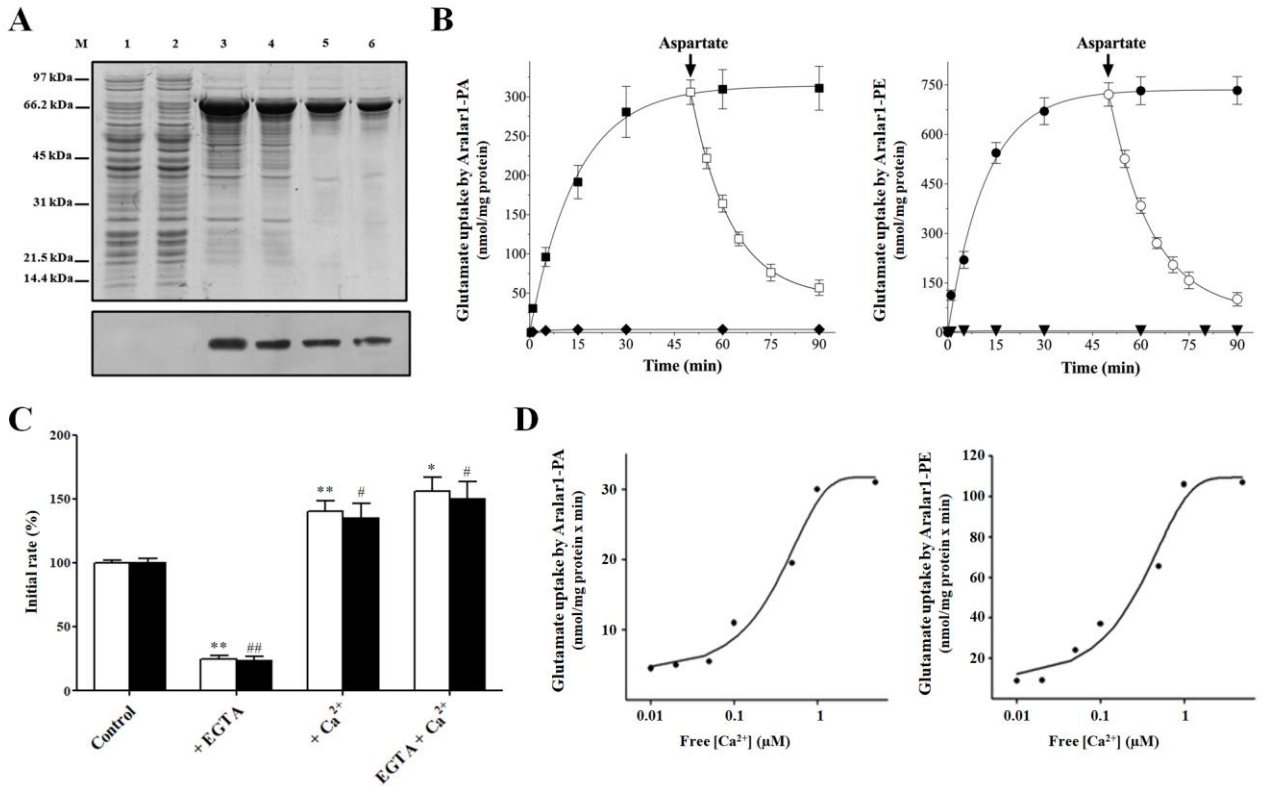

Figure 6. Bacterial expression and functionally characterization of Aralar1-PA and Aralar1-PE. (A) Expression in *Escherichia coli* of recombinant Aralar1. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye or transferred to nitrocellulose and immunodetected with an anti-V5 monoclonal antibody. Lane M, markers; lanes 1-6, *E. coli* BL21(DE3) containing the expression vector with the coding sequence for Aralar1-PA (lanes 1, 3 and 5) and Aralar1-PE (lanes 2, 4 and 6). Samples were taken at the time of the induction (lanes 1 and 2) and 4 h later (lanes 3 and 4). Lanes 5 and 6, Aralar1-PA and Aralar1-PE originating from bacteria shown in lanes 3 and 4, respectively, purified by centrifugation on sucrose gradient. The same number of bacteria was analyzed in each sample. **(B) Kinetics of [^{14}C]glutamate/glutamate exchange by Aralar1-PA and Aralar1-PE.** Proteoliposomes were reconstituted with Aralar1-PA and Aralar1-PE. 0.5 mM [^{14}C]glutamate was added to proteoliposomes containing 10 mM glutamate (■ and ●) or 10 mM NaCl (◆ and ▼). When the uptake approached equilibrium (50 min), 20 mM of aspartate was added outside the proteoliposomes (□ and ○). Similar results were obtained in four independent experiments. **(C-D) Effect of Ca^{2+} on transport activities of Aralar1-PA and Aralar1-PE. (C)** Recombinant Aralar1-PA (open bars) and Aralar1-PE (filled bars), were reconstituted in proteoliposomes, the [^{14}C]glutamate/glutamate transport rates were measured in de-ionized ultrapure water without additions (Control) or with the addition of 0.5 mM EGTA, or 1 mM $CaCl_2$, or 0.5 mM EGTA in the presence of 1 mM $CaCl_2$. Uptake rates of glutamate/glutamate exchange were measured at 60 s. Results are expressed as a percentage of the Aralar1-PA and Aralar1-PE values, which were on average 35.31 ± 1.24 and 110.58 ± 3.76 nmol/min x mg of protein, respectively. The average transport rates were calculated from the average of at least five independent experiments and displayed with the standard error. Different symbols indicate statistical differences, when occurring, between means of values as indicated by ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Aralar1-PA control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Aralar1-PE control). **(D)** Dependence of Aralar1-PA and Aralar1-PE activities on the free Ca^{2+} concentration. The [^{14}C] glutamate transport rates were measured in de-ionized ultrapure water in the presence of 0.5 mM EGTA and various concentrations of Ca^{2+}. Similar results were obtained in at least three independent experiments.

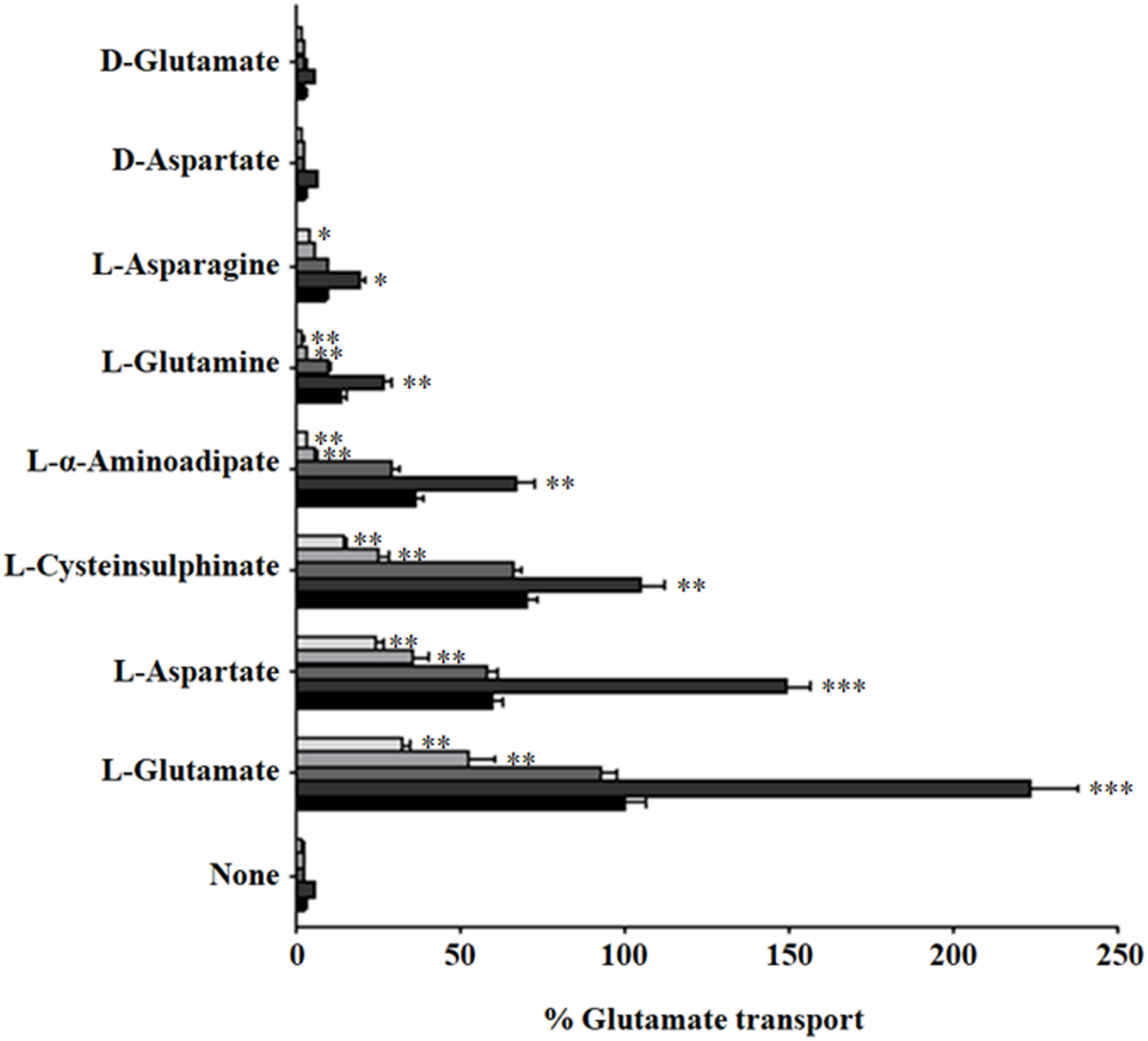

Figure 7. Dependence on internal substrate of the transport properties of proteoliposomes reconstituted with recombinant Aralar1-PE, 5ALA, Δ5-Aralar1-PE, Δ8-Aralar1-PE and Δ12-Aralar1-PE mutants. Proteoliposomes reconstituted with Aralar1-PE (black bars), 5ALA (very dark grey bars), Δ5-Aralar1-PE (dark grey bars), Δ8-Aralar1-PE (light grey bars) and Δ12-Aralar1-PE (very light grey bars) were preloaded internally with various substrates (concentration 10 mM). Transport was started by adding 0.5 mM [^{14}C]glutamate to proteoliposomes and terminated after 1 min. Data represent the mean of three independent experiments reported as the percent of glutamate transport with respect to the rate of [^{14}C]glutamate/glutamate exchange by Aralar1-PE (110 nmol/min/mg protein) setting at 100%. Asterisks indicate the level of statistical significance calculated comparing Aralar1-PE transport activity with that of its mutants in presence of each internal substrate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, using ANOVA.

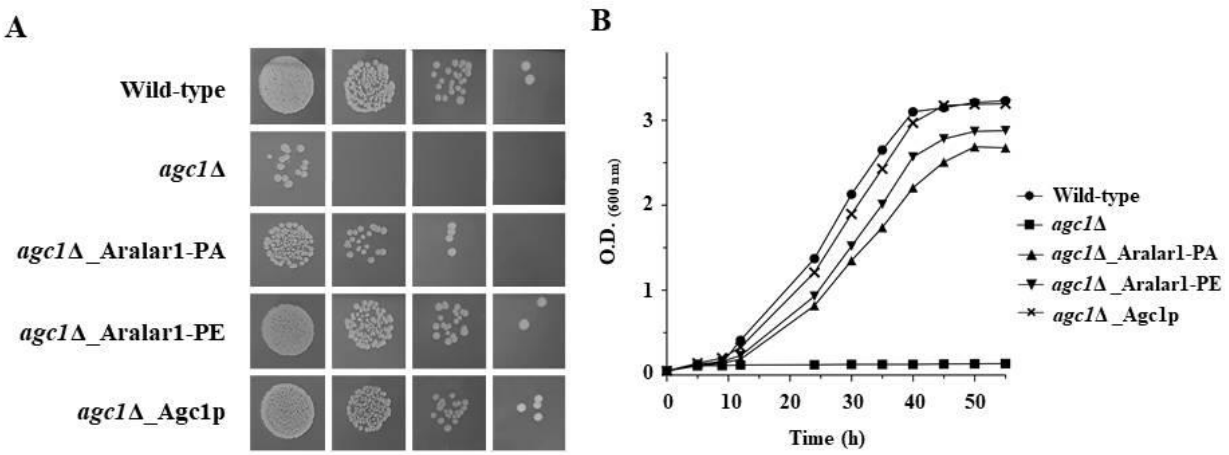

Figure 8. Complementation of the growth defect of *agc1*Δ yeast strain by Aralar1-PA and Aralar1-PE. Wild-type, *agc1*Δ cells transformed with the empty vector (*agc1*Δ) and *agc1*Δ cells expressing drosophila Aralar1-PA, Aralar1-PE and yeast Agc1p were plated by using a four-fold serial dilution (A) or inoculated (B) in YP medium supplemented with 0.5 mM oleate. Plates and cell cultures were placed at 30°C. Picture given in *A* was taken after 3 days to show yeast growth performance, whereas the optical density (*O.D.*) values at 600 nm given in *B* refer to cell cultures after the indicated growth times.

TABLE I

Dependence on internal substrate of the transport properties of proteoliposomes reconstituted with recombinant Aralar1-PA andAralar1-PE.

Proteoliposomes were preloaded internally with various substrates (concentration 10 mM). Transport was started by adding 0.5 mM [^{14}C]glutamate to proteoliposomes reconstituted with Aralar1-PAandAralar1-PE, respectively, and terminated after 1 min. For each internal substrates four indipendent experiments have been carried out. The means and the standard deviations of Aralar1-PA and Aralar1-PE for each substrate are reported. Different symbols indicate statistical differences between means of values as indicated by ANOVA ($^{*}P \leq 0.05$, $^{***}P \leq 0.001$ *vs* uniport reaction by Aralar1-PA, $^{\#}P \leq 0.05$,$^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* uniport reaction by Aralar1-PE).

Internal substrate	**[^{14}C]Glutamate transport**	
	Aralar1-PA	**Aralar1-PE**
	nmol/min x mg of protein	
None (Cl^- present)	1.63 ± 0.11	2.85 ± 0.19
L-glutamate	31.27 ± 1.31***	105.5 ± 6.89$^{\#\#\#}$
L-aspartate	23.14 ± 1.21***	63.34 ± 2.78$^{\#\#\#}$
L-cysteinesulfinate	13.76 ± 0.78***	73.91 ± 3.23$^{\#\#\#}$
L-α-aminoadipate	2.34 ± 0.13*	38.05 ± 2.98$^{\#\#\#}$
L-glutamine	1.84 ± 0.16	14.68 ± 1.24$^{\#\#\#}$
L-asparagine	3.63 ± 0.27*	9.51 ± 1.02$^{\#}$
D-aspartate	1.65 ± 0.10	2.99 ± 0.22
D-glutamate	1.34 ± 0.12	2.77 ± 0.25

TABLE II

Influence of the membrane potential on the activity of recombinant Aralar1-PA and Aralar1-PE. The exchange was started by the addition of 50 μM [^{14}C]aspartate to proteoliposomes containing 10 mM of the indicated internal substrate. K^+_{in} was included as KCl in the reconstitution mixture, whereas K^+_{out} was added as KCl together with the labeled substrate. The differences in osmolarity were compensated for by the addition of appropriate concentrations of sucrose in the opposite compartment. Valinomycin (1.0 μg/mg phospholipid) was added in 10 μl ethanol/ml of proteoliposomes (+ valinomycin). In the samples without valinomycin (– valinomycin) the only ethanol was added. The exchange reactions were stopped after 1 min. Similar results were obtained in three independent experiments. Asterisks indicate values that are significantly different from those obtained in the same experiment but in absence of valinomycin (**$P \leq 0.01$, using Student's *t*-test)

Internal substrate	**K^+_{in}/k^+_{out} (mM/mM)**	**[^{14}C]Aspartate uptake** (*nmol/min x mg protein*)			
		Aralar1-PA		**Aralar1-PE**	
		- valinomycin	**+ valinomycin**	**- valinomycin**	**+ valinomycin**
Aspartate	1/1	28.75 ± 2.11	30.05 ± 2.96	100.75 ± 8.55	102.21 ± 8.40
	1/50	27.37 ± 2.41	28.64 ± 2.05	103.25 ± 7.20	103.89 ± 9.10
Glutamate	1/1	21.52 ± 1.70	22.24 ± 1.45	76.33 ± 5.88	77.14 ± 6.39
	1/50	23.95 ± 2.35	57.48 ± 5.74**	75.68 ± 6.28	154.86 ± 7.50**

TABLE III

Kinetic constants of recombinant Aralar1-PA, Aralar1-PE, 5ALA, Δ5-Aralar1-PE, Δ8-Aralar1-PE *and* Δ12-Aralar1-PE *mutants*.

The values were calculated from double reciprocal plots of the rate of [^{14}C]glutamate uptake versus substrate concentrations. Transport was started by adding 125-750 μM [^{14}C]glutamate to proteoliposomes containing 10 mM glutamate, and terminated after 1 min. Similar results were obtained in at least three independent experiments.

Carrier	**K_m** (*mM*)	**V_{max}** (*nmol/min x mg protein*)
Aralar1-PA	0.26 ± 0.03	34.60 ± 2.11
Aralar1-PE	0.29 ± 0.07	92.08 ± 4.96
5ALA	0.31 ± 0.062	189.21 ± 10.50
Δ5-Aralar1-PE	0.33 ± 0.075	82.22 ± 7.55
Δ8-Aralar1-PE	0.31 ± 0.072	58.75 ± 5.10
Δ12-Aralar1-PE	0.30 ±0.055	37.32 ± 3.15

The mitochondrial aspartate/glutamate carrier (AGC or Aralar1) isoforms in *D. melanogaster*: biochemical characterization, gene structure, and evolutionary analysis

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Abstract

Background

In man two mitochondrial aspartate/glutamate carrier (AGC) isoforms, known as aralar and citrin, are required to accomplish several metabolic pathways. In order to fill the existing gap of knowledge in *Drosophila melanogaster*, we have studied *aralar1* gene, orthologue of human AGC-encoding genes in this organism.

Methods

The blastp algorithm and the ‘‘reciprocal best hit” approach have been used to identify the human orthologue of AGCs in Drosophilidae and non-Drosophilidae. Aralar1 proteins have been overexpressed in *Escherichia coli* and functionally reconstituted in liposomes for transport assays.

Results

The transcriptional organization of *aralar1* comprises six isoforms, three constitutively expressed (*aralar1-RA, RD and RF*), and the remaining three distributed during the development or in different tissues (*aralar1-RB, RC and RE*). Aralar1-PA and Aralar1-PE, representative of all isoforms, have been biochemically characterized. Recombinant Aralar1-PA and Aralar1-PE proteins share similar efficiency to exchange glutamate against aspartate, and same substrate affinities than the human isoforms. Interestingly, although Aralar1-PA and Aralar1-PE diverge only in their EF-hand 8, they greatly differ in their specific activities and substrate specificity.

Conclusions

The tight regulation of aralar1 transcripts expression and the high request of aspartate and glutamate during early embryogenesis suggest a crucial role of Aralar1 in this Drosophila developmental stage. Furthermore, biochemical characterization and calcium sensitivity have identified Aralar1-PA and Aralar1-PE as the human aralar and citrin counterparts, respectively.

General Significance

The functional characterization of the fruit fly mitochondrial AGC transporter represents a crucial step toward a complete understanding of the metabolic events acting during early embryogenesis.

Keywords: *Drosophila melanogaster*; *CG2139*; aspartate/glutamate carrier (AGC); aralar; citrin; phylogenetic footprint;

Abbreviations: AGC, aspartate/glutamate carrier; MAS, malate-aspartate shuttle; MCF, mitochondrial carrier family; NRG, nuclear respiratory gene; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TSS, transcriptional start site.

1.Introduction

The mitochondrial carrier family (MCF)represents one of the largest families of transporters widespread across all species. MCF members share a common structural organization, consisting of three tandemly repeated sequences of approximately 100 amino acids in length, and mediate the transport of metabolites across the inner mitochondrial membrane [1-3]. The aspartate/glutamate carrier (AGC) catalyzes an electrogenic exchange of cytosolic glutamate plus a proton for mitochondrial aspartate [4], and it is one of the most important family member, since it is a key component of the malate-aspartate shuttle (MAS),which transfers the reducing equivalents of NADH from cytosol to mitochondria in order to regenerate NAD^+ necessary for glycolysis. Furthermore, AGC plays an essential role in many other metabolic processes including the synthesis of urea and nitrogen-containing metabolites [5], gluconeogenesis from lactate [5], calcium-mediated regulation of mitochondrial respiration [4, 6-9], insulin secretion from islet cells [10], zygote development [11, 12], N-acetylaspartate (a myelin precursor) synthesis [13-16] and glial synthesis of glutamate and glutamine[17]. Increasing evidences support the hypothesis that MAS activity is crucial for breast cancer cell proliferation [18]. In this regard, it might divert energetic cell metabolism towards glycolysis, promoting cancer cell growth [19]. Furthermore, AGC is involved in taurine metabolism, since it can efficiently transport also cysteinesulfinate [4, 20], an intermediate of cysteine degradation that serves as a precursor of taurine [21].

In *Homo sapiens,* two isoforms named AGC1 and AGC2 (also known as aralar and citrin) and encoded by the *SLC25A12* and *SLC25A13* genes, respectively, have been identified and characterized [4, 22]. They belong to a subfamily of calcium-binding mitochondrial carrier sharing a characteristic bipartite structure, which consists of a C-terminal domain responsible for transport activity, and an N-terminal domain containing 8 EF-hands that regulates transport activity [4, 23].

Aralar is mainly expressed in the heart, retina, skeletal muscle and brain [24, 25], whereas citrin is widely expressed in the liver, epithelial cells and breast [4]. Aralar is involved in retinal visual function [26, 27], in myelination process [13] and in glutamate-induced excitotoxicity [28, 29]. Mutations in the *SLC25A12* gene have been found in autism spectrum disorders [25, 30-32], as well as in a rare human disease (OMIM number 612949) implying developmental delay, epilepsy and hypotonia [15, 33]. *SLC25A13* mutations are responsible for citrin deficiency that causes adult-onset type II citrullinemia (CTLN2, OMIM number 603471), neonatal intrahepatic cholestasis (NICCD, OMIM number 605814) [29, 34-39], as well as failure to thrive and dyslipidemia caused by citrin deficiency (FTTDCD) [40].

In *D. melanogaster,* a single gene annotated in FlyBase as *CG2139* or *aralar1*encodes a ortholog of the human AGC isoforms [41].A*ralar1* gene is involved in high energy demanding processes, together with many other genes, such as those responsible for embryonic epithelial repair [42]. Previous studies indicated that *aralar1* locus is involved in the determination of bristle number in *D. melanogaster*, which are structures that may also have a neurosensory function [43], as well as this locus may influence wing size phenotype [44].

Differently from other fruit fly mitochondrial transporters, such as adenine nucleotide translocase [45], uncoupling protein [46, 47] dicarboxylate [48], thiamine pyrophosphate [49, 50], and glutamate carriers [51], a genomic analysis of this insect has highlighted the presence of a single gene (*CG2139*) encoding various *aralar1* spliced isoforms. The presence of so many alternatively spliced isoforms in mitochondrial carriers [47, 52, 53] and more in general in this organism is not surprising, since alternative splicing is a peculiar mechanism very often used by *D. melanogaster* to tightly control tissue- and stage-specific protein isoforms with different functions in development, as highlighted by Venables et co-workers *"Drosophila uses every alternative splicing strategy imaginable with an elegance and complexity that often eclipses mammals"* [54].

Despite the high metabolic relevance of this transporter, very few data are available on its biological role in fruit fly cellular processes, and no data at all are available on its transport properties in Drosophila.

In the present study, we have described (i) the expression profile of *D. melanogasteraralar1*gene in different developmental stages and tissues; (ii) the evolutionary changes that have shaped *aralar1* gene structure in arthropods; (iii) the functional characterization of recombinant Aralar1-PA and Aralar1-PE and their identification as the fruit fly mitochondrial aspartate/glutamate carriers.

2. Material and methods

2.1. Blast search of AGC orthologs in *D. melanogaster* and other species

The *D. melanogaster* ortholog of human AGCs was identified using the blastp algorithm (http://flybase.org/blast/). A BLAST search strategy, using *D. melanogaster* CDS and/or peptides as queries against genomic and ESTs databases, was also adopted to identify putative genes encoding AGC in the genome of other Drosophilidae and several non-Drosophilidae species [55]. A complete list of the species investigated in this study and the accession number in which *aralar1* genes have been identified is shown in Supplementary Table 1. These searches were performed using organism-specific (www.flybase.org,www.vectorbase.org and http://hymenopteragenome.org) and generalist genomic repositories (NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ‘‘reciprocal best

hit" approach was used to identify *aralar1* genes in different species. In this approach, a common evolutionary origin is supposed when in the compared genomes two gene sequences represent each other the best BLAST hit[56].For each genomic sequence identified by using the above-mentioned criteria, exon/intron boundaries were carefully annotated after prediction *in silico* carried out with the aid of ESTs and cDNA sequences, if available, or predicted by the Genscan tool [57], or inferred by sequence similarity with transcript sequences of closely related species. Multiple alignments of amino acids, as well as coding and non-coding DNA sequences, were obtained using the MultAlin 5.4.1 software available at the MultAlin server (http://multalin.toulouse.inra.fr/multalin/) [58, 59]. The matrix-scan pattern matching tool of the Regulatory Sequence Analysis Tools from the RSAT server (http://rsat.ulb.ac.be/) was used, in combination with multiple alignments to identify Nuclear Respiratory Gene (NRG) elements [60] in the non-coding sequences of Drosophilidae and non-Drosophilidae *aralar1* genes.

2.2. Reverse-transcription analyses of *aralar1* transcriptional isoforms

Oregon-R flies were raised on standard culture medium at 24 °C. Total RNA for gene expression analysis was achieved from 0.5- to 1-g samples of Oregon-R individuals at different developmental stages (embryo, larvae, pupae and adult flies). Except for ovaries and testes, male and female tissues equally contributed to each dissection. Total RNA from all the developmental stages and tissues was extractedby employing Trizol. Purified RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and diluted to 1 mg/ml for reverse transcription. RNA (1 µg) was reverse transcribed by employing the Quantitect reverse transcription kit (QIAGEN) [61, 62] following the manifacturer's protocol. Amplification was carried out using the Platinum Taq DNA Polymerase (Thermo Fisher).

Primers used for amplification were:

aralar_RA_F ATTAGACGCGGGAATTGCTC
aralar_RA_R CTCCTCGTGAAAGTCGTGCAG
aralar_RB_F ATAGTGCGAACGTGCCTGA
aralar_RB_R ATGGCGAGATGAACCCAGTG
aralar_RC_F CCGATGCCAAGAATCTCCGT
aralar_RC_R TCCTCGTGAAAGTCGTGCAG
aralar_RE_F CAAATCACGCCGCTGGAGAT
aralar_RE_F GGCCCCTTTATCAAGCGCTA

2.3. Construction of the expression plasmids encoding *D. melanogaster*Aralar1 isoforms and mutants in *E. coli* and *S. cerevisiae*

The two protein isoforms Aralar1-PA and Aralar1-PE were considered for the functional characterization of D. melanogaster *CG2139* gene. Aralar1-RA and Aralar1-RE were amplified by polymerase chain reaction (PCR) from the cDNA of the fruit fly clones LD35441 and GH21613, respectively. Both clones were provided by Drosophila Genomics Resource Center (Indiana University 1001 East Third St., Bloomington IN, 47405-7107). The oligonucleotide primers (sense and antisense) used in PCR reactions carried suitable restriction sites at their 5′ ends for the further cloning of the amplified inserts in the pET-21b/V5-His *E. coli* expression vector [51]. The absence of a stop codon in the reverse primer sequence led to the expression of Aralar1 proteins containing V5/His-tag at their C-termini [63].

The WT Aralar1-RE cDNA was employed as a template to generate mutants named Δ5 (deletion from residue 301 to 305), Δ8 (deletion from residue 301 to 308), Δ12 (deletion from residue 299 to 310), and 5Ala (replacement of residues $K_{301}RRRK_{305}$ by consecutive alanines). All of the mutations were introduced by the overlap extension PCR method [64, 65], using oligonucleotides with suitable mutations in their sequences. Primers used for amplification were:

aralar_PE_FTAGGAATTCACCAATGCACATCCCGTTTCC and

aralar_PE_R CGAAAGCTTGGATCCCGTGGCCGTCG for all mutants.

The following specific primers were used to generate the above-mentioned mutants:

aralar_PE_ Δ5 _F CCATCAAGCAGGCTGGTGGATACCTCCGAGTAGCCGCA

aralar_ PE _ Δ5 _R TGCGGCTACTCGGAGGTATCCACCAGCCTGCTTGATGG

aralar_ PE _ Δ8 _F CCATCAAGCAGGCTGGTGGAGTAGCCGCATCGACTATAGTGAC

aralar_ PE _ Δ8 _R GTCACTATAGTCGATGCGGCTACTCCACCAGCCTGCTTGATGG

aralar_ PE _ Δ12 _F GCGCCGTCCATCAAGCAGGCCGCATCGACTATAGTGACCTGAGCAA

aralar_ PE _ Δ12 _R TTGCTCAGGTCACTATAGTCGATGCGGCCTGCTTGATGGACGGCGC

aralar_ PE _5Ala_F
CCATCAAGCAGGCTGGTGGGCGGCTGCCGCAGCGATACCTCCGAGTAGCCGCA

aralar_RE_5Ala_R
TGCGGCTACTCGGAGGTATCGCTGCGGCAGCCGCCCACCAGCCTGCTTGATGG

The aralar1-RA and aralar1-RE ORFs were recovered from pET-21b/V5-His clones by a EcoRI/AgeI digestion and cloned in the modified yeast expression vector pYES2/ V5-His [49], in which the inducible GAL1 promoter had been replaced with the constitutive TDH3 promoter. The

frameshift of transcripts was avoided cloning a KpnI/EcoRI fragment carrying the ATG start codon at '5-end. Fragments used were: GGTACCAGGATCCGATCTTGAACTGACTGAGATGGCGAATTC and GGTACCAGGATCCGATCTTGAACTGACTGAGATGGCGAATTC for aralar1-RA and for aralar1-RE, respectively.

2.4. Bacterial expression and purification of the recombinant proteins

Aralar1 proteins were overexpressed at high levels in *E. coli* BL21(DE3) [49].Their identities were assessed by MALDI-TOFMS of trypsin digests of the corresponding bands excised from a Coomassie blue-stained polyacrylamide gel [66, 67]. Inclusion bodies were purified using sucrose density gradient centrifugation, next they were washed at 4ºC, firstly with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8), then twice with a buffer containing Triton X-114 (2%, w/v) and 10 mM HEPES (pH 8), and finally with 10 mM PIPES pH 6.5. Aralar1-PA and Aralar1-PE were solubilized in 2% sarkosyl (w/v), and a small residue was removed by centrifugation (258000 g, 30 min). Solubilized proteins were diluted 10-fold with 10 mM PIPES pH 6.5 and reconstituted into liposomes [68].

2.5. Reconstitution into liposomes and transport assays

Reconstitution mixture (700 µl) contained 0.5-1 µg of solubilized proteins, Triton X-114 (1,3 % w/v), L-α-phosphatidylcholine from egg yolk (1,3 % w/v), as sonicated liposomes, 10 mM glutamate (except where otherwise indicated), and 20 mM PIPES at pH 6.5 [69]. These components were carefully blended, and the blend was recycled 13 times through the same Amberlite column (Bio-Rad) [69]. External substrate was eliminated from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with 50 mM NaCl and 10 mM PIPES at pH 6.5. Transport at 25ºC was started by adding L-[^{14}C]glutamate (Scopus Research BV, Wageningen, Netherlands) at the indicated concentrations to substrate-loaded proteoliposomes (exchange reaction), or to empty proteoliposomes (uniport reaction). In both cases, transport was terminated by adding 30 mM pyridoxal phosphate (PLP). In control samples, inhibitor was added together with the external radioactive substrate based on the inhibitor stop method [70, 71]. Finally, external substrate was removed and radioactivity intoproteoliposomes was measured. The experimental values were adjusted by subtracting control values. The initial transport rate was measured from the radioactivity taken up by proteoliposomes after 1 min (in the initial linear range of substrate uptake). The free Ca^{2+} concentrations were determined fluorimetrically with fura-2-acetoxymethyl ester (Fura-2 AM) (Thermo Fischer Scientific). Fura-2 AM is a membrane-permeable, non-invasive

derivative of the ratiometric calcium indicator fura-2. The excitation wavelengths used are 340 nm and 380 nm for Ca^{2+}-bound and Ca^{2+}-free fura-2 AM, respectively. In both states, the emission maximum is about 510 nm. The ratios 510 nm/340 nm and 510 nm/380 nm are directly related to the amount of Ca^{2+} [72]. Calcium dependence on transport activity was determined using de-ionized ultrapure water. In experiments for evaluating the influence of the membrane potential on the activity of the recombinant proteins, valinomycin was added to proteoliposomes in order to provide de-energized conditions. K^+ diffusion potentials were generated using valinomycin and K^+ gradients. In these experiments, substrate and buffer were neutralized with NaOH [4].

2.6. Yeast strains, growth conditions and functional complementation of yeast AGC1Δ by ARALAR1-PA and ARALAR1-PE

W303 (wild-type) (*MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}*) yeast strain was provided by the EUROFAN resource center EUROSCARF (Frankfurt, Germany). The yeast AGC1gene deletion (*agc*1Δ) and the cloning of endogenous yeast deleted gene AGC1 were achieved as described before [73].

The recombinant aralar1-RA-pYES2 and aralar1-RE-pYES2 plasmids were introduced into the agc1Δ yeast strain using the lithium acetate method [74], and transformants were selected on minimal medium lacking uracil, supplemented with 2% glucose.

Functional complementation was achieved growing cells on liquid complete medium (1% Bacto yeast extract, 2% Bacto Peptone, pH 5.0) supplemented with 2% glucose (YPD) or 0.5 mM oleic acid dissolved in 10% Tween40 (YPO) as carbon sources. Growths were started from medium log precultures grown on complete medium YPD and diluted with YPO to an optical density of 0.01 at 600 nm.

Simultaneously,washed cells weredilutedand spotted on complete solid medium YPO, then plates were incubated for 72 h at 30 °C. Four-fold serial dilutions of both the transformed strains, wild-type and AGC1Δ cells were analyzed.

2.7. Other methods

Proteins were resolved by SDS-PAGE and stained with Coomassie blue dye [75]. The amounts of recombinant pure Aralar1-PA and Aralar1-PE incorporated into liposomes were measured as previously described [76] and proved to be approximately 20% of the protein amount added to the reconstitution mixture. Moreover, recombinant proteins were resolved by SDS-PAGE analysis [77, 78], then transferred to a nitrocellulose membrane and analyzed by Western blotting employinga mouse anti-V5 monoclonal antibody (Sigma-Aldrich) [79, 80]. A multiple sequence alignment

(MSA) of the fruit fly Aralar1 proteins, human aralar and citrin isoforms was obtained by using ClustalW to insert gaps in the MSA [81]. All data were analyzed using the statistical software GraphPad Prism Software (7.0 version). Pairwise comparisons between means of different groups were performed using a Student's t-test (two tailed, unpaired). Multiple comparisons were performed using a univariate ANOVA. Values at $P \leq 0.05$ were considered statistically significant, while values at $P \leq 0.01$ and $P < 0.001$ were considered very significant [82, 83]. Orientation of functional Aralar-1 in the membrane of reconstituted proteoliposomes was investigated by ELISA assay performed as previously described [84]. ELISA tests were carried out on intact and permeabilized proteoliposomes by using either the rabbit polyclonal antiserum generated against 35-177 amino acids of Drosophila Aralar-1 proteins as described in [85] or the anti-V5 antibody directed against the V5 epitope placed at their C-termini (Sigma-Aldrich).

3. Results

3.1. Identification of genes encoding the aspartate/glutamate carrier in Drosophilaspecies

A single gene, annotated in FlyBaseas *CG2139* or *aralar1*, encodes a *D. melanogaster* ortholog of the human AGC isoforms (also known as aralar and citrin) [4], with no additional hits obtained throughout database mining. A*ralar1* locus spans roughly 10 Kbp on the third chromosome (3R:30,445,635..30,455,867 [-]) in the 99F4-99F5 cytogenetic band of polytene chromosomes of *D. melanogaster*. The transcriptional organization of *aralar1* reported in FlyBase (Figure 1A) comprises sixtranscriptional isoforms. The utilization of alternative transcriptional start sites, alternative splicing and alternative UTRs, produces five different transcriptional isoforms (namely RA, RB, RC, RD, RE), which in turn can be translated into four different proteins (PA/PD, PB, PC, PE). An additional translational isoform is reported in FlyBase, Aralar1-PF, which is translated from the *aralar1-RF* transcript that is structurally identical to *aralar1-RA* isoform by mean of a read-through translational mechanism [86]. In this regard, *aralar1-RA* transcript is translated into a 682 amino acids long protein (Aralar1-PA), whereas *aralar1-RF* transcriptional isoform is translated into a polypeptide having 12 additional aminoacids at its C-terminus due to translation progression beyond the canonical stop codon. Three different promoters contribute to the transcription of *aralar1* locus. *Aralar1-RA* (identical to *aralar1-RF*) and *aralar1-RD* are transcribed from the same downstream promoter, with *aralar1-RD* transcript possessing a shorter 3'UTR if compared to that of*aralar1-RA/RF* isoforms. *Aralar1-RB* arises from the central promoter,whereas *aralar1-RC* and *aralar1-RE* are originated from the upstream promoter. The two latter isoforms differ by a short, 36 bp long exon, which is specifically incorporated into the *aralar1-RE* transcript. Given the availability of a wide range of sequenced Arthropoda genomes, we have surveyed *aralar1* gene structure in Arthropoda.

Orthologous genes in Drosophilidae have been retrieved using a BLAST strategy, and locus structure has been inferred with the aid of ESTs and cDNA sequences annotated in FlyBaseand NCBI databases.Sequences with significant similarity to *D. melanogasteraralar1* gene have been detected in the genome of 22 additional Drosophila species (Supplementary Table 1), and their organization has been compared. Similarly to *D. melanogaster* annotation, in all the investigated Drosophilaspecies three transcriptional start sitescan be inferred from ESTs and cDNAs comparison and from inter-species similarity. In order to give a better snapshot of *aralar1* locus, its organization has been divided into three schemes highlighting the exon/intron organization relative to the three TSSs (Transcriptional Start Site) (Supplementary Tables 2-5). The comparison clearly suggests that the exon-intron structure of the locus is highly conserved in the 23 analyzed species. As can be observed, exon length is poorly variable. The first exon of all transcriptional isoforms is the most variable in length. Indeed, they encode the 5' UTRs and the leader peptides that are expected to be divergent in sequence, even if closely related species are compared. The length of the coding region of the last exon, encoding the protein C-terminus, also presents variable length and sequence in the analyzed Drosophila species. Internal coding exons are instead extremely conserved in length. As expected, introns are the most plastic regions of eukaryotic genes, and they are longer in species distantly related to *D. melanogaster*. It is worth to note that although the presence of the*aralar1-RE* specific exon is not supported by ESTs or cDNAs in the vast majority of the Drosophilidae species (not shown), it can be inferred by sequence similarity, suggesting that it could be also a functional exon in other species. In support of this hypothesis, the *aralar1-RE*specific exon, which can be 33-39 bp long depending upon the species, is flanked by a non-canonical donor splice site (GC)conserved in all the Drosophila species, suggesting the usage of an atypical splice site in the common ancestor of Drosophila and Sophophora genera (Supplementary Figure 1).

3.2. Conservation of regulatory elements

Inter-species DNA sequence comparison is an excellent tool for the identification of cis-regulatory DNA sequences interested in eukaryotic gene expression regulation, especially in non-coding DNA sequence. In the noncoding sequences of Drosophilidae *aralar1* genes we have searched for conserved motifs by using phylogenetic footprinting and DNA pattern discovery softwares. The nuclear respiratory gene (NRG) element has been previouslyreported as a palindromic 8-bp motif (TTAYRTAA) that is shared by all nuclear OXPHOS genes [87], as well as by many other nuclear genes involved in biogenesis and function of mitochondria in insects [60]. In *D. melanogaster*, NRG elements are usually located within an intron, in close proximity to the transcription start site (TSS), but it can be found in different gene locations, such as upstream the TSS. A NRG element

(TTATATAA) associated with high weight and low P value in the RSAT output (8,2 and $6.0e^{-05}$ respectively) is located in the first intron, 518 bp downstream of the transcription start site related to *D. melanogaster aralar1-RB* transcriptional isoform. This 8-bp motif is extremely conserved and located in the first intron in all the Drosophilidae *aralar1* orthologs with high frequency (Figure 1B). An additional NRG element, associated with a slightly lower score and a moderately higher P-value in the RSAT output (6,7 and $1,5e^{-04}$ respectively), is located downstream within the same intron, 686 bp upstream the TSS related to the RA/RD/RF transcriptional isoforms (Figure 1C).

The functional importance of the NRG element in *aralar1* genes is suggested by its presence in the non-coding regions of *aralar1* genes belonging to the 32 non-Drosophilidae Arthropoda species investigated in this work (Supplementary Table 1). Notwithstanding, the very long divergence times (divergence between Drosophilaand Parasteatoda/Limulus genera is estimated approximately 600 MYA, from Treebase http://www.timetree.org) [88], single or multiple NRG elements have been detected in *aralar1* genes of the majority of Arthropoda species, and their intragenic localization is often strictly conserved (not shown). However, standing to the availability of transcriptional data in the vast majority of the studied cases, we have reconstructed the complete exon/intron structure of *aralar1* related to the shortest transcriptional isoform, which we have arbitrarily assumed to be homologous to *D. melanogaster* RA-RD-RF transcripts. In many of these species, the NRG elements have been found in the first intron related to the shortest transcriptional isoform, suggesting that their positions could have been remodeled during insects' evolution. Also the length of the first intron, with respect to the most proximal TSS, is on average longer in non-Drosophilidae insects (mean length=15149 bp, 29 species) than in *D. melanogaster* homologs (mean length=245 bp, 23 species),supporting the hypothesis of a functional role of the NRG elements, although in a different position.

3.3. All eukaryotic *aralar1* genes have a common evolutionary origin.

Sequences homologous to *D. melanogaster aralar1* gene have been found in the genomes of 23 Drosophilidae species. They share not only significant sequence similarity with *D. melanogaster* genes, but also a strikingly conserved exon/intron organization (Figure 2 and Supplementary Tables 2-5). As a first step towards understanding the evolutionary history of *aralar1* genes, we have compared the Drosophilidae gene organization with their counterparts in an informative range of 32 Arthropod species consisting of 30 insects species (namely 7 Diptera, two Lepidoptera 1 Coleoptera, 18 Hymenoptera and 2 Hemiptera), an Arachnida (*Parasteatoda tepidariorum*) and a Xiphosurida (*Limulus polyphemus*).

Similarly to what found in Drosophilidae, a single *aralar1* gene has been detected in the genome of all these species. The complete list of the identified *aralar1* genes and the accession numbers in which they have been found in the respective genomes is shown in Supplementary Table 1. Variations in the exon/intron organization of the investigated genes are reported in Supplementary Table 6. Comparison of all the investigated genes, in particular those in insects and Arachnida (*Parasteatoda tepidariorum*), as well as in Xiphosura (*Limulus polyphemus*), suggests the presence of a common ancestor before Arthropods' divergence. Intron loss seemingly occurred in Diptera-Lepidoptera-Coleoptera, while intron gain suggests a lineage-specific process.

The existence of an internal exon, specific for a transcriptional isoform homologous to *D. melanogasteraralar1-RE,* can be inferred in a subset of the analyzed speciesincluding Hymenoptera (14 out of 18 species) and Diptera (6 out of 7 non-Drosophilid species), whereas we have not detected it in Coleoptera (one species) and Lepidoptera (two species), as well as in the evolutionary distant Hemiptera species.

In humans, the nearly identical exon/intron organization of *ARALAR* (*SLC25A12*) and *CITRIN* (*SLC25A13*) genes (19 and 21 coding exons, respectively)is consistent with a duplication of an ancestral gene, which was probably present in the genome of the Vertebrate's ancestor, due to the existence of two paralogue genes in the genome of extant vertebrate species so far sequenced (not shown). Alternatively spliced transcript variants have been reported for the human *ARALAR* gene (as provided by ac.nos NM_003705, NR_047549, XM_011512070). While the NM_003705 is translated into the Aralar1 protein (NP_003696), the functional roles of the predicted (XM_011512070) and the putatively non-coding (NR_047549) transcriptional isoformsare currently unknown. Comparisons of exon phase, exon length, and intron positions, suggest that Arthropod and human genes have a unique intron-rich ancestor predating the divergence of such lineages. Since position conservation in humansand in numerous Arthropod lineages can be supposedin order to identify retained ancestral introns,we have deducedthat such common ancestor gene containedat least 15 introns.

3.4. Expression pattern of *Drosophila melanogasteraralar1* gene

The developmental expression pattern of *aralar1* in *D. melanogaster* is widely described in FlyBase, which reports the modENCODE mRNA-Seq temporal expression data (mRNA-Seq_U, [89]. Relative data are summarized in Figure 3. In order to integrate these data with the expression pattern of *aralar1* transcriptional isoforms, we have carried out a Reverse Transcriptase-PCR analysis (RT-PCR) on total RNA samples isolated from distinct Drosophila developmental stages (Figure 4A) or from dissected adult tissues (Figure 4B).

With this aim, we have devised a set of primers specific to easily discriminate the five reported isoforms. Unfortunately, we were not able to find oligos suitable for performing quantitative analysis, so we could only conduct semiquantitative analyses based on RT-PCR. However, RA/RF and RD, cannot be distinguished, due to their nearly identical sequence, as described above. We will refer to *aralar1-RA* to indicate the RA/RD/RF isoforms through this paragraph.

The obtained results have revealed a constitutive expression of *aralar1-RA* transcript throughout development (Figure 4A). By contrast, the expression of the other isoforms is patchy distributed during the development or in different tissues. *Aralar1-RB* isoforms detected in early embryo development, during all the pupal stages and in adults, being only limited to females. Finally, *aralar1-RC*is detected in embryos (at least until 12-15 hours after egg laying), in all the pupal stages and in both sexes during the adult stage, while *aralar1-RE* is expressed in the early-embryo development and in both sexes during the adult stage. Consistent with the expression pattern detected in whole adult males and females, we have found that *aralar1-RA*, *aralar1-RC* and *aralar1-RE* are ubiquitously expressed in all the main fruit fly body regions (namely head, thorax and abdomen), whereas *aralar1-RB* expression is limited to the abdomens of females, due to its ovary-specific expression (Figure 4B).

3.5. Bacterial expression and functional characterisation of recombinant Aralar1 proteins

A ClustalW alignment of Drosophila Aralar1 with the human AGC isoforms has revealed that all the proteins conserve the same domain structure organization consisting of an N-terminal domain with 8 EF-hands, a carrier domain and a C-terminal domain with unknown function (Figure 5) [23]. Five out of the six Drosophila Aralar1 protein isoforms do not present any difference in their N-terminal EF-hands and carrier domains (Figure 5). Aralar1-PE diverges from the other isoforms because of an insertion of 12 additional amino acid residues in the loop of its eighth EF-hand (EF-hand 8) [23]. In order to check if this peculiarity may affect its transport activity, we have chosen Aralar1-PE to be biochemically characterized together with Aralar1-PA that also shows a shorter N-terminus similar to that found in Aralar1-PD and Aralar1-PF (Figure 5).

Aralar1-PA and Aralar1-PE have been overexpressed at high levels in *E. coli* BL21(DE3) (Figure 6A, lane 3 for Aralar1-PA, lane 4 for Aralar1-PE). They accumulate as inclusion bodies and have been purified by centrifugation on sucrose gradient and washing (Figure 6A, lane 5 for Aralar1-PA, lane 6 for Aralar1-PE), with a yield of 50 – 60 mg/ml bacterial culture. These proteins have been not revealed in bacterial cells harvested just before the induction of expression (Figure 6A, lane 1 for Aralar1-PA, lane 2 for Aralar1-PE), or in cells harvested after induction but do not carrying the coding sequence for Aralar1-PA and Aralar1-PE in the expression vector (data not shown). Their

identities have been assessed by western blot analysis employing a mouse anti-V5 monoclonal antibody (Figure 6A, lanes 1-6).

Aralar1-PA and Aralar1-PE have been functionally reconstituted into liposomes and the orientation of their N- and C-terminal regions in the proteoliposomes was performed by ELISA assay, indicating that both termini of Aralar1 proteins reconstituted into the proteoliposomal membrane protrude toward the outside, which in intact proteoliposomes is the only side of the membrane accessible to antibodies (Supplementary Figure 2). Aralar1-PA and Aralar1-PE transport activity have been tested in homo-exchange experiments (same substrate inside and outside). Using external and internal substrate concentrations of 1 and 10 mM, respectively, both proteins efficiently catalyze [^{14}C]glutamate/glutamate and [^{14}C]aspartate/aspartate exchange reactions. A very active uptake of [^{14}C]glutamate and [^{14}C]aspartate has also been observed when proteoliposomes had been preloaded with 10 mM aspartate or glutamate, respectively (hetero-exchange reactions). Both proteins do not catalyze any homo-exchange of phosphate, ADP, ATP, malonate, malate, oxoglutarate, ketoisocaproate, citrate, carnitine, ornithine, lysine, arginine, glutathione, choline, proline, and threonine (data not shown). No [^{14}C]glutamate/aspartate exchange has been observed using boiled Aralar1-PA and Aralar1-PE for incorporation into liposomes or reconstituting sarcosyl-solubilized bacterial material deriving either from cells not carryingany expression vector for Aralar1-PA and Aralar1-PEor from cells collected just before the induction of expression.

We have displayed kinetics of Aralar1-PA and Aralar1-PE uptake in Figure 6B. Each protein reconstituted into proteoliposomes contains 10 mM internal glutamate, and uptake has been measured in the presence of external 0.5 mM [^{14}C]glutamate (exchange). Exchange reactions follow first-order kinetics,and isotopic equilibrium is approached exponentially, maximum uptake is reached after 90 min (rate constants 0.066 and 0.080 min^{-1} and initial rates of 20.78 and 59.51 nmol/min x mg protein for Aralar1-PA and Aralar1-PE, respectively). Conversely, no [^{14}C]glutamate uptake is observed in the absence of an internal substrate (uniport), highlighting that both proteins are unable to catalyze a unidirectional transport of glutamate (Figure 6B). After 50 min incubation, when radioactive uptake by proteoliposomes has approached equilibrium, the addition of 20 mM unlabeled aspartate leads to an extensive efflux of radiolabeled glutamate from glutamate-loaded proteoliposomes (Figure 6B). A similar efflux of labeled substrate is observed by adding 20 mM unlabeled glutamate (not shown). This efflux further confirms the strict exchange mechanism catalyzed by Aralar1-PA and Aralar1-PE. The unidirectional transporthas been further investigated in backward experiment, as it provides a more convenient assay [53], i.e., by measuring the efflux of [^{14}C]glutamate from prelabeled active proteoliposomes; in the absence of an external substrate no efflux of [^{14}C]glutamate has been observed, even after 60 minutes of

incubation,but an extensive efflux has occurred upon the addition of 10 mM external glutamate or aspartate (data not shown).

Substrate specificity of recombinant Aralar1-PA and Aralar1-PE has been examined in detail by measuring the uptake of [^{14}C]glutamate into proteoliposomes preloaded with different possible substrates. Table I reports the results of our explorative statistical analysis. Both proteins efficiently exchange external [^{14}C]glutamate for internal L-glutamate or L-aspartate. Aralar1-PA also catalyzes a lower but significant uptake of [^{14}C]glutamate in the presence of internal L-cysteinesulfinate, whereas no transport activity has been found with L-α-aminoadipate, L-glutamine and L-asparagine (Table I). Differently from Aralar1-PA and yeast AGC [90], but similarly to the human AGC isoforms [4], Aralar1-PE exchanges external [^{14}C]glutamate for internal cysteinesulfinate at the same rate as aspartate, and it shows a significantly high [^{14}C]glutamate uptake in the presence of internal L-α-aminoadipate and L-glutamine. Both isoforms are virtually unable to exchange [^{14}C]glutamate for internal D-aspartate and D-glutamate, indicating a high degree of stereo specificity.

The [^{14}C]glutamate/glutamate exchange reactions catalized by Aralar1-PA and Aralar1-PE are hampered by many well-known inhibitors of several MCF members, being completely inhibited by 50 μM *p*-chloromercuribenzoate [4] and 1 mM pyrocarbonate [90] (100 and 96% inhibition, respectively for both proteins) and, to a slightly lesser extent by 50 μM mersalyl [76] and 0.1% tannic acid [53] (80 and 85% inhibition, respectively for both proteins), and also by 1 mM *N*-ethylmaleimide [53] (40 and 50% inhibitionfor Aralar1-PA and Aralar1-PE, respectively). Moreover, 10 mM bathophenanthroline or 0.1 mM bromocresol purple [53] are unable to exert a significant inhibition. Additionally, no inhibition has been observed with 10 μM bongkrekic acid [68]and 2.5 mM 1,2,3-benzenetricarboxylate [81], which are specific inhibitors of the mitochondrial ADP/ATP and citrate carrier, respectively.

Considering that the AGC-catalyzed aspartate/glutamate antiport reaction is electrogenic [4], the effect of membrane potential has been examined on the aspartate/glutamate exchange reaction mediated by our Aralar1 carriers. A K^+diffusion potential has been created across the membrane of proteoliposomes using valinomycin/KCl (calculated value ∼100 mV, positive inside). In such conditions, the rates of the [^{14}C]aspartate$_{out}$/glutamate$_{in}$exchanges of Aralar1-PA and Aralar1-PE are stimulated (Table II). In the absence of a membrane potential or when homo-exchanges have been measured, no effect has been observed. As a consequence, Aralar1-PA and Aralar1-PE are able to catalyze an electrogenic exchange of aspartate for glutamate.

We have determined the kinetic constants of the recombinant purified Aralar1-PA and Aralar1-PEby measuring the initial transport rate at various external [^{14}C]glutamate or [^{14}C]aspartate

concentrations, in the presence of a constant internal saturating concentration (10 mM) of unlabeled substrates. In five independent experiments carried out at the same internal and external pH (6.5), the half-saturation constants (K_m) of reconstitutedAralar1-PA and Aralar1-PE for glutamate are 0.26 ± 0.03 and 0.29 ± 0.07 mM, respectively, and for aspartate are 47 ± 2.3 and 51 ± 2.6 μM, respectively.Interestingly, the two isoforms significantly differ in their V_{max} values, which are 32.25 ± 1.69 and 94.29 ± 5.40 nmol/min/mg protein, for Aralar1-PA and Aralar1-PE, respectively for both substrates. The activity has been normalized by determining the amount of recombinant proteins recovered into proteoliposomes after reconstitution. We have further analyzed whether the differences in transport rates (Table I) and Vmax values found between the two isoforms could arise from the 12 additional amino acid residues present in the loop of the helix-loop-helix structure of the eighth EF-hand (EF-hand 8) of Aralar1-PE. First of all, we have checked, in de-ionized ultrapure water, the effect of Ca^{2+} or EGTA (a divalent ion chelator) on Aralar1-PA and Aralar1-PE transport activity (Figure 6C). The activities of both isoforms are increased of about 35% by the addition of 1 mM $CaCl_2$ and diminished by the addition of 0.5 mM EGTA. The inhibitory effect of EGTA is abolished in the presence of 1 mM $CaCl_2$ (Figure 6C). These results indicate that both isoforms are highly sensitive to calcium, since low calcium contamination in the reconstitution mixture is able to activate transport activity of both isoforms, whereas its removal by a Ca^{2+} chelator exerts an inhibitory effect. The Ca^{2+}-dependence of the transport activities of Aralar1-PA and Aralar1-PE has been further studied by measuring their transport rates as a function of the free Ca^{2+} concentration (Figure 6D). Both isoforms are highly Ca^{2+}-sensitive, since Aralar1-PA and Aralar1-PE have a half-maximal activation of about 0.34 and 0.15 μM, respectively.

3.6. Influence of the eighth EF-hand on the activity of ARALAR1 PE isoform

Once established that in our recostituted system calcium is able to regulate transport activity of both isoforms, we have checked if the shortening of the insertion consisting of 12 additional residues in Aralar1-PE or the replacement of some residues might affect its transport activity. In particular, the five positive charged residues (KRRRK from 301 to 305) have been replaced by consecutive alanine residues (5Ala mutant) or deleted (Δ5 mutant). Two further deletions have also been investigated, the first encompassing residues 301-308 (Δ8 mutant), and the second including residues 299-310 (Δ12mutant); in this latter mutant the insertion characterizing Aralar1-PE has been completely removed. Substrate specificity of recombinant Aralar1-PE mutants has been examined in detail by measuring the uptake of [14C]glutamate into proteoliposomes preloaded with different possible substrates. As reported in figure 7, the substitution of the five charged residues with Ala deeply increases transport activity of all the tested substrates, whereas its deletion

decreases tranport activity proportionally with the size of the deletion gradually leading Aralar1-PE to have the same substrate specificity of Aralar1-PA. The kinetic constants of the recombinant Aralar1-PE mutants are reported in Table III.The half-saturation constants (K_m) of reconstituted mutants do not vary with respect to the wild type, whereas as regards transport acitivity a significant change has been observed. In particular, 5Ala mutant shows a transport activity three-fold higher than that of the wild-type protein, whereas deletions negatively affect transport activity, which decreases proportionally with the size of the deletion. Interestingly, Δ12 mutant exhibits the same transport activity as Aralar1-PA.

3.7. Aralar1-PA and Aralar1-PE function as an AGC transporter in *S. cerevisiae*

The yeast *AGC1* null mutant does not grow on oleate even in rich medium [90]. This phenotype is explained since MAS is required for reduction of the cytosolic NADH derived from peroxisomal oleate oxidation [90]. Thus, the expression of a mitochondrial carrier protein able to exchange aspartate with glutamate should abolish the growth defect of the *agc1*Δ knockout. Aralar1-PA and Aralar1-PE expressed in *AGC1*null cells have fully restored growth of the *agc1*Δ strain on oleate, similarly to the yeast Agc1p (Figure 8), indicating that both Drosophila proteins function as AGC transporters. By contrast,in *agc1*Δ cells transformed with the empty vectorno growth rescue has been observed.

Discussion

By allowing the mitochondrial re-oxidation of the cytosolic NADH, the MAS plays a central role in the redox homeostasis and in different metabolisms such as glucose homeostasis and nitrogen metabolism. The MAS requires the concerted functioning of six proteins, two located in the cytosol, the malate dehydrogenase 1 (MDH1) and the aspartate aminotransferase 1 (GOT1), two located in the mitochondrial matrix, the malate dehydrogenase 2 (MDH2) and the aspartate aminotransferase 2 (GOT2), and the last two located in the inner mitochondrial membrane, the aspartate/glutamate and the oxoglutarate/malate (OGC) carriers, which connect MAS cytosolic reactions to the mitochondrial ones. Despite the central role in energetic metabolism, very little is known about MAS components in the fruit fly, and up to date no functional data are available on AGC and OGC. In mammals, there are two genes encoding AGC, *ARALAR*, also known as AGC1, more widely expressed but absent in the liver, and *CITRIN* also known as AGC2, expressed in fewer tissues but present in the liver [24]. By searching the FlyBase database (http://flybase.org/) for human AGC homologs, we have found 6 alternatively spliced-isoforms annotated as *aralar1* and none as citrin.

The expression profile of *aralar1* transcriptional variants studied in this work suggests that three isoforms (namely *aralar1-RA/RF* and *aralar1-RD*) are constitutively transcribed throughout the development and in adult tissues. Due to their overlapping sequences, it is not possible to discriminate the expression profile of these transcriptional isoforms, which are transcribed from the innermost promoter. The slight differences observed in their organization suggest that they might have a different stability (i.e. half-life of their mRNA molecules) due to the length of the 3' UTR (*aralar1-RD* and *aralar1-RA* isoforms). Similarly, the stability of Aralar1-PF protein, encoded by *aralar1*-RF transcript (identical to *aralar1-RA* transcript), could be affected by the presence of the additional 12 amino acids introduced upon the translational read-through process [86].

Conversely, *aralar1-RB*, *aralar1-RC* and *aralar1-RE* transcriptional isoforms have a more complex expression pattern, as described in the Results section. While *aralar1-RB* is undetectable by RT-PCR in adult males, it can be specifically detected in adult ovary, suggesting its role in oogenesis. We do not know whether *aralar1-RB* detected during the early embryo development is the result of a maternal contribution or of the active transcription in embryos, as instead appears evident for *aralar1-RC* and *aralar1-RE*, which are undetectable in female germline and are present in early embryo developmental stages. Furthermore, while *aralar1-RC* expression persists in the whole adult tissues with the exclusion of the germline tissues, *aralar1-RE* expression is confined in the early stages of embryo development and in somatic adult tissues. The expression of the more active *aralar1-RE* in the early embryogenesis might be related to the very active aspartate and glutamate metabolism occurring in this stage [91]. *Aralar1-RB* and *aralar1-RC*, together with the constitutively expressed isoforms, seem to be also important during metamorphosis. It is noteworthy that all the *aralar1* transcriptional isoforms contribute to the strong *aralar1* expression observed in adult fat bodies (mainly located in the abdomen), as can be inferred by the combination of our results (see Figure 4) and the modENCODE Tissue Expression Data. Fat bodies are important energy storages and utilization organs in Drosophila, as well as in other insects [92], and they play a fundamental role in the post-metamorphic energy metabolism and egg development. Taken together, these results suggest that an isoform-specific expression pattern becomes defined after the initial embryo development, when all the isoforms are transcribed, and its establishment appears to be important for certain developmental stages or in specific adult tissues.

It can be speculated that the observed transcriptional pattern could be related to the presence of two evolutionary conserved DNA motifs, located between the TSSs of *aralar1-RB* and *aralar1-RA*/*RF*/*RD* transcripts. Although we have not performed functional studies aimed to demonstrate their involvement in transcriptional regulation, it could be hypothesized that NRG elements might influence *aralar1* gene expression for two reasons. Firstly, its evolutionary conservation in 23

Drosophila species belonging to two distinct sub-genera indicates a constrained role. Indeed, intron sequences are less conserved, even in related species, and only functionally constrained motifs are maintained. The divergence between *D. melanogaster* and D. *grimshawi* is thought to have occurred 50Mya (Russo et al. 2003), and the conservation of such putative *cis*-acting sequence suggests a functional role. The NRG element can also be detected in non-Drosophilidae insects (Supplementary Table 6), thus enforcing its putative functional role, although its position related to the expressed transcriptional isoforms in these organisms is not easy to be determined.

Secondly, previous studies reported that NRG elements are conserved in a subset of Drosophila genes encoding mitochondrial proteins, while they have been lost in duplicated genes, which in turn have acquired testis-specific transcription patterns. Since *alarar1* gene lacks paralogues in *D. melanogaster* (and in all the analyzed Drosophila species), the presence of two NRG elements within it might have acquired a different role, such as the establishment of a regulated expression pattern of all, or at least some, of the transcriptional *aralar1*isoforms. In this view, the two NRG elements detected could act either cooperatively or independently, as positive or negative regulators. Whether or not the two NRG elements are functional to *aralar1* expression cannot be determined without the aid of mutant or transgenic strains carrying *aralar1* genes containing disrupted NRG elements, which necessarily calls for future analyses.

In order to verify if this complex tissue- and developmental stage-specific transcriptional regulation is associated to the expression of functionally different isoforms, the transport properties of Aralar1-PA and Aralar1-PE, representing the two main encoded products of all *aralar1* transcripts, have been determined. The two recombinant proteins, once reconstituted into liposomes, catalyze a very efficient aspartate/glutamate electrogenic exchange reaction and are able to restore the yeast defect of the *AGC1* null gene (Figure 8), confirming their identity as the *D. melanogaster* mitochondrial AGC. Similarly to the human isoforms [4], fruit fly Aralar1 isoforms do not show any significant difference in their substrate affinity, moreover, as the human isoforms, the Km value for aspartate was significantly lower than that for glutamate. Interestingly, although Aralar1-PA and Aralar1-PE differ only in their N-terminal halves, i.e., region known to interact with calcium [23], they showed a significant different specific activity, being Aralar1-PE more active than Aralar1-PA. A similar situation had been previously found in *H. sapiens*, where citrin resulted more active than aralar [4].

Although fruit fly and human AGC isoforms showed this overlapping behavior, it should be emphasized that the different specific activities found in the latter are mainly due to their different carrier domains [4], whereas all *D. melanogaster* AGC isoforms share the same carrier domain and differ in their N-terminal domains. In fact, the recombinant carrier domain reconstituted into liposomes, shows the same kinetic properties of Aralar1-PA (data not shown).

Interestingly, this N-terminal domain was also able to alter substrate specificity of the fruit fly AGC isoforms; since Aralar1-PE, similarly to human citrin [4], is able to exchange glutamate against cysteinesulfinate at the same rate as that of aspartate, while Aralar1-PA, similarly to human aralar, shows a glutamate/cysteinesulfinate exchange rate about one half lower than that of glutamate/aspartate. Furthermore, Aralar1-PE, differently from Aralar1-PA, significantly exchanges glutamate for L-α-aminoadipate and L-glutamine, suggesting a wider range of substrate specificity if compared to the latter.

A ClustalW alignment of Drosophila Aralar1 with human AGC isoforms has clarified that all proteins conserved the same structural organization including an N-terminal domain having 8 EF-hands with different functions (i.e. EF-hands 1-3 constitute a calcium-responsive unit, whereas EF-hands 4-8 are part of a static unit), a carrier domain and a C-terminal domain with unknown role (Figure 5) [23].Furthermore, calcium (EF-hands 1-3) and substrate binding sites are almost completely conserved between man and Drosophila, with a single conservative variation present in the calcium-binding site, where a threonine was replaced by a serine in the fruit fly isoforms (Figure 5). The most variable regions in the fruit fly Aralar1 isoforms are located in the N-terminus and into the loop of the helix-loop-helix structure of EF-hand 8 (Figure 5, green shaded) [23], where Aralar1-PE has an extra highly positive charged amino acid sequence. In the light of this observation, it is evident that N-terminus variations could play a fundamental role on the functional differences found between Aralar1-PA and Aralar1-PE. Site-direct mutagenesis experiments conducted on this additional region in EF-hand 8 have showed that the substitution of the charged residues by alanine can significantly affect substrate specificity and increase initial transport rate, whereas the progressive removal of these extra amino acids reduce initial transport rate and change substrate specificity until making Aralar1-PE similar to Aralar1-PA. Furthermore, this data are in agreement with the role proposed for the human AGC isoforms, in which the EF-hands 1–3 form a calcium-responsive mobile unit, EF-hands 4–8 form a static unit while the N-terminus region, external to the first EF-hand motif, seems not to be involved in any crucial regulatory function [23]. Finally, we have demonstrated that both proteins are Ca^{2+}-dependent, and similarly to citrin, Aralar1-PE shows a higher sensitivity to calcium being S0.5 about 2 times lower than that of Aralar1-PA. Hence, biochemical characterization and calcium sensitivity have identified Aralar1-PA and Aralar1-PE as the human aralar and citrin counterparts, respectively (Figure 6 C-D).

The functional characterization of the fruit fly mitochondrial AGC transporter represents a crucial step toward a complete understanding of the metabolic events acting during early embryogenesis, and it might strengthen the use of *D. melanogaster* as a model for genetics and developmental biology. Nevertheless, further studies of gain and loss of function are required to better understand

how the complex transcriptional and expression patterns of Aralar1 regulates specific metabolic pathways in the different developmental stages of the fruit fly.

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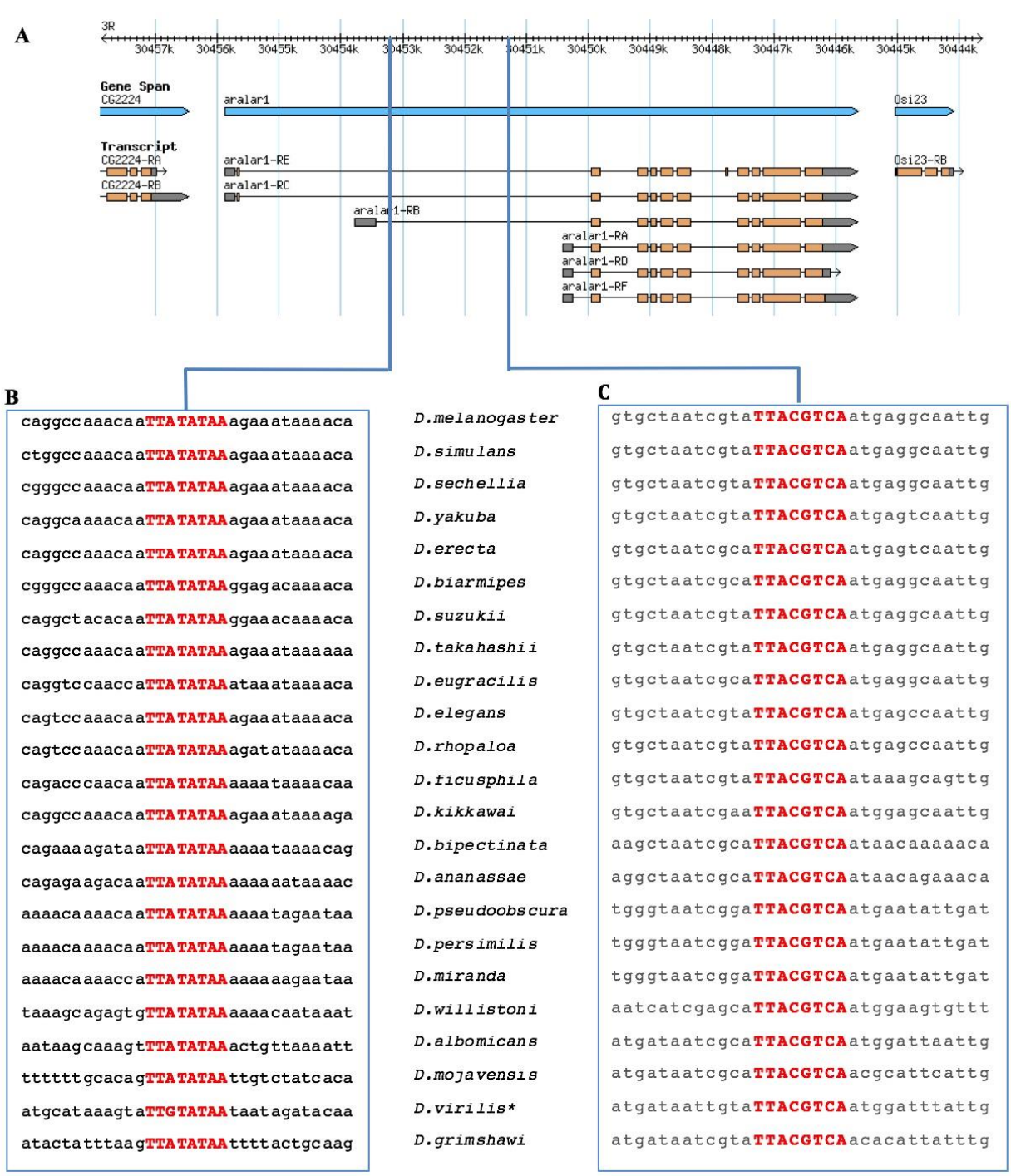


Figure 1 Chromosomal organization of the *aralar1* gene in *D. melanogaster*. A) Overall organization of *aralar1* locus and its map position in *D. melanogaster*. B) and C) multiple sequence alignments encompassing the two conserved NRG elements in the *aralar1* gene of 23 Drosophila species. * variant NRG motif in homologous position.

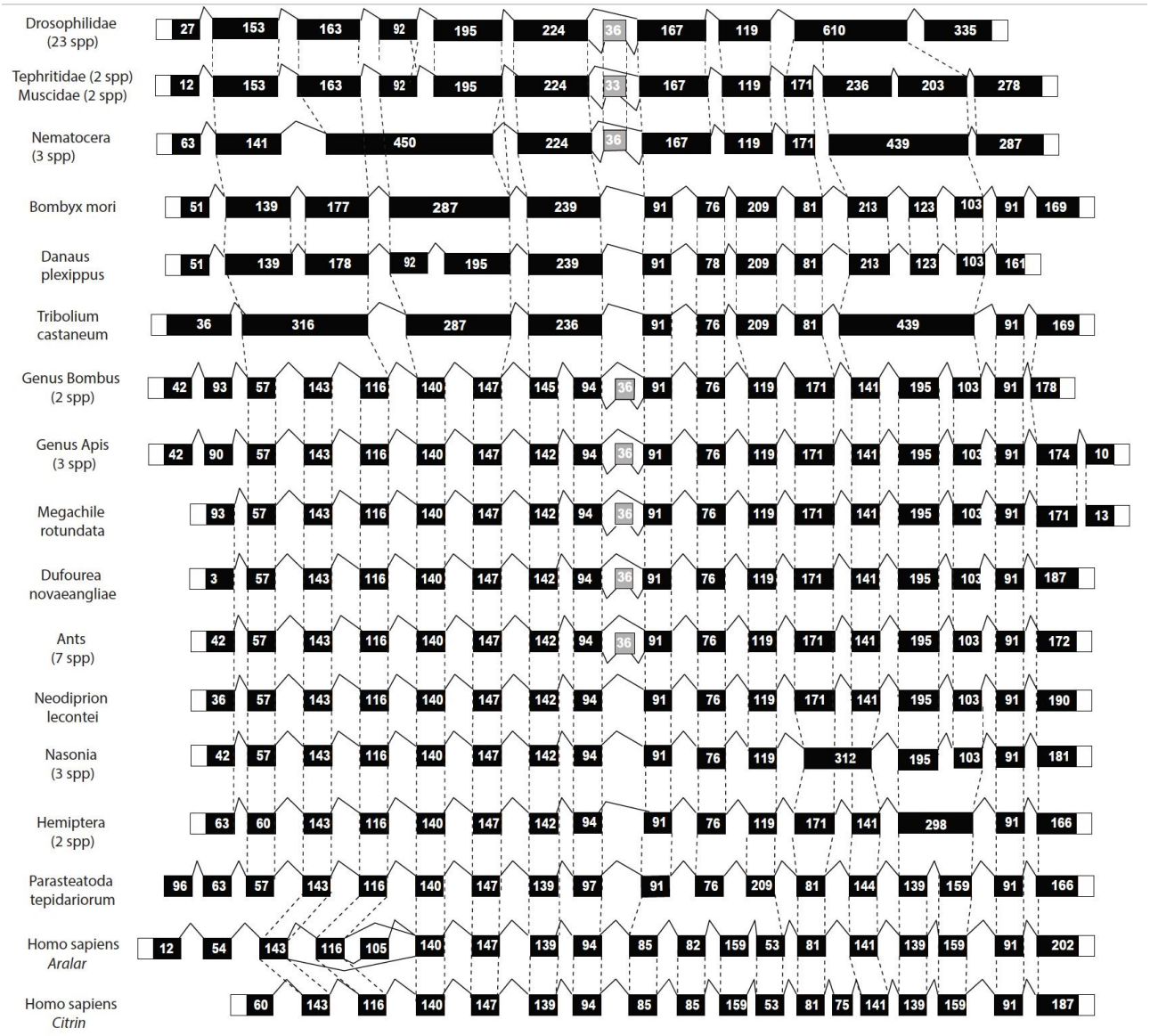


Figure 2. Eukaryotic AGC-encoding genes share an ancient intron-rich ancestor. The exon/intron structure of orthologous AGC-encoding genes of representative eukaryotic species is compared considering intron position, exon phase and length. Dashed lines indicate conservation of intron position. Translated AGC1-encoding regions are indicated in black, UTRs as white boxes. Internal *aralar1-RE* specific exons are indicated as gray boxes. Boxes are not in scale.

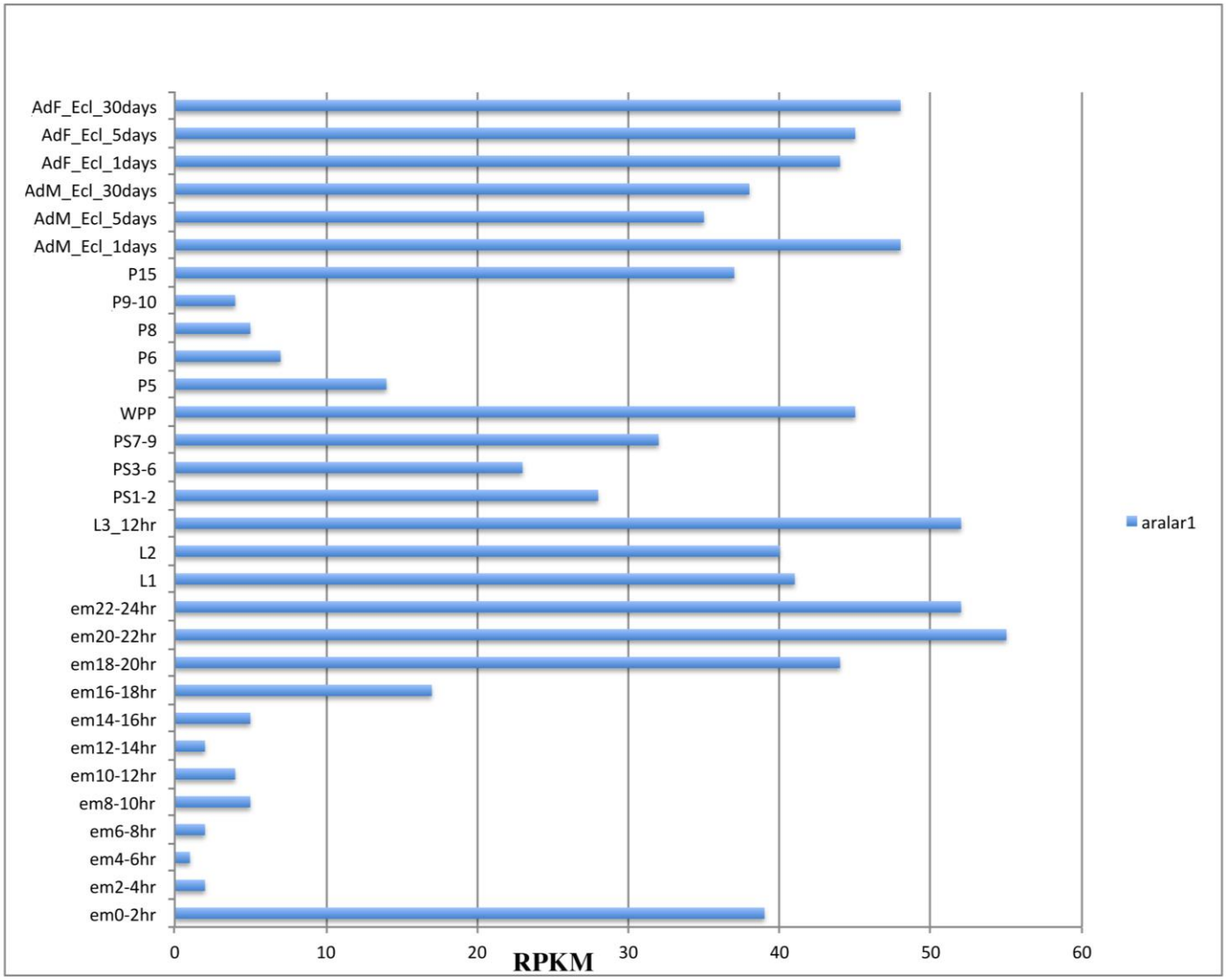


Figure 3. modEncode expression of *aralar1* through the development. Cumulative expression of all *aralar1* isoforms through the development is shown. Data were collected from modENCODE. Abbreviations legend. em: embryo stages; L: larval stages: P:pupae stages; AdM: adult males, various ages; AdF: adult females, various ages; RPKM: reads per kilobase million.

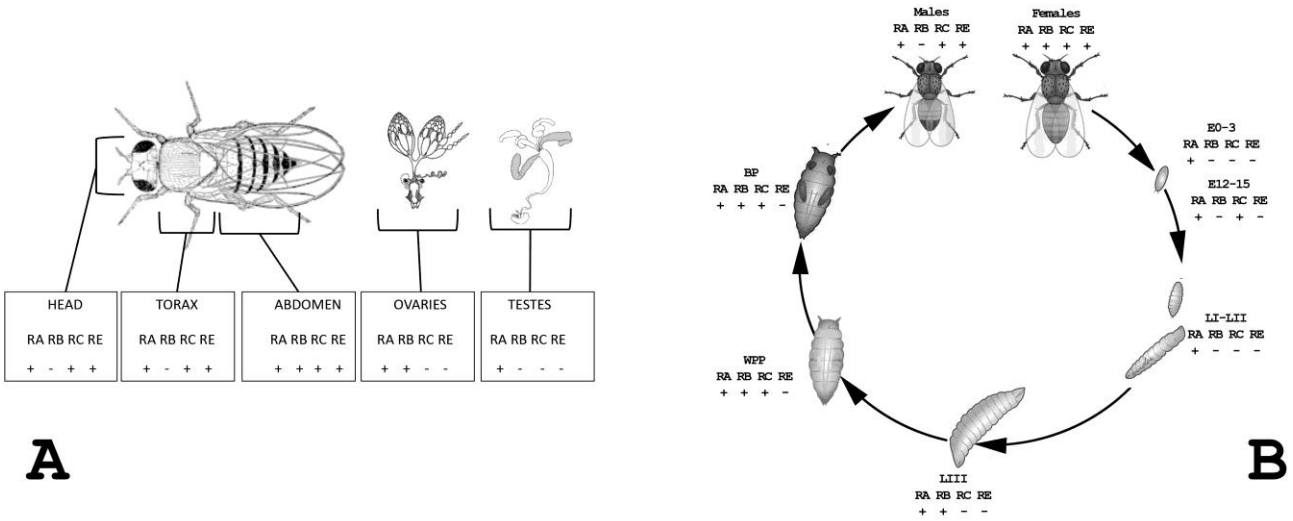


Figure 4. Transcriptional analysis of *aralar1* in Drosophila adult tissues.

The presence/absence of the indicated*aralar1* isoforms revealed by the detection of RT-PCR fragment in each analyzed RNA sample is indicated with "+" or "–", respectively.

Panel A. The impact of the four transcriptional isoforms of *aralar1* analyzed in adult heads, thoraces and abdomens, and in the reproductive organs of males and females of *D. melanogaster*.

Panel B. The presence/absence of four transcriptional isoforms of *aralar1* was analyzed by RT-PCR through development in the indicated stages. RNA samples were collected from embryos at two stages of embryonic development (0–3, 12–15 after egg lying), mixed first and second instar larvae (LI-LII), third instar larvae (LIII), white prepupae (WP), black pupae (BP), and adults (females and males).

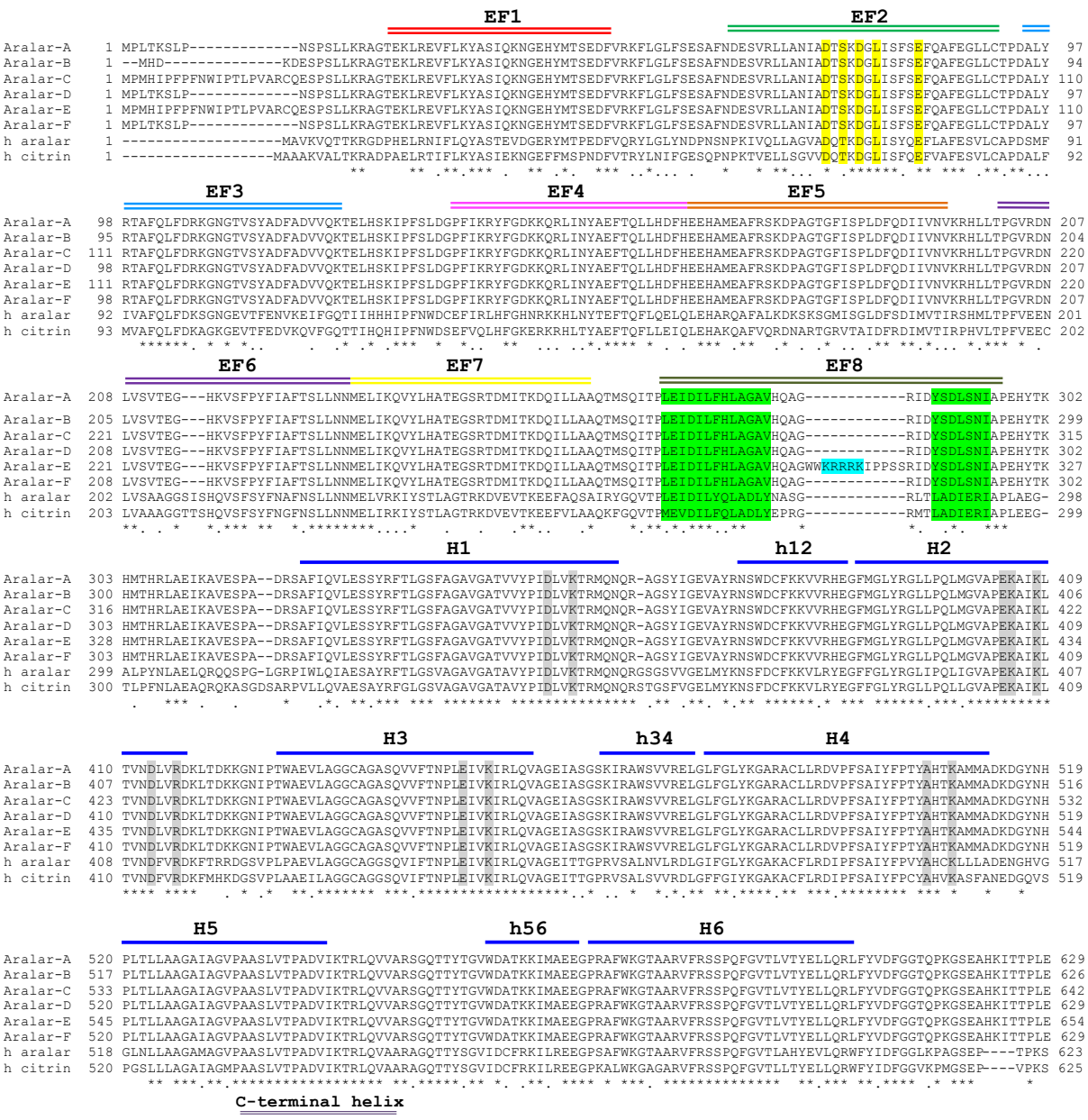


Figure 5. Alignment of Drosophila Aralar1-PA-PF and human aralar and citrin. Sequence conservation is indicated with an asterisk for identical residues, a dot for conserved substitutions, and a gap for non-conserved residues. The EF-hands, the six transmembrane helices H1-H6, the three small helices parallel to the membrane plane h12-h56, and the C-terminal α-helix are indicated by coloured bars and labels. The two α-helices of EF-hand 8 and the positive residues in

the loop of Aralar-1-PE are shaded in green and cyan, respectively. Residues that are involved in the coordination of calcium and the contact points of the substrate-binding site are shaded in yellow and gray, respectively. The alignment was obtained by using ClustalW.

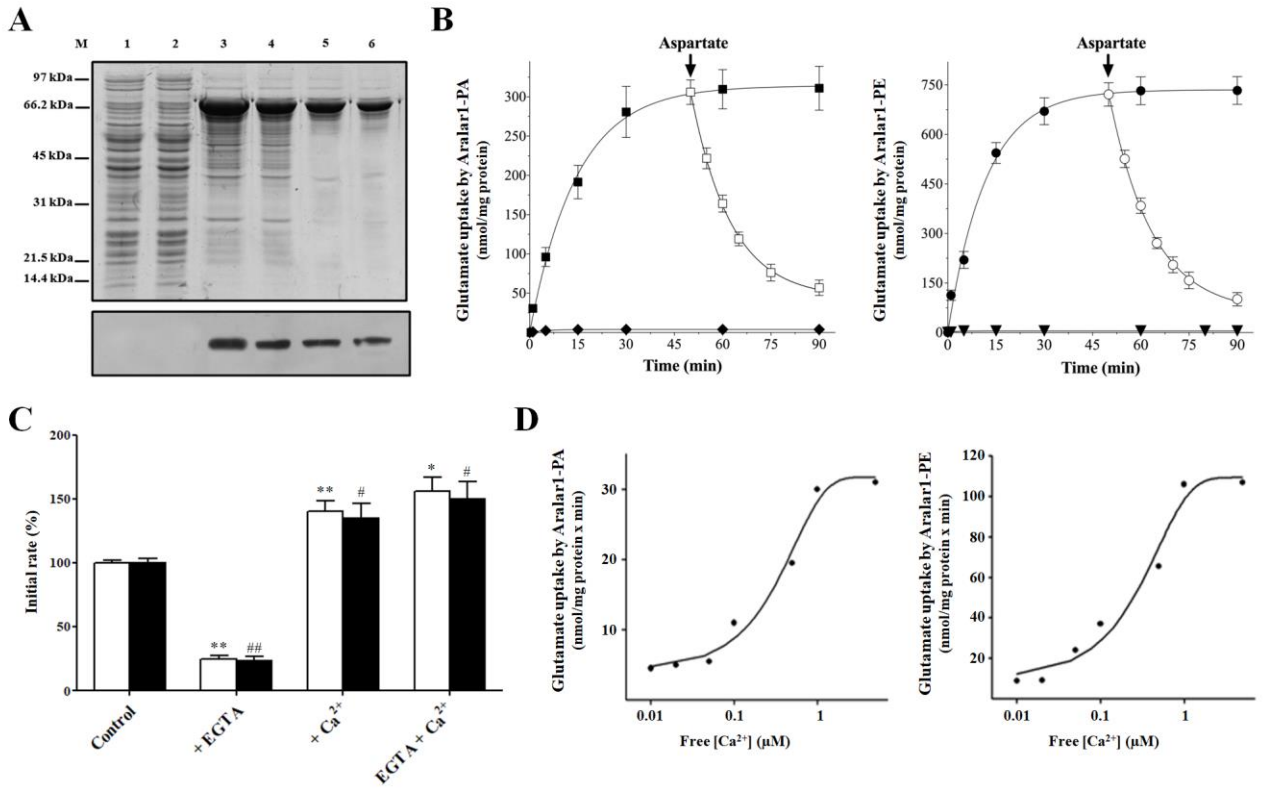


Figure 6. Bacterial expression and functionally characterization of Aralar1-PA and Aralar1-PE. (A) Expression in *Escherichia coli* of recombinant Aralar1. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye or transferred to nitrocellulose and immunodetected with an anti-V5 monoclonal antibody. Lane M, markers; lanes 1-6, *E. coli* BL21(DE3) containing the expression vector with the coding sequence for Aralar1-PA (lanes 1, 3 and 5) and Aralar1-PE (lanes 2, 4 and 6). Samples were taken at the time of the induction (lanes 1 and 2) and 4 h later (lanes 3 and 4). Lanes 5 and 6, Aralar1-PA and Aralar1-PE originating from bacteria shown in lanes 3 and 4, respectively, purified by centrifugation on sucrose gradient. The same number of bacteria was analyzed in each sample. **(B) Kinetics of [^{14}C]glutamate/glutamate exchange by Aralar1-PA and Aralar1-PE.** Proteoliposomes were reconstituted with Aralar1-PA and Aralar1-PE. 0.5 mM [^{14}C]glutamate was added to proteoliposomes containing 10 mM glutamate (■ and ●) or 10 mM NaCl (◆ and ▼). When the uptake approached equilibrium (50 min), 20 mM of aspartate was added outside the proteoliposomes (□ and ○). Similar results were obtained in four independent experiments. **(C-D) Effect of Ca^{2+} on transport activities of Aralar1-PA and Aralar1-PE. (C)** Recombinant Aralar1-PA (open bars) and Aralar1-PE (filled bars), were reconstituted in proteoliposomes, the [^{14}C]glutamate/glutamate transport rates were measured in de-ionized ultrapure water without additions (Control) or with the addition of 0.5 mM EGTA, or 1 mM $CaCl_2$, or 0.5 mM EGTA in the presence of 1 mM $CaCl_2$. Uptake rates of glutamate/glutamate exchange were measured at 60 s. Results are expressed as a percentage of the Aralar1-PA and Aralar1-PE values, which were on average 35.31 ± 1.24 and 110.58 ± 3.76 nmol/min x mg of protein, respectively. The average transport rates were calculated from the average of at least five independent experiments and displayed with the standard error. Different symbols indicate statistical differences, when occurring, between means of values as indicated by ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Aralar1-PA control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Aralar1-PE control). **(D)** Dependence of Aralar1-PA and Aralar1-PE activities on the free Ca^{2+} concentration. The [^{14}C] glutamate transport rates were measured in de-ionized ultrapure water in the presence of 0.5 mM EGTA and various concentrations of Ca^{2+}. Similar results were obtained in at least three independent experiments.

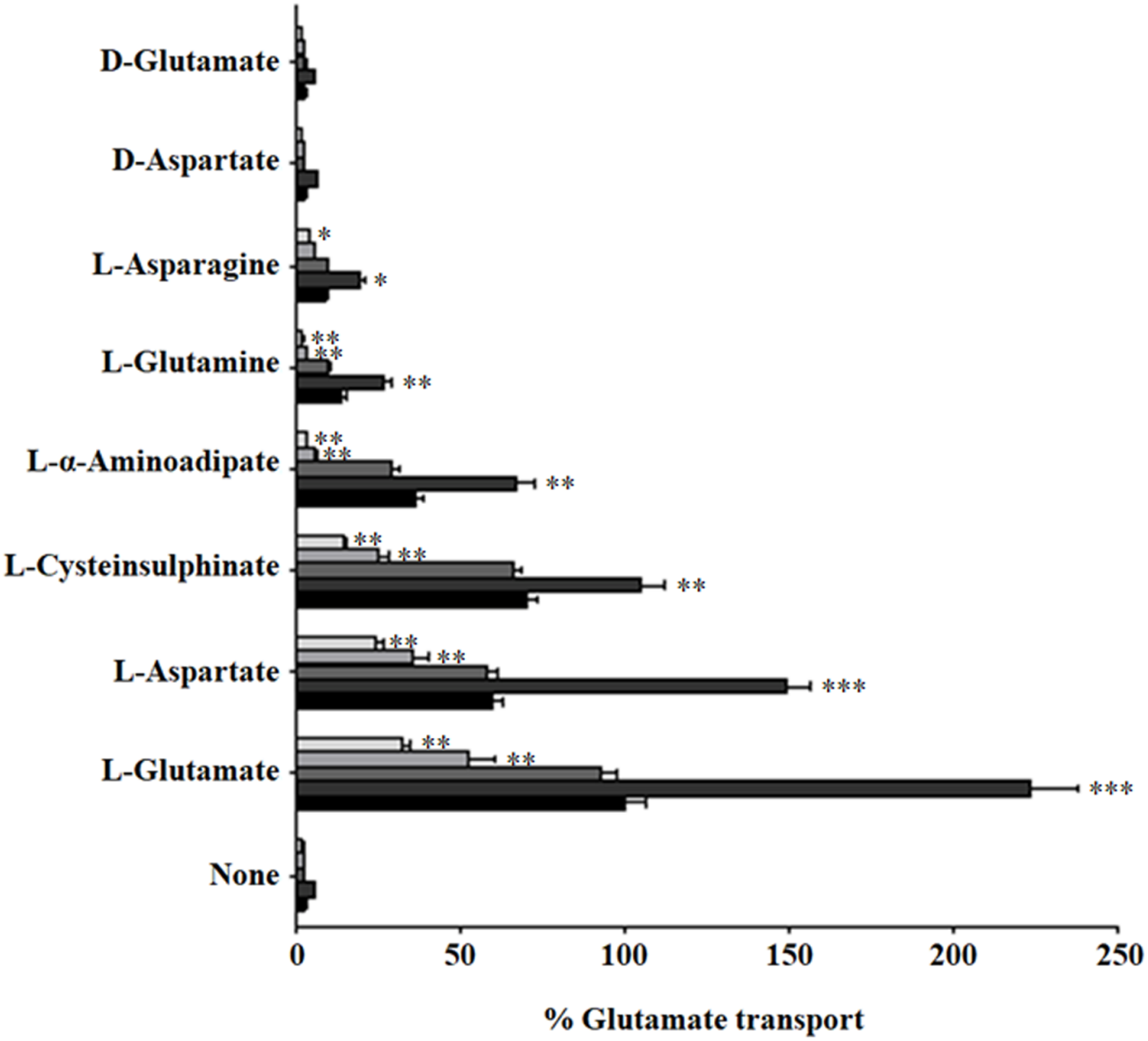


Figure 7. Dependence on internal substrate of the transport properties of proteoliposomes reconstituted with recombinant Aralar1-PE, 5ALA, Δ5-Aralar1-PE, Δ8-Aralar1-PE and Δ12-Aralar1-PE mutants. Proteoliposomes reconstituted with Aralar1-PE (black bars), 5ALA (very dark grey bars), Δ5-Aralar1-PE (dark grey bars), Δ8-Aralar1-PE (light grey bars) and Δ12-Aralar1-PE (very light grey bars) were preloaded internally with various substrates (concentration 10 mM). Transport was started by adding 0.5 mM [^{14}C]glutamate to proteoliposomes and terminated after 1 min. Data represent the mean of three independent experiments reported as the percent of glutamate transport with respect to the rate of [^{14}C]glutamate/glutamate exchange by Aralar1-PE (110 nmol/min/mg protein) setting at 100%. Asterisks indicate the level of statistical significance calculated comparing Aralar1-PE transport activity with that of its mutants in presence of each internal substrate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, using ANOVA.

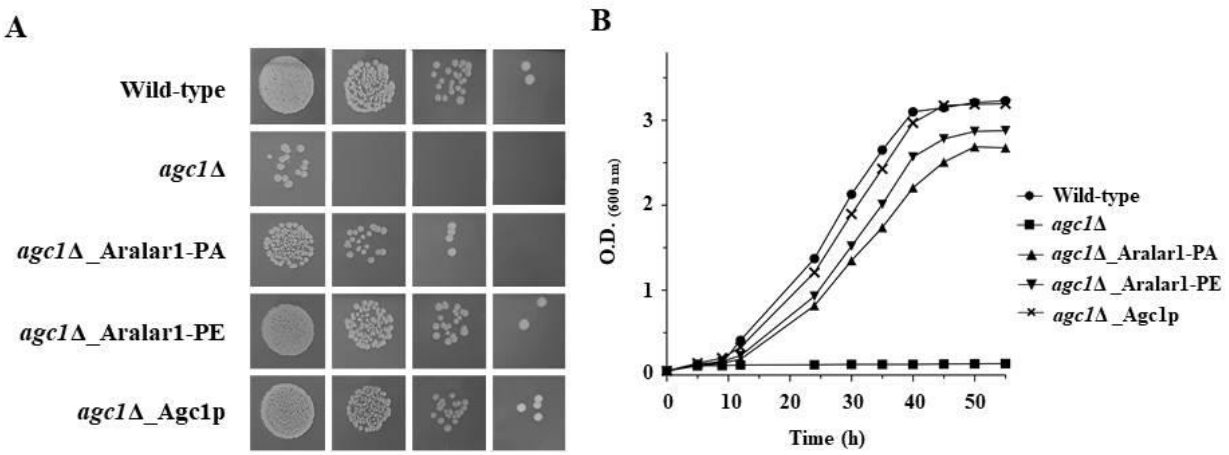


Figure 8. Complementation of the growth defect of *agc1*Δ yeast strain by Aralar1-PA and Aralar1-PE. Wild-type, *agc1*Δ cells transformed with the empty vector (*agc1*Δ) and *agc1*Δ cells expressing drosophila Aralar1-PA, Aralar1-PE and yeast Agc1p were plated by using a four-fold serial dilution (A) or inoculated (B) in YP medium supplemented with 0.5 mM oleate. Plates and cell cultures were placed at 30°C. Picture given in *A* was taken after 3 days to show yeast growth performance, whereas the optical density (*O.D.*) values at 600 nm given in *B* refer to cell cultures after the indicated growth times.

TABLE I

Dependence on internal substrate of the transport properties of proteoliposomes reconstituted with recombinant Aralar1-PA andAralar1-PE.

Proteoliposomes were preloaded internally with various substrates (concentration 10 mM). Transport was started by adding 0.5 mM [^{14}C]glutamate to proteoliposomes reconstituted with Aralar1-PAandAralar1-PE, respectively, and terminated after 1 min. For each internal substrates four indipendent experiments have been carried out. The means and the standard deviations of Aralar1-PA and Aralar1-PE for each substrate are reported. Different symbols indicate statistical differences between means of values as indicated by ANOVA ($^{*}P \leq 0.05$, $^{***}P \leq 0.001$ *vs* uniport reaction by Aralar1-PA, $^{\#}P \leq 0.05$,$^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* uniport reaction by Aralar1-PE).

Internal substrate	**[^{14}C]Glutamate transport**	
	Aralar1-PA	**Aralar1-PE**
	nmol/min x mg of protein	
None (Cl^- present)	1.63 ± 0.11	2.85 ± 0.19
L-glutamate	31.27 ± 1.31***	105.5 ± 6.89$^{\#\#\#}$
L-aspartate	23.14 ± 1.21***	63.34 ± 2.78$^{\#\#\#}$
L-cysteinesulfinate	13.76 ± 0.78***	73.91 ± 3.23$^{\#\#\#}$
L-α-aminoadipate	2.34 ± 0.13*	38.05 ± 2.98$^{\#\#\#}$
L-glutamine	1.84 ± 0.16	14.68 ± 1.24$^{\#\#\#}$
L-asparagine	3.63 ± 0.27*	9.51 ± 1.02$^{\#}$
D-aspartate	1.65 ± 0.10	2.99 ± 0.22
D-glutamate	1.34 ± 0.12	2.77 ± 0.25

TABLE II

Influence of the membrane potential on the activity of recombinant Aralar1-PA and Aralar1-PE. The exchange was started by the addition of 50 μM [^{14}C]aspartate to proteoliposomes containing 10 mM of the indicated internal substrate. K^+_{in} was included as KCl in the reconstitution mixture, whereas K^+_{out} was added as KCl together with the labeled substrate. The differences in osmolarity were compensated for by the addition of appropriate concentrations of sucrose in the opposite compartment. Valinomycin (1.0 μg/mg phospholipid) was added in 10 μl ethanol/ml of proteoliposomes (+ valinomycin). In the samples without valinomycin (– valinomycin) the only ethanol was added. The exchange reactions were stopped after 1 min. Similar results were obtained in three independent experiments. Asterisks indicate values that are significantly different from those obtained in the same experiment but in absence of valinomycin (**$P \leq 0.01$, using Student's *t*-test)

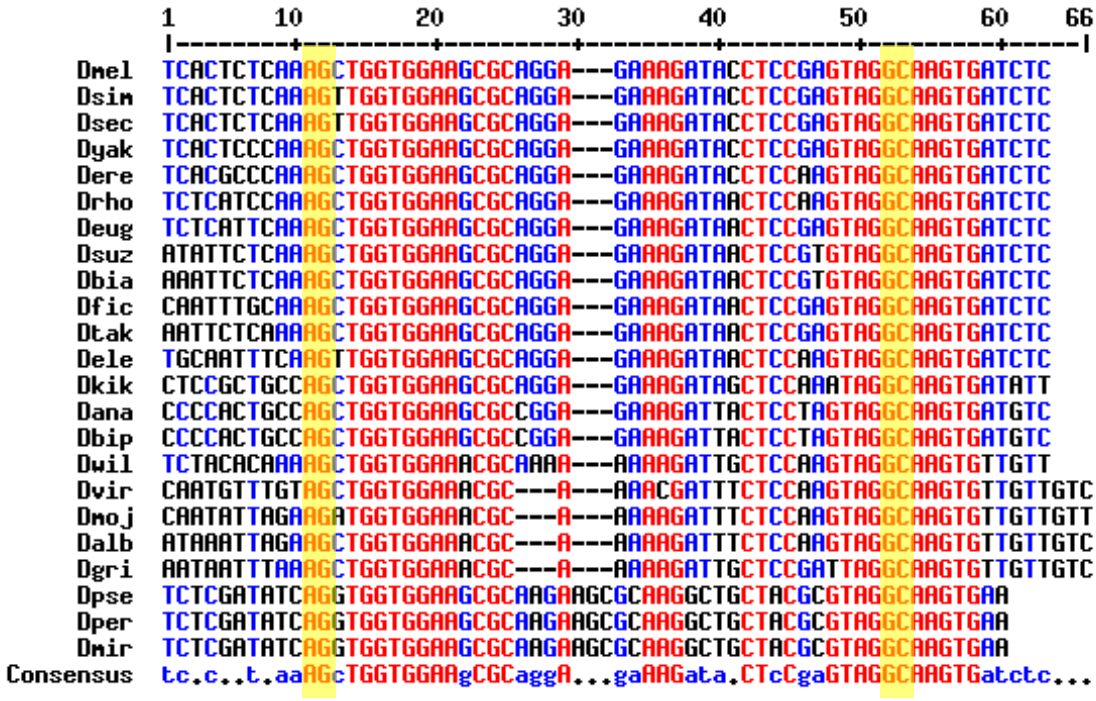
Internal substrate	**K^+_{in}/k^+_{out} (mM/mM)**	**[^{14}C]Aspartate uptake** (*nmol/min x mg protein*)			
		Aralar1-PA		**Aralar1-PE**	
		- valinomycin	**+ valinomycin**	**- valinomycin**	**+ valinomycin**
Aspartate	1/1	28.75 ± 2.11	30.05 ± 2.96	100.75 ± 8.55	102.21 ± 8.40
	1/50	27.37 ± 2.41	28.64 ± 2.05	103.25 ± 7.20	103.89 ± 9.10
Glutamate	1/1	21.52 ± 1.70	22.24 ± 1.45	76.33 ± 5.88	77.14 ± 6.39
	1/50	23.95 ± 2.35	57.48 ± 5.74**	75.68 ± 6.28	154.86 ± 7.50**

TABLE III

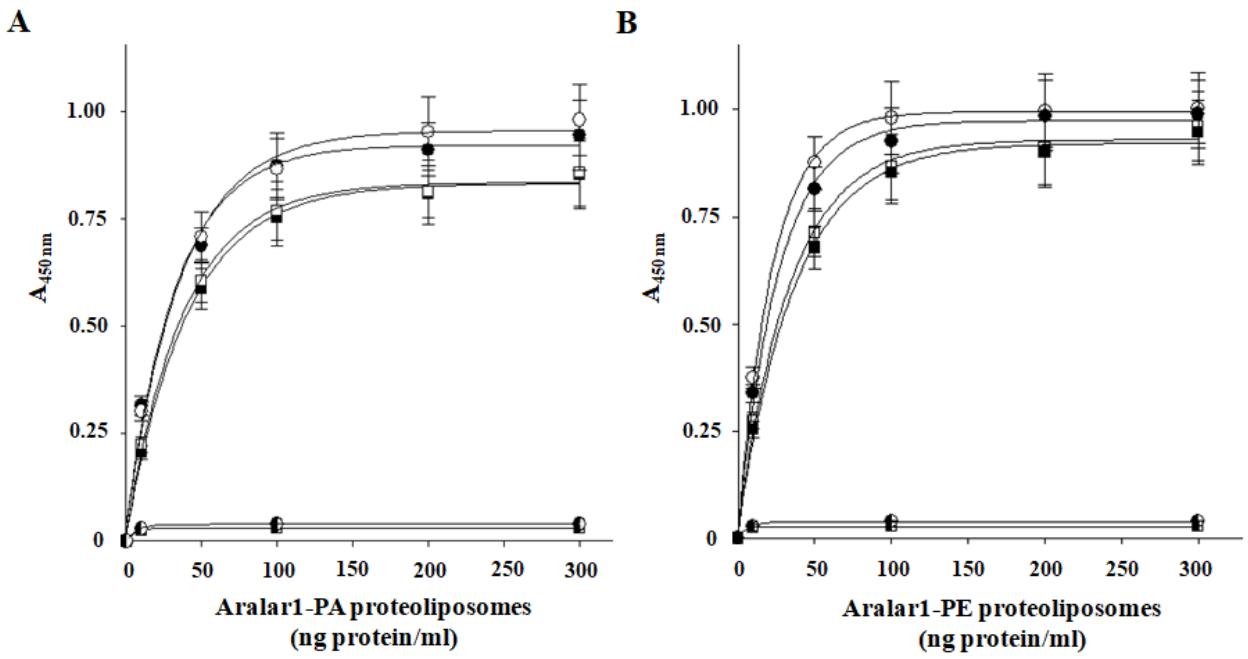
Kinetic constants of recombinant Aralar1-PA, Aralar1-PE, 5ALA, Δ5-Aralar1-PE, Δ8-Aralar1-PE *and* Δ12-Aralar1-PE *mutants*.

The values were calculated from double reciprocal plots of the rate of [^{14}C]glutamate uptake versus substrate concentrations. Transport was started by adding 125-750 μM [^{14}C]glutamate to proteoliposomes containing 10 mM glutamate, and terminated after 1 min. Similar results were obtained in at least three independent experiments.

Carrier	**K_m** (*mM*)	**V_{max}** (*nmol/min x mg protein*)
Aralar1-PA	0.26 ± 0.03	34.60 ± 2.11
Aralar1-PE	0.29 ± 0.07	92.08 ± 4.96
5ALA	0.31 ± 0.062	189.21 ± 10.50
Δ5-Aralar1-PE	0.33 ± 0.075	82.22 ± 7.55
Δ8-Aralar1-PE	0.31 ± 0.072	58.75 ± 5.10
Δ12-Aralar1-PE	0.30 ±0.055	37.32 ± 3.15



Supplementary Figure 1. Multiple alignment of the aralar1-RE specific exon. the conservation of the acceptor (AG) and donor splice sites (GC) respectively of the upstream and downstream flanking introns are highlighted in yellow.



Supplementary Figure 2. Reactivity of the anti-N-terminal amino acids 35-177 of Aralar1 and anti-V5 epitope tag antisera to the reconstituted Aralar1 proteins assessed by ELISA. Reactivity was evaluated for both recombinant Aralar1-PA (A) and Aralar1-PE (B) isoforms by using either the anti-N-terminal peptide antiserum direct against 35-177 amino acids of Aralar1 proteins (●, ○ and ◐), or the anti-V5 antibody direct against the V5 epitope placed at their C-termini (■, □ and ◧). Microtiter plates were coated with the indicated amounts of intact (● and ■) or permeabilized (○ and □) proteoliposomes. In (◐ and ◧), each antibody, in presence of only not reconstituted liposomes, was used as a negative control.

Supplementary Table S1. List of the *aralar1* genes investigated in this study with their positions in the respective genome.

Species	Accession number	RA-RD-RF transcript Coding exons	RB Coding exons	RC(RE) Coding exons
Drosophila melanogaster	FBgn0028646	10	11	12(13)
Drosophila simulans	JMCE01000007.1	10	11	12(13)
Drosophila sechellia	AAKO01001326.1	10	11	12(13)
Drosophila yakuba	AAEU02000259	10	11	12(13)
Drosophila erecta	AAPQ01006792	10	11	12(13)
Drosophila ficusphila	AFFG02008687	10	11	12(13)
Drosophila eugracilis	AFPQ02005951	10	11	12(13)
Drosophila biarmipes	AFFD02006539.1	10	11	12(13)
Drosophila takahashii	AFFI02007371	10	11	12(13)
Drosophila suzukii	CAKG01025788	10	11	12(13)
Drosophila elegans	AFFF02008080	10	11	12(13)
Drosophila rhopaloa	AFPP02032380	10	11	12(13)
Drosophila kikkawai	AFFH02006507	10	11	12(13)
Drosophila bipectinata	AFFE02007958	10	11	12(13)
Drosophila ananassae	AAPP01015636	10	11	12(13)
Drosophila miranda	JXPA01005888	10	11	12(13)
Drosophila pseudoobscura	AAFS01000152	10	11	12(13)
Drosophila persimilis	AAIZ01003247	10	11	12(13)
Drosophila willistoni	AAQB01009418	10	11	12(13)
Drosophila albomicans	JXOX01003054	10	11	12(13)
Drosophila mojavensis	AAPU01011085	10	11	12(13)
Drosophila virilis	AANI01017206	10	11	12(13)
Drosophila grimshawi	AAPT01018777	10	11	12(13)
Ceratitis capitata	AOHK01020840	12	>=13	>=14(<=15)
Musca domestica	NW_004765104.1	12	>=13	>=14(<=15)
Bactrocera cucurbitae	NW_011863679	12	>=13	>=14(<=15)
Stomoxys calcitrans	NW_013172444.1	12	>=13	>=14(<=15)
Anopheles gambiae	AAAB01008811	9	ND	ND
Culex quinquefasciatus	NW_001886742 AAWU01004063	9	ND	ND
Aedes aegypti	NC_035107.1	9	ND	ND

Bombyx mori	NW_004582021	14	ND	ND
Danaus plexippus	AGBW02014362.1	14	ND	ND
Tribolium castaneum	NC_007417	11	ND	ND
Apis mellifera	NW_003378056	18	ND	ND
Apis florea	NW_003790556	18	ND	ND
Apis dorsata	NW_006263665	18	ND	ND
Bombus impatiens	NT_177753	18	ND	ND
Bombus terrestris	NC_015777	18	ND	ND
Megachile rotundata	NW_003797109	18	ND	ND
Dufourea novaeangliae	NW_015374267	17	ND	ND
Acromyrmex echinatior	NW_011627086	18	ND	ND
Atta cephalotes	NW_012130091	16	ND	ND
Solenopsis invicta	NW_011795700	17	ND	ND
Pogonomyrmex barbatus	NW_011933718	18	ND	ND
Harpegnathos saltator	NW_011655019	17	ND	ND
Linepithema humile	NW_012160777	17	ND	ND
Camponotus floridanus	NW_011880404	16*	ND	ND
Neodiprion lecontei	NW_015383904	17	ND	ND
Nasonia giraulti	ADAO01244945	15**	ND	ND
Nasonia longicornis	ADAP01028838	15**	ND	ND
Nasonia vitripennis	NC_015869	16	ND	ND
Acyrthosiphon pisum	NW_003383605			
Diuraphis noxia	JOTR01000078			
Limulus poliphemus	NW_013667734			
Parasteatoda tepidariorum	NW_015458343			
Homo sapiens aralar1	NG_011781.1			
Homo sapiens citrin	NG_012247			

* lacks exon 8 due to sequence gap

** 1st exon not detected due to sequence limit

Supplementary Table S2. aralar1 gene structure comparisons in Drosophila species.
Isoforms RA-RD-RF

Key: UTR, CDS , intron *length inferred by similarity, RA-RD-RF transcript specific exon

Subgenus	subgroup	Species	RA-RD-RF											
Sophophora	melanogaster subgroup	*Drosophila_melanogaster*	151	27	269	153	598	163	58	92	66	195	65	224
		Drosophila_simulans	151	27	264	153	585	163	58	92	64	195	63	224
		Drosophila_yakuba	156	27	306	153	595	163	59	92	64	195	63	224
		Drosophila_erecta	150	27	270	153	557	163	66	92	64	195	64	224
		Drosophila_sechellia	151	27	264	153	582	163	55	92	64	195	63	224
	suzukii subgroup	*Drosophila_biarmipes*	152*	27	237	153	601	163	63	92	78	195	64	224
		Drosophila_takahashii	145*	27	284	153	901	163	61	92	80	195	63	224
		Drosophila_suzukii	145*	27	240	153	744	163	66	92	68	195	64	224
	eugracilis subgroup	*Drosophila_eugracilis*	147*	24	284	153	657	163	67	92	75	195	62	224
	ficusphila subgroup	*Drosophila_ficusphila*	145*	27	309	153	514	163	63	92	68	195	62	224
	elegans subgroup	*Drosophila_elegans*	150*	27	261	153	818	163	59	92	66	195	64	224
	montium subgroup	*Drosophila_rhopaloa*	154*	27	282	153	863	163	59	92	67	195	64	224

		Drosophila_kikkawai	155*	27	470	153	698	163	65	92	72	195	65	224
	ananassae subgroup	*Drosophila_bipectinata*	154*	27	147	153	611	163	62	92	67	195	58	224
		Drosophila_ananassae	154*	27	142	153	631	163	59	92	61	195	58	224
	willistoni subgroup	*Drosophila_willistoni*	156	27	169	153	476	163	65	92	167	195	66	224
Drosophila	pseudoobscura subgroup	*Drosophila_miranda*	ND	27	177	153	535	163	50	92	67	195	62	224
		Drosophila_pseudoobscura	ND	27	177	153	504	163	57	92	67	195	62	224
		Drosophila_persimilis	ND	27	177	153	534	163	57	92	67	195	62	224
	nasuta subgroup	*Drosophila_albomicans*	136	18	233	153	1188	163	57	92	63	195	55	224
	mulleri subgroup	*Drosophila_mojavensis*	652	18	217	153	531	163	80	92	97	195	65	224
	virilis subgroup	*Drosophila_virilis*	139	18	215	153	521	163	69	92	70	195	68	224

	grimshawi subgroup	*Drosophila_grimshawi*	65	18	254	153	466	163	92	92	75	195	154	224

Supplementary Table S3. aralar1 gene structure comparisons in Drosophila species.
Isoform RB

Key: UTR, CDS , intron *length inferred by similarity, RB transcript specific exon

Subgenus	Subgroup		RB											
Sophophora	melanogaster	*Drosophila_melanogaster*	335	18	3468	153	598	163	58	92	66	195	65	22
		Drosophila_simulans	330	18	3548	153	585	163	58	92	64	195	63	22
		Drosophila_yakuba	376	18	3654	153	595	163	59	92	64	195	63	22
		Drosophila_erecta	353	18	6209	153	557	163	66	92	64	195	64	22
		Drosophila_sechellia	321	18	3445	153	582	163	55	92	64	195	63	22
	suzukii	*Drosophila_biarmipes*	393*	18	3545	153	601	163	63	92	78	195	64	22
		Drosophila_takahashii	389*	18	5299	153	901	163	61	92	80	195	63	22
		Drosophila_suzukii	386*	18	3806	153	744	163	66	92	68	195	64	22
	eugracilis	*Drosophila_eugracilis*	366*	18	3900	153	657	163	67	92	75	195	62	22
	ficusphila	*Drosophila_ficusphila*	385*	18	3582	153	514	163	63	92	68	195	62	22
	elegans	*Drosophila_elegans*	413*	18	3973	153	818	163	59	92	66	195	64	22
	montium	*Drosophila_rhopaloa*	254*	18	3963	153	863	163	59	92	67	195	64	22

		Drosophila_kikkawai	270*	18	3898	153	698	163	65	92	72	195	65	22
	ananassae	*Drosophila_bipectinata*	ND	18	3703	153	611	163	62	92	67	195	58	22
		Drosophila_ananassae	129*	18	3695	153	631	163	59	92	61	195	58	22
	willistoni	*Drosophila_willistoni*	ND	18	3858	153	476	163	65	92	167	195	66	22
Drosophila	pseudoobscura	*Drosophila_miranda*	ND	18	3469	153	535	163	50	92	67	195	62	22
		Drosophila_pseudoobscura	ND	18	3396	153	504	163	57	92	67	195	62	22
		Drosophila_persimilis	ND	18	3181	153	534	163	57	92	67	195	62	22
	nasuta	*Drosophila_albomicans*	483	9	3585	153	1188	163	57	92	63	195	55	22
	mulleri	*Drosophila_mojavensis*	ND	9	3521	153	531	163	80	92	97	195	65	22
	virilis	*Drosophila_virilis*	471	9	3319	153	521	163	69	92	70	195	68	22
	grimshawi	*Drosophila_grimshawi*	477	9	3448	153	466	163	92	92	75	195	154	22

Supplementary Table S4. aralar1 gene structure comparisons in Drosophila species.
Isoform RC

Key: UTR, CDS , intron *length inferred by similarity, RC transcript specific exon

Subgenus	Subgroup	Species	RC				
Sophophora	melanogaster	*Drosophila_melanogaster*	151	66	5691	153	5
		Drosophila_simulans	151	66	5758	153	5
		Drosophila_yakuba	152	66	5910	153	5
		Drosophila_erecta	152	66	8315	153	5
		Drosophila_sechellia	151	66	5666	153	5
	suzukii	*Drosophila_biarmipes*	ND	60	6048	153	6
		Drosophila_takahashii	ND	63	7836	153	9
		Drosophila_suzukii	ND	60	6250	153	7
	eugracilis	*Drosophila_eugracilis*	ND	60	6186	153	6
	ficusphila	*Drosophila_ficusphila*	ND	60	5728	153	5
	elegans	*Drosophila_elegans*	ND	60	6552	153	8
	montium	*Drosophila_rhopaloa*	ND	66	6572	153	8
		Drosophila_kikkawai	ND	66	6262	153	6
	ananassae	*Drosophila_bipectinata*	ND	66	5787	153	6

		Drosophila_ananassae	ND	66	5914	153	6
	willistoni	*Drosophila_willistoni*	ND	60	7358	153	4
Drosophila	pseudoobscura	*Drosophila_miranda*	ND	66	5108	153	5
		Drosophila_pseudoobscura	ND	66	5037	153	5
		Drosophila_persimilis	ND	66	4831	153	5
	nasuta	*Drosophila_albomicans*	ND	66	6144	153	1
	mulleri	*Drosophila_mojavensis*	ND	66	6767	153	5
	virilis	*Drosophila_virilis*	ND	66	6175	153	5
	grimshawi	*Drosophila_grimshawi*	ND	66	6438	153	4

Supplementary Table S5. aralar1 gene structure comparisons in Drosophila species.
Isoform RE

Key: UTR, CDS , intron *length inferred by similarity, RE transcript specific exons

Subgenus	Subgroup		RE						
Sophophora	melanogaster	*Drosophila_melanogaster*	151	66	5691	153	598	163	58
		Drosophila_simulans	151	66	5758	153	585	163	58
		Drosophila_yakuba	152	66	5910	153	595	163	59
		Drosophila_erecta	152	66	8315	153	557	163	66
		Drosophila_sechellia	151	66	5666	153	582	163	55
	suzukii	*Drosophila_biarmipes*	ND	60	6048	153	601	163	63
		Drosophila_takahashii	ND	63	7836	153	901	163	61
		Drosophila_suzukii	ND	60	6250	153	744	163	66
	eugracilis	*Drosophila_eugracilis*	ND	60	6186	153	657	163	67
	ficusphila	*Drosophila_ficusphila*	ND	60	5728	153	514	163	63
	elegans	*Drosophila_elegans*	ND	60	6552	153	818	163	59
	montium	*Drosophila_rhopaloa*	ND	66	6572	153	863	163	59
		Drosophila_kikkawai	ND	66	6262	153	698	163	65
	ananassae	*Drosophila_bipectinata*	ND	66	5787	153	611	163	62

		Drosophila_ananassae	ND	66	5914	153	631	163	59	
	willistoni	*Drosophila_willistoni*	ND	60	7358	153	476	163	65	
Drosophila	pseudoobscura	*Drosophila_miranda*	ND	66	5108	153	535	163	50	
		Drosophila_pseudoobscura	ND	66	5037	153	504	163	57	
		Drosophila_persimilis	ND	66	4831	153	534	163	57	
	nasuta	*Drosophila_albomicans*	ND	66	6144	153	1188	163	57	
	mulleri	*Drosophila_mojavensis*	ND	66	6767	153	531	163	80	
	virilis	*Drosophila_virilis*	ND	66	6175	153	521	163	69	
	grimshawi	*Drosophila_grimshawi*	ND	66	6438	153	466	163	92	

Supplementary Table S6. aralar1 gene structure comparison in Arthropoda.
Key: UTR, CDS , intron ***sequence gap;** ND: Not detected

			first exon of RB isoform									first exon RA, RF, RD is		
Order (Family)	Species	other TSSs	exon	intron	exon	intron	exon	intron	exon	intron	exon	intron	exon	
Diptera (Drosophilidae)	*Drosophila_melanogaster*	+ 1 alternative TSS	335	3021	151	27	269				153	598	163	
Diptera (Tephritidae)	*Ceratitis_capitata OK*	+ 1 alternative TSS	361	7381	218	12	470				153	1334	163	
	Bactrocera_cucurbitae		ND	ND	328+ND+292	27	24806*				153	3360	163	
Diptera (Muscidae)	*Stomoxys_calcitrans*	+ 1 alternative TSS	564	34157*	314	24	10080*				153	>=9702	163	

	Musca_domestica	+ 1 alternative TSS	141+481+137	24	28126*	309	24	4072	153	7984	163	
Diptera (Culicidae)	*Anopheles_gambiae*		ND	ND	ND	136	30	3507	141	297		
	Culex_pipiens_quinq.		ND	ND	ND		63	2117*	141	93		
	Aedes_aegypti		ND	ND	ND	520	63	50083*	141	70		

Lepidoptera	*Bombyx_mori*	+ 1 alternative TSS	ND	ND	ND	283	51	5746				139	1689	177	
	Danaus_plexippus		ND	ND	ND	ND	51	130482				139	70	177	
Coleoptera	*Tribolium_castaneum*		247	36	1516	54	36	81			316				
Hymenoptera	*Apis_mellifera*		ND	ND	ND	426	42	1720	90	8900	57	199	143	78	116
	Apis_florea		ND	ND	ND	306	42	10509			57	206	143	75	116
	Apis_dorsata		ND	ND	ND	375	42	1680	93	6893	57	207	143	75	116

	Bombus_impatiens		ND	ND	ND	288	42	12665	57	118	143	106	116			
	Bombus_terrestris		ND	ND	ND	284	42	12543	57	111	143	104	116			
	Megachile_rotundata		182	44	2986	1108	93	85	57	70	143	121	116			

Dufourea_novae angliae		294	26	3798	275	3	5655		57	97	143	71	116	
Acromyrmex_echinatior		673	15	54214*	84	76	10608*	106	1930	60	4118	143	34109	116
Atta_cephalotes		ND	ND	ND	ND	69	3014					143	38399	116
Solenopsis_invicta		180	42	5951	11	33	7490*		57	267	143	1077	116	
Pogonomyrmex_barbatus		248	42	4501	915	36	9493		57	457	143	277	116	

	Harpegnathos_saltator		ND	ND	ND	78	42	93341	57	147	143	1907	116	
	Linepithema_humile		ND	ND	ND	250	42	7691	57	152	143	151	116	
	Camponotus_floridanus		ND	ND	ND	386	42	13515	57	1156	143	980	116	
	Neodiprion_lecontei		ND	ND		25	36	3862	57	808	143	172	116	
	Nasonia_vitripennis		253	339		75	42	10940	57	90	143	277*	116	
	Nasonia_giraulti		ND	ND	ND	ND	ND	ND	57	90	143	267	116	
	Nasonia_longicornis		ND	ND	ND	ND	ND	364*	57	90	143	277	116	
Hemiptera	*Acyrthosiphon_pisum*		ND	ND	ND	173	63	554	60	75	143	1612	116	

	Diuraphis_noxia		ND	ND	ND	643	63	570	60	67	143	1610	116		
Xiphosura (Limulidae)	*Limulus_polyphemus*		ND	ND	ND	105	42	3641	60	8494	143	2534	116		
Arachnida	*Parasteatoda_tepidariorum*		ND	ND	ND	ND	96	103	63	5434	57	2636	143	1018	116
	Homo_sapiens\aralar1					92	12	944	54	24381	143	12731	116	75	

	Homo_sapiens\citrin	+ 1 alternative TSS	94	48	12587	54	12173	51	60	7279		143	42278	116		

NOTE: the CITRIN locus has been depicted using the X1-X4 mRNA isoforms annotated in NCBI. The first exc

Credit Author Statement

P.L., R.C, L.C. and V.D. conceived the project, designed experiments, and wrote the manuscript. P.L., R.C, F.M, Y.L., G.L. and F.D. performed the biochemical and molecular biology experiments. R.M.M., R.M. performed the genetic experiments. D.A. performed the calcium experiments. A. A. M. provided antiserum and contributed to ELISA experiments. P. L. and F.M. analyzed the yeast phenotype. A.R.C. A.F. and G.F. assisted in data interpretation. G.F. and V. Z. participated in the discussion of the project and were involved in the revision of the manuscript. L.C. and V.D. directed and supervised the project. G.F. and V.Z. acquired the funding. All authors read and approved the final manuscript.