

# Mitochondrial targeting of farnesyl diphosphate synthase is a widespread phenomenon in eukaryotes

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## Abstract

The isoprenoid pathway is responsible for the generation of a wide range of products that are crucial for cellular processes; namely, cholesterol synthesis, protein glycosylation, growth control and synthesis of several hormones. Farnesyl diphosphate synthase (FPS), a key enzyme in this pathway, is usually considered to be cytosolic/peroxisomal. However, significant enzymatic activity has also been detected in rat liver mitochondria, although none of the mammalian FPS genes characterized to date contain sequences coding for mitochondrial transit peptides. Here, we describe the genomic organization of the human *FPS* gene and demonstrate that one of the two mRNAs expressed from this gene encodes an isoform with a 66 amino acid N-terminal extension containing a peptide that targets it to mitochondria. Previous studies suggested that the N-terminal extension of FPS in the plant *Arabidopsis thaliana* contains a mitochondrial targeting sequence. In this study, database analysis reveals that this is also the case in a number of mammals and insects. Finally, we provide functional proofs that the N-terminal sequence of *Drosophila melanogaster* FPS targets the protein to mitochondria. Taken together, these data suggest that mitochondrial targeting of FPS may be widespread among eukaryotes.

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## 1. Introduction

Farnesyl diphosphate synthase (FPS; EC 2.5.1.1/EC 2.5.1.10) catalyzes the sequential 1'–4 condensation of two molecules of isopentenyl diphosphate with the allylic phosphates dimethylallyl diphosphate and geranyl diphosphate to produce the 15-carbon prenyl diphosphate farnesyl diphosphate [1]. This compound is a key intermediate in the isoprenoid biosynthetic pathway, since it is the common precursor of a variety of essential isoprenoid end products, including sterols, dolichols, and the isoprenoid moieties of ubiquinone, heme a, and

prenylated proteins. The subcellular distribution of the enzymes involved in isoprenoid biosynthesis has been the subject of debate in recent years. It is generally accepted that in mammals, peroxisomes play a central role in isoprenoid/cholesterol biosynthesis, since the enzymes required to synthesize farnesyl diphosphate from acetyl-CoA have been localized predominantly in these organelles. However, it has recently been claimed that mevalonate kinase and mevalonate pyrophosphate decarboxylase are exclusively cytosolic enzymes, at least in humans [2,3]. These results, together with the observation that cholesterol biosynthesis is unaffected in human fibroblasts defective for peroxisome biogenesis, led scientists to challenge the dogma of the central role of peroxisomes in mammalian isoprenoid/cholesterol biosynthesis [4].

Although it does not contain any peroxisome targeting sequences, FPS has been reported to be predominantly peroxisomal in a number of mammalian cells [5]. Nevertheless, controversy still remains over its precise subcellular localization, as well as the possibility that significant amounts of the

*Abbreviations:* DmFPS, *Drosophila melanogaster* farnesyl diphosphate synthase; EGFP, enhance green fluorescent protein; FPS, farnesyl diphosphate synthase; GP, gold particles; HsFPS, human farnesyl diphosphate synthase; WSR, mutant strain of *Saccharomyces cerevisiae* carrying a disrupted *COXIV* gene

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enzyme could be localized in mitochondria. Thus, whereas no evidence in favour of mitochondrial localization of FPS was obtained in immunofluorescence or immunoelectron microscopy studies [6], subcellular fractionation and enzyme activity assays showed that in rat liver up to 13% of total cellular FPS activity is contained within mitochondria [7]. In contrast to the situation in animals, indirect evidence of FPS localization in mitochondria of plants has been provided. The genome of *Arabidopsis thaliana* contains two genes (*FPS1* and *FPS2*; [8]) encoding three FPS isoforms (FPS1S, FPS1L, and FPS2), one of which (FPS1L) has an N-terminal extension of 41 amino acids that has been shown to be a mitochondrial transit peptide [9]. However, the prevailing view is that mammalian genomes contain a single functional gene coding for FPS [10]. This raises the possibility that a single gene encodes more than one isoform, each of which could be localized and function in different cellular compartments.

Given that the precise subcellular distribution of FPS in mammals is still a matter of debate, particularly in terms of its putative mitochondrial localization, we examined whether mammals also contain a mitochondrial FPS isoform. In this paper, we describe the genomic organization and splicing pattern of the 5' region of the human FPS (HsFPS) gene, and demonstrate that one of the two mRNA species transcribed from this gene encodes an FPS isoform that is efficiently targeted to mitochondria. Moreover, we demonstrate the presence of putative mitochondrial targeting peptides in the N-terminal extension of FPS from several mammals and insects, and show that the N-terminal extension of FPS from *Drosophila melanogaster* conveys a passenger protein into mitochondria. Taken together, these data suggest that FPS targeting to mitochondria is widespread in eukaryotes.

## 2. Materials and methods

### 2.1. Sequence comparisons and analysis

The software package designed by the Genetics Computer Group of the University of Wisconsin [11] was used for sequence alignments and secondary structure prediction. Putative mitochondrial targeting sequences were predicted with the TargetP [12] and Predator (<http://www.inra.fr/predator/>) programs.

### 2.2. Mapping of the 5'-end of HsFPS and DmFPS mRNAs

The 5'-end of the HsFPS and *D. melanogaster* FPS (DmFPS) mRNAs were determined by 5' RACE, using the FirstChoice™ RLM-RACE kit (Ambion) and *Drosophila* S2 cells as a source of RNA. For HsFPS, the reverse primers used were as follows: outer primer (HUMO) 5'-CGGAATGCTACTACCACCGTCAA-3' (positions 1,731,921 to 1,731,943 from contig NT\_004487), and inner primer (HUMI), 5'-CGCGGATCCGAGCCGGGCAATAGCATCTCCTA-3' (1,729,801 to 1,729,823). Nested reverse primers used to amplify the 5'-end of the cDNA corresponding to the mitochondrial isoform were as follows: outer primer (HUMITO), 5'-TTCCTCTGTCCACGCTTGGCA-3' (1,729,564 to 1,729,584), and inner primer (HUMITI), 5'-CGCGGATCCGTCACGCTTGGCACCAGCA-3' (1,729,558 to 1,729,577). *Bam*HI restriction sites are underlined. In the case of DmFPS, the reverse primers were as follows: outer primer (Dro1), 5'-TCCATCACATCGTCTGAGATGAT-3' (positions 590 to 611 from sequence AF132554), and inner primer (Dro2), 5'-ACTTTGGAGCATTTCCACGCACCA-3' (560–583). In both cases, amplified fragments were cloned into pBluescript and sequenced.

### 2.3. Reverse transcription-PCR analysis

To detect the HsFPS cDNA that includes the mitochondrial targeting peptide, RNA from HepG2, CaCo-2, HT29 and HeLa human cells [13] was used. RNA was isolated with the GenElute™ Mammalian Total RNA kit (Sigma), and cDNA was obtained as described previously [14]. Primers used for detection of the HsFPS cDNA that includes the mitochondrial targeting peptide were as follows: forward, 5'-ATGCCCTGTCCCGCTGGTTGAGATCT-3', and reverse, 5'-CTGAGGGAGGAGCAAAGGGCTCGAGGT-3', encompassing amino acids 1–9 and 58–66, respectively, according to the sequence represented in Fig. 3. To amplify the DmFPS cDNA containing the mitochondrial targeting peptide we followed the same procedure, using *Drosophila* S2 cells as RNA source, and primers were as follows: forward, 5'-ATGTTTAAACTGGCCCGTATCCTC-3', and reverse, 5'-ACTTTGGAGCATTTCACGCACCA-3' encompassing amino acids 1–8 and 73–81, respectively, according to the sequence represented in Fig. 3. *D. melanogaster* ribosomal protein 49 (Rp49) was used as a control for amplification and loading.

### 2.4. Green fluorescent protein fusion constructs

Plasmid HsFPS(tp)-EGFP contains the first 66 amino acids of the HsFPS protein fused with EGFP. The sequence coding for the putative targeting peptide was obtained by PCR using the primer pair 5'-ATAAAGCTTATGCCCTGTCCCGCTGGTTGAGATCT-3' (forward) and 5'-ATAACC-GGTTTCTGAGGGAGGAGCAAAGGGCTCGAGGT-3' (reverse). *Hind*III and *Age*I restriction sites (underlined) were added to facilitate cloning of the fragments into the pEGFP-N1 vector (Clontech). The resulting plasmid, HsFPS(tp)-EGFP, expressed the fusion protein under the control of the CMV promoter. The pEGFP-N1 plasmid, expressing the native EGFP protein, was used as a control.

To construct DmFPS(tp)-EGFP, containing *D. melanogaster* FPS(tp), the sequence coding for the first 80 amino acids of the DmFPS was PCR amplified using primers that contained *Age*I restriction sites (underlined): 5'-ATA-ACCGGTATGTTAAACTGGCCCGTATCCTC-3' (forward) and 5'-ATA-ACCGGTTTCGACACCGTGACGCGAGCGGCAAT-3' (reverse). Prior to amplification, a PCR-based site directed mutagenesis was used to change the Met residues at positions 7 and 40 to Ile, using the mutagenic complementary primer pair 5'-ATCAAGTCCATCGACACCATT-3' (forward) and 5'-AATGG-TGTCGATGGACTTGAT-3' (reverse), and also using the mutated initial forward primer (mutations within the primers are underlined). The amplified fragment was cloned into the pEGFP-N1 plasmid. Afterwards, the 1050 base pair fragment containing DmFPS(tp) fused to EGFP was subcloned into the pAc5/V5-His(C) plasmid (Invitrogen) just downstream of the *D. melanogaster* actin5C promoter. As a control, plasmid pAc5-EGFP was constructed by subcloning the entire EGFP sequence from the pEGFP-N1 plasmid into pAc5/V5-His(C) plasmid. This construct expressed the EGFP protein under the control of *D. melanogaster* actin5C promoter. Preparation of all constructs was confirmed by restriction analysis and sequencing.

### 2.5. Cell culture and confocal microscopy

Human HeLa and *D. melanogaster* S2 cells were maintained and transfected as described elsewhere [15,16]. Forty-eight hours after transfection, HeLa and S2 cells were placed in fresh medium containing 100–500 nM of the mitochondria-specific stain MitoTracker Red CMXros (Molecular Probes) and incubated for 15–30 min. Cells were examined with appropriate filters on a Leica TCS confocal laser scanning microscope (Heidelberg, Germany).

### 2.6. Immunoelectron microscopy

HeLa cells were fixed and embedded in Lowicryl K4M. Ultrathin sections (50–70 nm) were obtained with a Leica Ultracut UCT ultramicrotome. For immunolabeling, a polyclonal antibody raised in rabbit against rat FPS (a gift of P. Edwards, UCLA) was used at a dilution of 1:500, and gold-conjugated goat anti-rabbit IgG was used as the secondary antibody at a dilution of 1:25. Control treatments using the secondary antibody alone were carried out in parallel. Sections were examined on a Jeol 1010 transmission electron microscope at

80 Kv. For quantitative studies, 50 micrographs were used (5 micrographs selected at random from the same cell  $\times 10$  cells studied). These micrographs were taken at a magnification between  $\times 20,000$  and  $\times 30,000$ , and immunolabeling was quantified by counting the gold particles observed in each cell compartment. An equivalent series of 50 micrographs of control cells, treated only with the secondary antibody, were also studied.

### 2.7. Functional complementation of mutant yeast

DNA fragments from *D. melanogaster* encoding putative targeting peptides of two different lengths, from Met 1 to Ser 80 and from Met 40 to Ser 80, were generated by RT-PCR and cloned in frame into the corresponding sites of plasmid pY $\Delta$ COX, which contains a truncated yeast *COXIV* (lacking the presequence) gene under the control of the alcohol dehydrogenase promoter, yielding plasmids pDm1- $\Delta$ COX and pDm2- $\Delta$ COX. The WSR mutant strain of *Saccharomyces cerevisiae* was used. This strain carries a disrupted *COXIV* gene encoding the CoxIV subunit of mitochondrial cytochrome *c* oxidase and cannot grow in medium containing glycerol as energy source [17]. Transformation of the WSR strain with pYCOX, pY $\Delta$ COX, pDm1- $\Delta$ COX, or pDm2- $\Delta$ COX, and functional complementation assays were carried out as described elsewhere [9,18].

## 3. Results

### 3.1. The human FPS gene encodes a putative mitochondrial isoform

A cDNA sequence coding for HsFPS has been reported previously [19], although the authors stated that the deduced protein might contain additional amino acids at the N-terminal region, since there was no stop codon upstream of the proposed ATG start codon. A search in the NCBI database revealed the occurrence of a human sequence (accession number, NM002004) containing an in-frame ATG codon located 198 bp upstream of the previously reported HsFPS start codon. Interestingly, conceptual translation from this ATG gives rise to an FPS isoform with a N-terminal extension of 66 amino acids when compared with the described HsFPS. This extension contains a putative mitochondrial targeting sequence, as determined by analysis with conventional prediction algorithms (TargetP, Predictor).

To precisely define the 5'-end of the HsFPS mRNAs we performed 5'-RACE analysis. Using primers HUMO and HUMI as nested PCR gene-specific primers (Fig. 1A), we amplified two different sets of products ranging in size from 196 to 252 nt, and from 324 to 337 nt. Sequence alignment of these fragments with the *HsFPS* gene (chromosome 1, contig NT\_079484) revealed the occurrence of mRNA species containing the as yet unreported exon 1 as the most 5' exon, joined with the exon encoding the N-terminal region of the previously described HsFPS [19] (further referred to here as exon 3, Fig. 1A). This analysis also showed that the *HsFPS* gene has multiple transcription start sites, with those located at positions -136, -36 and -29 with respect to the first ATG codon in exon 1 being the most frequently used (Fig. 1A). Finally, results additionally indicated that the intervening sequence between exons 1 and 3 can be spliced using alternative 5' donor (GT) sites (*a* and *b*), and 3' acceptor (AG) sites (*c* and *d*) (Fig. 1A). Nevertheless, sites *a* and *c* are preferentially used as donor and acceptor sites, respectively. Interestingly, if donor

site *b* is used, then exon 1 contains 111 additional nucleotides, including a short ORF coding for 32 amino acids that is located in the 5' untranslated region of the resulting mRNA (Fig. 1A). The use of the alternative acceptor sites *c* and *d* also leads to two forms of exon 3 differing in 21 nt, although in both cases the resulting exon includes the ATG start codon of the cytosolic/peroxisomal HsFPS [19]. Given that none of these mRNA splice variants would include the sequence coding for the putative mitochondrial targeting peptide identified in the aforementioned cDNA (NM002004), additional 5'-RACE experiments were carried out using a new pair of nested PCR gene-specific primers, HUMITO and HUMITI, complementary to sequences within the predicted exon 2 (Fig. 1A). Cloning and sequencing of the PCR products revealed the amplification of a set of fragments ranging in size from 225 to 335 nt. Alignment of these sequences with the *HsFPS* gene demonstrated the existence of novel mRNA splice variants in which exon 1 was joined with the novel exon 2 located between exons 1 and 3 (Fig. 1A). Given that both 5' donor sites (*a* and *b*) of intron 1 can be used to generate these transcripts, some of them also contain the long version of exon 1 including the ORF in the 5' untranslated sequence (Fig. 1A). As the sequence of exon 2 includes the aforementioned in-frame ATG codon upstream of the first ATG codon in exon 3, these novel splice variants would encode a longer novel FPS isoform with the predicted mitochondrial targeting peptide of 66 amino acids (Fig. 1B). The occurrence of transcripts encoding this novel isoform was further confirmed by RT-PCR using primers in exons 2 and 3, and RNA from human cell lines HepG2, CaCo-2, HT29, and HeLa. A PCR product of the expected size (225 bp) was detected in all samples (Fig. 1C). Taken together, all these results indicate that *HsFPS* expression generates multiple mRNA splice variants that encode two FPS isoforms: the previously reported short isoform [19], which should be cytosolic/peroxisomal, and a longer isoform with an N-terminal extension that is likely to function as a mitochondrial targeting peptide.

### 3.2. A Human FPS isoform is targeted to the mitochondria

To test the functionality of the targeting peptide, we first engineered an EGFP fusion vector expressing the first 66 amino acids of HsFPS, which should contain the putative mitochondrial targeting peptide, fused to the N-terminal end of EGFP. This construct, HsFPS(tp)-EGFP, as well as the control plasmid expressing EGFP itself, was transfected into HeLa cells and fluorescence was examined by confocal microscopy (Fig. 1D). In cells transfected with the control plasmid, green fluorescence was visible throughout the entire cell, including the nucleus. In contrast, cells expressing HsFPS(tp)-EGFP exhibited a network-like fluorescence pattern, perfectly matching the staining pattern of the mitochondria-specific dye MitoTracker Red CMXRos (Fig. 1D). These results strongly suggest that the N-terminal region of HsFPS functions as a mitochondrial targeting peptide.

To confirm the occurrence of FPS within human mitochondria, we examined its subcellular localization in HeLa cells by

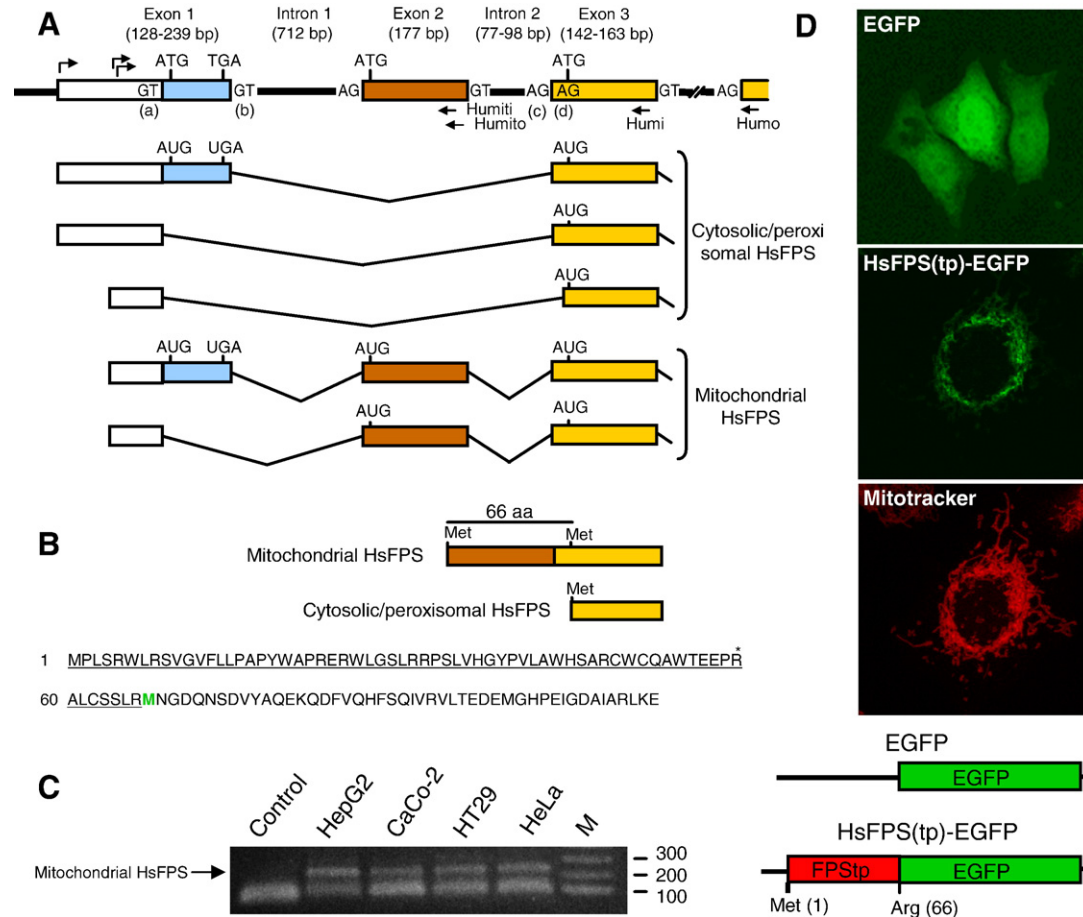


Fig. 1. Expression of FPS in human cells and properties of the N-terminal extension as mitochondrial targeting sequence. (A) Genomic organization and splicing pattern of the 5' region of the *HsFPS* gene. Exons are shown as boxes and introns as lines. The most frequently used transcription start sites of *HsFPS* are indicated by arrows. The conserved dinucleotides in the 5' donor (GT) and 3' acceptor (AG) splice sites of introns are shown. (a) and (b) indicate the two alternative donor sites of intron 1 whereas (c) and (d) indicate the alternative acceptor sites of intron 2. Arrows located below the schematic representation of the *HsFPS* gene represent nested gene-specific PCR primers (Humo/Humi and Humito/Humiti) used in 5'-RACE experiments. The two forms of exon 1 are represented by an open box (short form) and by an open box joined with a blue box (long form). Translation start codons in exons 1, 2 and 3, and the stop codon in exon 1 are also indicated. The predicted protein products are named to the right of the illustration. (B) Schematic representation of the N-terminal region of the HsFPS isoforms encoded by the mRNA splice variants shown in panel A. The sequence encoded by exon 2 is represented as a brown box, and that encoded by exon 3 is shown as a yellow box. The positions of the initiator Met residues for the mitochondrial and cytosolic/peroxisomal HsFPS isoforms are indicated, and the amino acid sequence of the predicted mitochondrial targeting peptide is underlined. An asterisk indicates the last amino acid encoded by exon 2, and the initiator Met residue of the cytosolic/peroxisomal HsFPS is shown in green. (C) RT-PCR analysis of mRNA variants containing exon 2 in different human cell lines (HepG2, CaCo-2, HT29 and HeLa). An arrow indicates the position of the 225-bp amplified fragment corresponding to mitochondrial HsFPS mRNA. Numbers on the right indicate the sizes in bp of DNA size markers in lane M. (D) Confocal laser scanning micrographs showing the distribution of green fluorescence in HeLa cells transiently expressing a fusion of the HsFPS mitochondrial targeting peptide with the EGFP (HsFPStp-EGFP) and EGFP alone. The pattern of red fluorescence obtained when HeLa cells were treated with the mitochondria-specific dye MitoTracker Red CMXRos is also shown. The scheme of the constructs is shown below the fluorescence images.

immunoelectron microscopy using a polyclonal antibody against rat FPS. Most HsFPS immunolabeling was detected in the cytoplasm, either free (48% of the gold particles, GP) or associated with the rough endoplasmic reticulum (11% GP) (Fig. 2A). Then, significant HsFPS immunolabeling was also observed in the mitochondria (26% GP), either within the matrix or associated with the mitochondrial membrane (Fig. 2A, B), and in the peroxisomes (12% GP). A small percentage of GP (3%) was observed in the nucleus and in extracellular spaces, which is taken to represent unspecific immunolabeling. In our samples, mitochondria were more abundant than peroxisomes, and the mean ratio of GP per organule was 1.17 in the case of mitochondria, and 1.27 for peroxisomes. Control cells, treated only with the secondary antibody, did not show any labeling.

### 3.3. Occurrence of FPS proteins with mitochondrial transit peptides is a widespread phenomenon in eukaryotes

Taken together with results reported for *A. thaliana* [9], the data presented above suggest that mitochondrial localization of FPS proteins is a general phenomenon in eukaryotes. To address this hypothesis, we performed multiple sequence alignments of the FPS proteins found in the literature and in databases. In this way, N-terminal extensions were identified in a number of mammals (in addition to *Homo sapiens*): *Rattus norvegicus* (AY\_321335) and *Mus musculus* (AK077979, not designated as FPS); and insects: *Agrotis ipsilon* [20] (AJ009962), *Bombyx mori* (BAB69490), *Choristoneura fumiferana* (AAY33486.1) [21], *Mythimna (=Pseudaletia) unipuncta* (AAY33487.1)

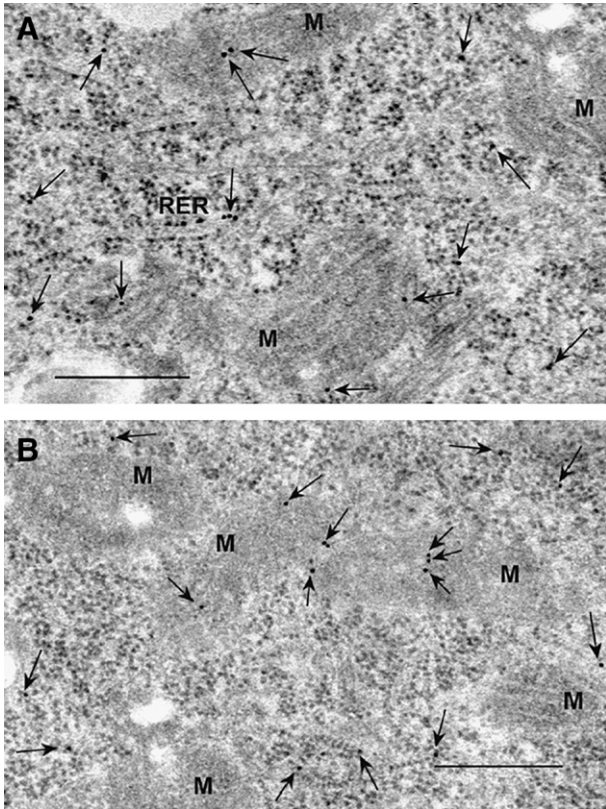


Fig. 2. Electron micrographs of HeLa cells immunolabeled for FPS using a polyclonal antibody against rat FPS. (A) Section showing HsFPS immunolocalization in mitochondria and in the cytoplasm, scattered and associated to rough endoplasmic reticulum, (B) immunolocalization in the matrix of mitochondria. Scale bars, 0.5 μm. Arrows indicate gold particles. M, mitochondria; RER, rough endoplasmic reticulum.

[21], *Myzus persicae* (AAY33491.1) [21], *Aphis fabae* (AAY33488.1) [21], *Ips pini* (AAX55631.1) [22], *Anthonomus grandis* (AAX78434.1) [22], *Dendroctonus jeffreyi*

(AAX78435.1) [22], *Anopheles gambiae* (XP\_308653, not designated as FPS) and *D. melanogaster* (AF132554), in addition to the plant *A. thaliana* (U80605). As in *H. sapiens* and *A. thaliana*, the N-terminal extensions of the FPS of these species are enriched in basic, hydroxylated and hydrophobic residues, have at least one RXXS tetrapeptide, which is a consensus cleavage motif in mitochondrial targeting peptides [23], and contain sequence portions that may form a positively charged amphiphilic α-helix. All of these features are typical of mitochondrial targeting peptides. A selection of these sequences have been included in Fig. 3, highlighting the features suggestive of mitochondrial targeting peptides.

### 3.4. An insect FPS gene also encodes a mitochondrial protein

Analysis of the *D. melanogaster* genome database revealed the occurrence of a single FPS gene (FlyBase ID: FBgn0025373), as occurs in humans. It has a length of 2,064 nt, comprises six exons, and encodes a predicted protein of 419 amino acids, which would include the N-terminal extension predicted to contain a mitochondrial targeting sequence (Fig. 4A). To assess the occurrence of this sequence in a native protein isoform, we first defined the structure of the 5'-end of the DmFPS mRNA by 5'-RACE using two primers, one located at a point within exon 4 (Dro1) and the other one at a point within intron 3 (Dro2) (Fig. 4A). This generated a PCR product of 590 bp. Alignment of this sequence with that of the *DmFPS* gene defined the 5'-end of the mRNA, and more importantly, showed that it included the putative mitochondrial targeting peptide located within exon 1 (Fig. 4A). Moreover, RT-PCR analysis (with a primer pair designed to amplify a fragment starting at a point within exon 1 and terminating at a point within exon 3) showed that this DmFPS isoform is expressed throughout the entire developmental cycle, being particularly

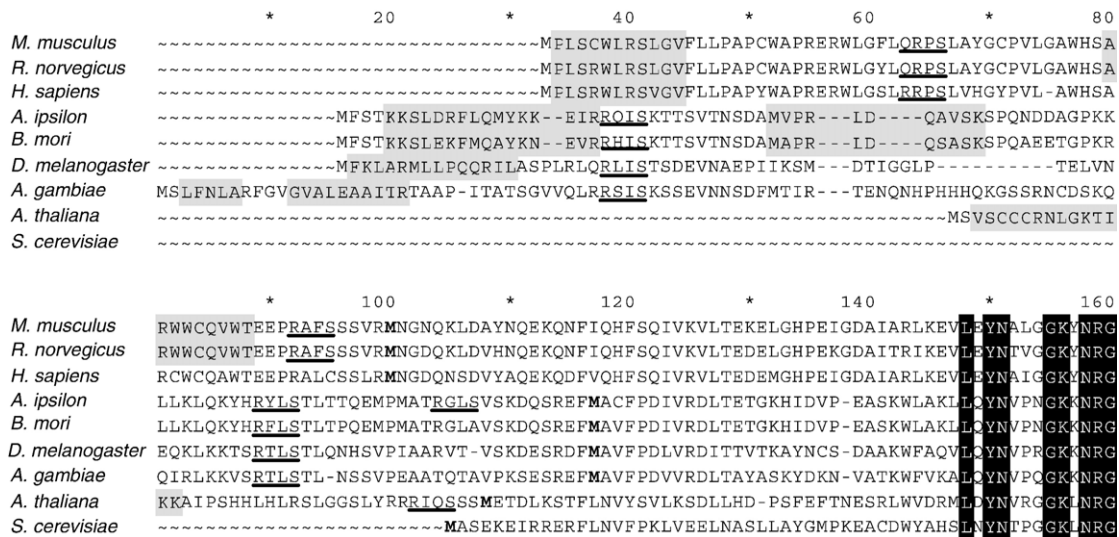


Fig. 3. Alignment of the 5' region of a selection of farnesyl diphosphate synthase sequences from mammals, insects and a plant that show a N-terminal extension. The yeast *S. cerevisiae* (AAA34606) was included as a reference of a FPS without such extension. These regions show a 65–113 amino acid extension with respect to the initial Met (indicated in bold) of the strict FPS molecule. RXXS motifs, suggestive of mitochondrial targeting cleavage sequence, are underlined. Sequences before the first RXXS motif that may form a positively charged amphiphilic α-helix are indicated in grey.

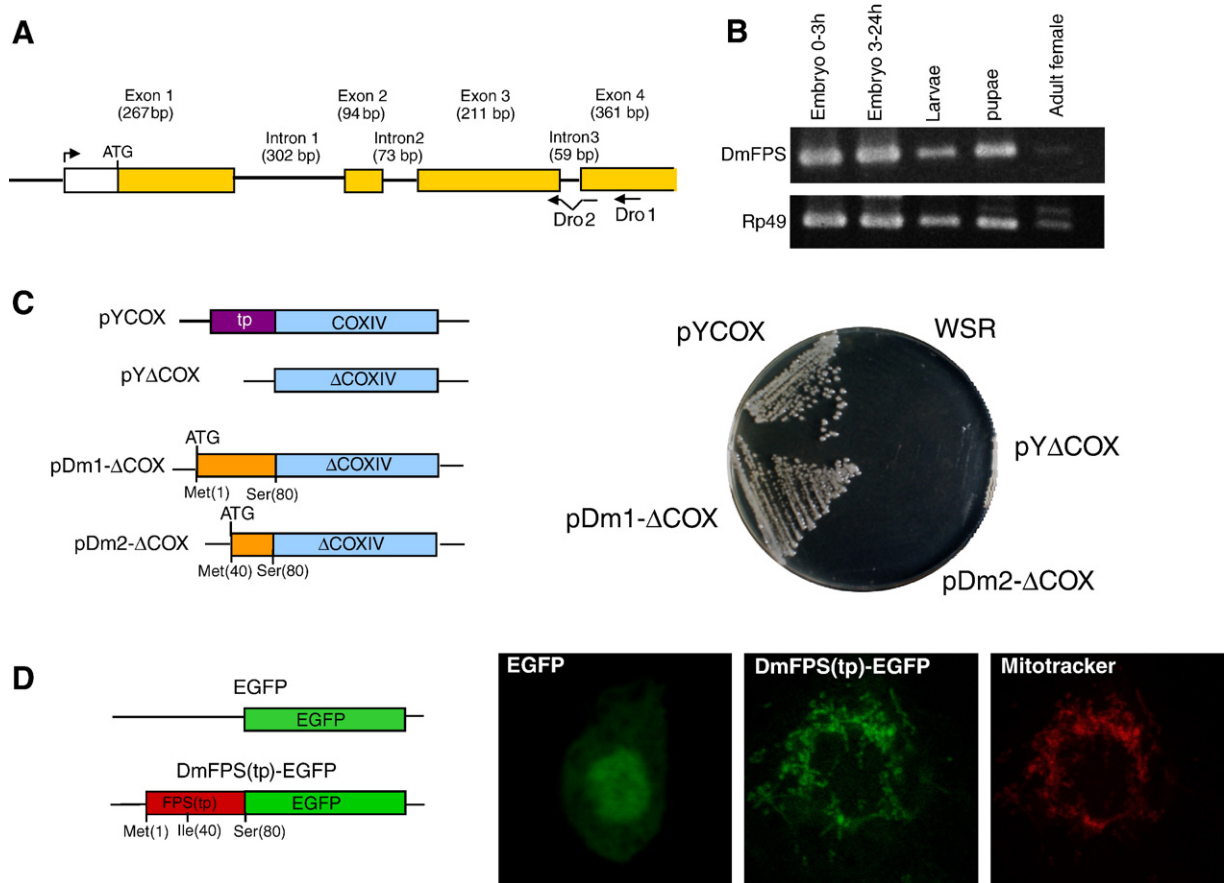


Fig. 4. Expression of *FPS* in fruitfly cells and properties of the N-terminal extension as mitochondrial targeting sequence. (A) Genomic organization of the 5' region of the *DmFPS* gene. Different exons and introns as well as their size are indicated. Primers used in 5'-RACE as well as the transcription start site are shown by arrows. (B) Expression of *DmFPS* containing the mitochondrial targeting peptide is detectable by RT-PCR throughout the whole development cycle. A band of the expected size (243 bp) was obtained in every case. *D. melanogaster* Rp49 was used as a control for amplification and loading. (C) Functional complementation of a CoxIV-deficient yeast strain, by plasmids pDm1- and pDm2- $\Delta$ COX. Left: schematic representation of the plasmids used. Plasmid pYCOX contains the wild-type *COXIV* gene from *S. cerevisiae*, including the region encoding its own mitochondrial targeting peptide (tp), which has been deleted in plasmid pY $\Delta$ COX. Plasmids pDm1- $\Delta$ COX and pDm2- $\Delta$ COX contain putative targeting peptides Dm1 (from Met 1) and Dm2 (from Met 40) from N-terminal extension of fruitfly *FPS*, ligated upstream of the partially deleted *COXIV* gene from yeast. Right: complementation analysis of yeast strain WSR transformed with the plasmids described above. Strains WSR, WSR [pY $\Delta$ COX], WSR[pYCOX], WSR[pDm1- $\Delta$ COX] and WSR[pDm2- $\Delta$ COX] were streaked onto N3 medium containing glycerol as energy source, and incubated at 28 °C for 2 days. (D) Subcellular localization of the N-terminal fragment of the DmFPS (amino acids 1–80) fused to EGFP. Fluorescence images of S2 cells (right), transiently transfected with the respective constructs (left). Cells expressing EGFP showed strong fluorescence in the nucleus and in the cytoplasm ("EGFP" panel) whereas cells expressing the fusion protein showed a network-like fluorescence pattern ("DmFPS(tp)-EGFP" panel) virtually identical to that obtained with the mitochondria-specific dye MitoTracker Red CMXRos ("Mitotracker" panel).

abundant in embryos and pupae, two stages characterized by high metabolic activity (Fig. 4B).

To determine whether the DmFPS N-terminal extension functions as a mitochondrial targeting peptide, we studied its ability to convey a passenger protein into mitochondria, using a heterologous complementation assay in mutant WSR yeast [9]. We followed this approach first because the case of DmFPS is more complex than that of HsFPS, given that DmFPS N-terminal extension contains two in-frame Met and two RXXS motifs, which cast doubts on the localization of the putative mitochondrial targeting peptide. Therefore, the amino acid sequences from Met 1 (Dm1) and Met 40 (Dm2) to Ser 80, were fused in frame with a truncated *S. cerevisiae* *COXIV* gene lacking the region coding for its own mitochondrial targeting peptide ( $\Delta$ COXIV). The plasmids (pDm1- $\Delta$ COX, pDm2- $\Delta$ COX; Fig. 4C) were then assayed for their ability to com-

plement the mutant yeast strain WSR. Only pDm1- $\Delta$ COX restored the respiratory function of the WSR strain (Fig. 4C), indicating that the yeast CoxIV subunit was effectively targeted into yeast mitochondria by Dm1, and that the mitochondrial targeting peptide is located between Met 1 and Met 40 of DmFPS.

To assess the functionality of the DmFPS N-terminal extension in an homologous system, we transfected *Drosophila* S2 cells with the DmFPS(tp)-EGFP fusion construct. This construct contains an EGFP fusion of the first N-terminal 80 amino acids of DmFPS, with Met 40 and Met 7 mutated to Ile, under the control of the *D. melanogaster* actin5C promoter (Fig. 4D). As a control, a plasmid expressing EGFP alone was also transfected. Confocal microscopy revealed that in DmFPS(tp)-EGFP-transfected cells fluorescence appears in a network-like pattern virtually identical to that obtained with the specific

mitochondrial marker MitoTracker Red CMXRos. In contrast, fluorescence is uniform throughout the whole cell in control, EGFP-transfected cells (Fig. 4D).

#### 4. Discussion

Traditionally, the enzyme FPS has been considered as cytosolic/peroxisomal. However, the results of our database analysis and 5'-RACE experiments indicate that transcription of *HsFPS* gives rise to a heterogeneous population of mRNAs encoding two FPS isoforms: the cytosolic/peroxisomal isoform, which has been reported elsewhere [19], and a novel isoform containing an N-terminal extension of 66 amino acids. This previously unidentified isoform contains structural features suggestive of a mitochondrial targeting peptide.

Our transfection and confocal microscopy studies showed the ability of the N-terminal extension to convey a passenger protein into mitochondria. In addition, our immunoelectron microscopy observations indicated the occurrence of FPS within the mitochondria. Taken together, these data demonstrate for the first time that an HsFPS isoform has an N-terminal extension containing a functional mitochondrial targeting sequence, and that FPS localizes in human mitochondria. These results are consistent with the FPS enzymatic activity found in rat liver by Runquist et al. [7], and are at odds with those of Krisans et al. [6], who reported an absence of FPS localization in mitochondria analyzed by microscopy.

Although several enzymes of the presqualene segment of the isoprenoid biosynthetic pathway have been reported to be localized partially or totally in peroxisomes, Waterham and colleagues have clearly established that functional peroxisomes are not required for the normal functioning of such enzymes [4]. Indeed, in recent years three of these enzymes, mevalonate kinase, mevalonate pyrophosphate decarboxylase and phosphomevalonate kinase, have been shown unambiguously to be localized in the cytosol [2,3,24].

Interestingly, the structure of the 5' region of the *M. musculus* FPS gene (contig NT\_039234) is similar to that of *HsFPS* and, in fact, a cDNA sequence encoding a form of *M. musculus* FPS containing a putative mitochondrial targeting sequence is found in the NCBI database (AY399923). This observation suggests that the complex splicing pattern in the 5' region of *HsFPS* also occurs in other mammalian FPS genes, and that mitochondrial targeting of FPS is not exclusive to humans. Further literature searches and database analysis revealed a number of lepidopteran, homopteran, coleopteran and dipteran insect species in which FPS has an N-terminal extension containing typical features of a mitochondrial targeting peptide. This suggests that targeting of FPS to mitochondria is widespread in eukaryotes. This hypothesis is further supported by our functional analyses using the insect *D. melanogaster* as a model system. The isoprenoid pathway in insects has two relevant peculiarities: the absence of the sterol branch, which makes insects unable to synthesize cholesterol de novo, and the synthesis of juvenile hormone, an insect-specific sesquiterpenoid molecule that regulates a number of physiological processes, including embryogenesis, metamorphosis,

and reproduction [25]. These two constraints have led to the occurrence of specific regulatory mechanisms controlling the whole pathway, and make insects an especially interesting model in which to analyze the regulation of the mevalonate pathway and its organization into subcellular compartments. Our analyses in both yeast and *Drosophila* S2 cells indicate that the N-terminal extension of FPS from *D. melanogaster* functions to target the protein to mitochondria.

Previous studies based on WSR complementation assays suggested that the N-terminal extension of FPS from the plant *A. thaliana* contains a mitochondrial targeting sequence [9]. The data obtained here indicate that this is also true of some vertebrate (mammals) and invertebrate (insects) animals, thus suggesting that it is a general phenomenon in eukaryotes. The machinery for protein import into mitochondria requires a transit peptide that binds to receptors on the mitochondrial surface, a membrane potential across the inner membrane, and ATP to maintain the precursors in an unfolded state. When the precursor has penetrated the mitochondrial matrix, the NH<sub>2</sub>-extension is removed by additional processing machinery, and the protein is directed to the corresponding submitochondrial compartment [26,27]. Our results indicate that, for FPS, such an exquisitely complex translocation system is conserved not only in plants, but also in animals.

In both humans and fruit flies (present results), as in the plant *A. thaliana* [9], FPS is an alternatively spliced gene, encoding mitochondrial and non-mitochondrial isoforms. This is consistent with the reported localization of FPS in peroxisomes in mammal cells [5], which would be predicted to use the non-mitochondrial isoform. Targeting of FPS to mitochondria may offer greater versatility for regulation of the entire isoprenoid pathway by subcellular compartmentalization. In particular, it could facilitate a tighter regulation of those isoprenoid derivatives that are needed in the mitochondrion itself, such as ubiquinone, the isoprenoid moiety of heme a, and prenylated proteins.

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