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# Stress-Induced Expression of a Novel Variant of Human Fumarate Hydratase (FH)

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Fumarate hydratase (FH) is an enzyme of the mitochondrial tricarboxylic acid cycle (TCAC). Here we report the characterization of a novel *FH* variant (*FHv*) that contains an alternative exon 1b, thus lacking the mitochondrial signal sequence. Distinct from mitochondrial FH, FHv localized to cytosol and nucleus and lacked FH enzyme activity. *FHv* was expressed ubiquitously in human fetal and adult tissues. Heat shock and prolonged hypoxia increased *FHv* expression in a cell line (HTB115) by nine- and fourfold, respectively. These results suggest that FHv has an alternative function outside the TCAC related to cellular stress response.

Key words: Tricarboxylic acid cycle (TCAC); Fumarate hydratase (FH); Alternative exon; Hereditary leiomyomatosis and renal cell cancer (HLRCC); Hypoxia; Heat shock; Stress

## INTRODUCTION

Mitochondrial fumarate hydratase (FH) acts in the tricarboxylic acid cycle (TCAC, Krebs cycle), catalyzing the conversion of fumarate to malate. The FH gene, predicted to encode a 511-amino acid peptide, consists of at least 10 exons of which the first encodes a mitochondrial signal peptide. Mature, processed FH (without the signal peptide) is also present in the cytosol and is known to be involved in the urea cycle.

Recently, heterozygous germline mutations in this gene crucial for cellular energy metabolism have been shown to underlie a tumor susceptibility syndrome, hereditary leiomyomatosis and renal cell cancer (HLRCC; MIM 605839). This syndrome is characterized by benign cutaneous and uterine leiomyomas, renal cell cancer (RCC), and uterine leiomyosarcoma (ULMS) (15,28). Biallelic inactivation of *FH* has been detected in almost all HLRCC tumors, and therefore FH is suggested to function as a tumor suppressor (12,15,28). However, the link between the TCAC defect and tumorigenesis is still obscure. Several mechanisms have been suggested, but evidence supporting the pseudohypoxia pathway induction in particular has expanded. The accumulation of fumarate in cells due to the FH defect has been shown to inhibit hypoxia-inducible factor 1 (HIF1) prolyl hydroxylation, resulting in HIF1 stabilization that in turn increases the expression of HIF1-regulated genes, such as *vascular endothelial growth factor* (*VEGF*) (11,23).

In this study, we report the characterization of a novel *FH* variant (*FHv*) that includes an alternative exon 1b, and exons 2–10 identical to the *FH* transcript (*FH*). The strategy of two primary transcripts has been seen, for example, in the production of protein isoforms that localize to more than one intracellular compartment (18). According to our studies, exon 1b seems to be an untranslated 5′ sequence. The FHv protein localizes to the cytosol and the nucleus. The expression of *FHv* was clearly lower when compared to mitochondrial *FH* but it can be induced by heat shock and prolonged hypoxia.

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## MATERIALS AND METHODS

### *mRNA Expression of* FHv *in Human Tissues*

The relative expression of *FHv* exon 1b in different tissues (Human MTC™ Panel 1, BD Biosciences Clontech, Palo Alto, CA, USA and Human Fetal MTC™ Panel), and the *FH* and *FHv* expression levels in cell experiments were determined using TaqMan chemistry and the GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All primer and probe combinations were designed to span exon–exon junctions using Primer Express 3.0 (AB) (Fig. 1, Table 1). The human housekeeping genes phosphoglycerokinase (PGK) or β-actin (BA) were used to normalize the relative mRNA copy numbers in each sample (ABI PRISM 5700 Sequence Detection System User's Manual, AB).

### *Mutation Analysis of* FH *Exon 1b*

A total of 139 human DNA samples were screened for *FH* exon 1b mutations, including 21 cell lines (12 renal cell cancer, 4 prostate cancer, 5 sarcoma cell lines), 99 nonsyndromic HLRCC-associated tumors (39 ULMs, 43 RCCs, and 17 ULMSs), 9 HLRCC patient-derived tumors without detected second hits (16,30) as well as 10 blood samples from *FH* mutation-negative individuals (6 cases with early-onset RCC, and 4 cases with RCC or leiomyomatosis). The primer sequences for the genomic fragment were 5′-GGCTGTCAGAGAGGGTCCTA-3′ (forward) and 5′-TACGGGGGAAACCATAGTCA-3′ (reverse). Sequencing was performed using the Big Dye Terminator v.3.1 kit (Applied Biosystems) and an ABI3730 Automatic DNA sequencer (Applied Biosystems).

### *Constructs*

The cDNA of FHv was cloned into a pCI-neo Mammalian Expression Vector (Promega Corporation, Madison, WI, USA) and a pEGFP-N3 vector (BD Biosciences Clontech, Mountain View, CA, USA). Cloning primer sequences were 5′-TTTCTC GAGGCCGCCCTGAACTGTGGTTTGTTTC-3′ (forward) and 5′-TTTGTCGACTCACTTTGGACCCA

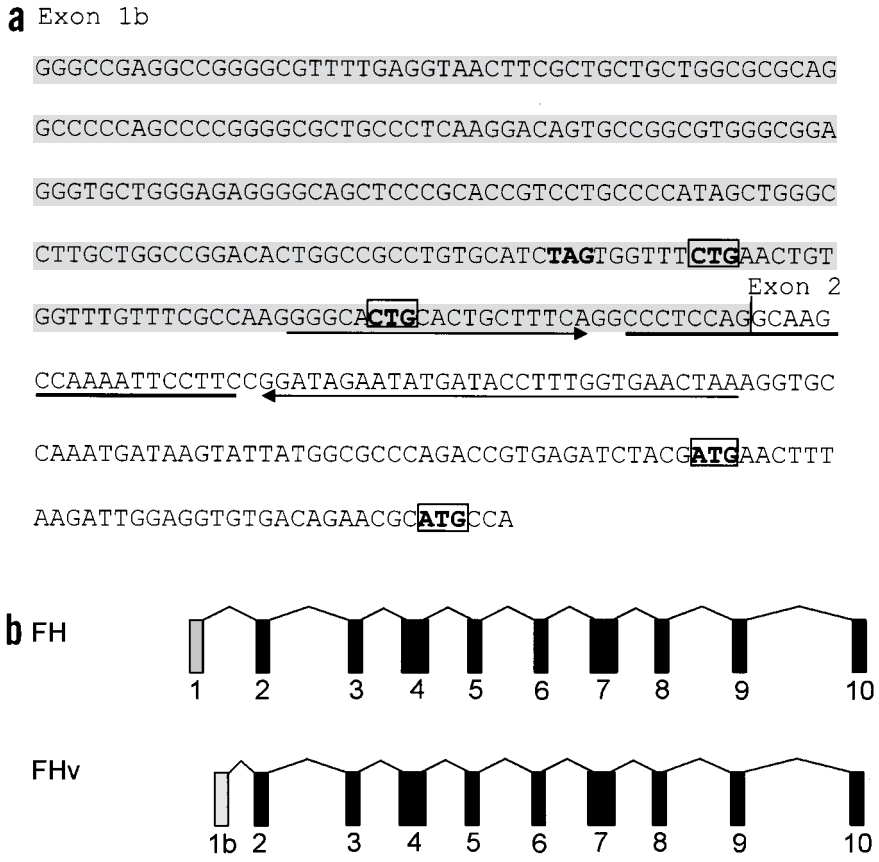


Figure 1. Exon organization of *FH* and *FHv*. (a) Schematic presentation of a 414-bp *FH* variant transcript (highlighted) (AI971779.1; http://ncbi.nih.gov/IEB/Research/Acembly/index.html). The putative translation initiation codons are boxed, and an in-frame stop codon (TAG) is in bold. The lines and arrows indicate the quantitative PCR probe and primer sequences, respectively. (b) Exon 1 encoding the mitochondrial targeting signal (MTS) of *FH* is in *FHv* replaced by alternative exon 1b. Exons 2–10 are identical in the transcripts.

TABLE 1
PRIMERS AND PROBES FOR RT QUANTITATIVE PCR OF *FHv* (EXON 1b) AND *FH* (EXON 1)

| Fragment | cDNA-Specific Primers (5′-3′) | Taqman Probes |
|---|---|---|
| Exon 1 | F: GCCGTGCCCTCGTTTTG | CCTCCGAACGCGGCTCGAATG |
| | R: TTCTATCCGGAAGGAATTTTGG | |
| Exon 1b | F: GGGCACTGCACTGCTTTCA | CCCTCCAGGCAAGCCAAAATTCCTTC |
| | R: TTAGTTCACCAAAGGTATCATATTCTATCC | |

GCATG-3′ (reverse) for pCI-neo and 5′-TTTCTC GAGGCCGCCCTGAACTGTGGTTTGTTTC-3′ (forward) and 5′-TTTGGATCCCTTTGGACCCAGCA TGTCCTT-3′ (reverse) for pEGFP-N3. With these primers the FHv sequence starts from the first CTG in exon 1b (Fig. 1). To identify the actual translation initiation codon, several constructs with different putative initiation sites were created in the basic FHv-pEGFP vector ($FHv_{ctg1ccc}$-GFP, $FHv_{ctg2ccc}$-GFP, $FHv_{ctg1atg}$-GFP, $FHv_{ctg2atg}$-GFP, $FHv_{atg1ccc}$-GFP, $FHv_{atg2ccc}$-GFP, and $FHv_{atg3ccc}$-GFP) by site-directed mutagenesis (QuickChange® Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). The sequences of the mutagenesis primers are available from the authors upon request. In addition, a construct without the sequence of the signal peptide and an additional ATG at the beginning of the exon 2 was created ($FH_{no\text{-}signal}$-GFP). All constructs were verified by direct sequencing.

### *Cell Culture*

Cell lines HEK293 (CRL-1573, ATCC) and HeLa (CCL-2, ATCC) were cultured in DMEM, and SK-UT-1B (HTB115, ATCC) in MEM. The culture medias were supplemented with penicillin-streptomycin (100 U/ml and 0.1 mg/ml) (Sigma-Aldrich) and with 5% FBS for HEK293 and 10% FBS for HeLa and SK-UT-1B. Culture conditions were normally set to 37°C, 5% $CO_2$, and 21% $O_2$. Additionally, 12 cell lines (originating from ULMS, RCC, prostate cancer, colorectal cancer, and immortalized kidney cells) were used in stress experiments.

### *Immunofluorescence and Cell Imaging*

Transfection of the cells in approximately 70% confluence was performed using FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN, USA). As a mitochondrial marker we used 100 pmol/ml of MitoTracker (MitoTracker Red CMXRos; Molecular Probes/Invitrogen, Carlsbad, CA, USA) and cells were fixed in 4% paraformaldehyde 24 h after transfection. Immunodetection of the endogenous FH protein was performed with porcine FH antibody (1:100; Nordic Immunology, Tilburg, The Netherlands) and FITC-conjugated goat anti-rabbit IgG. Nuclei were visualized with Hoechst (33258) stain. Localization of the proteins was monitored using a Leica TCS SP1 confocal microscope or an Axioplan upright epifluorescence microscope.

### *Stress Experiments*

Cell lines were cultured under different stress conditions including glucose or serum deprivation, hydrogen peroxide exposure, hypoxia, and heat shock. For glucose and serum deprivation, the cells were cultured in DMEM without glucose and either with or without 0.1% FBS for 24 and 48 h. Hydrogen peroxide exposure was performed by incubating the cells with 200 μM $H_2O_2$ for 30 min, after which the medium was replaced with normal medium, and incubation was continued for 24 and 48 h. The cells were exposed to hypoxia (37°C, 1% $O_2$, 5% $CO_2$, 94% $N_2$) for 24, 48, 72, and 96 h in a hypoxia incubator (Invivo2 Hypoxia Workstation 400, Ruskinn Technology Ltd, Leeds, UK). The heat stress was generated by incubating the cells at 42°C in a regular culture incubator for 0.5, 1, and 2 h. After the treatments total RNA was extracted from the cells and analyzed by quantitative PCR as described above. β-Actin was used for normalization of the expression levels.

### *Western Blot*

For Western blotting 25 μg of protein from whole cell extract was loaded into a gel, and the FH protein was detected using porcine FH antibody (1:500; Nordic Immunology, Tilburg, The Netherlands) and the ECL plus Western blotting detection system (Amersham Biosciences UK Ltd, Buckinghamshire, UK).

### *FH Enzyme Activity Assay*

HEK293 cells were transfected with FH and FHv-pCi-neo constructs with FuGENE 6 and cultured for 24 h. FH enzyme activities were measured from the

cell lysates as described previously (31). Nontransfected cells were used as a negative control.

### *In Silico Tools*

Genomatix tools (www.genomatix.de), Eldorado, GEMS Launcher, Gene2Promoter, and MatInspector, were used in the promoter and transcription binding site analyses. The conservation of genomic sequences was inspected using the ERC Browser (http://ecrbrowser.dcode.org/, http://www.ensembl.org/index.html, http://www.ncbi.nlm.nih.gov/dbEST/index.html) (3,21). CpG islands were predicted using Webgene (http://www.itb.cnr.it/sun/webgene//). The cage cap database (http://gerg01.gsc.riken.jp/cage/hg17prmtr/, http://fantom3.gsc.riken.jp/) (5), transcription start site database (http://dbtss.hgc.jp) (26), or variant transcript databases (http://statgen.ncsu.edu/asg/index.php, http://www.ebi.ac.uk/asd/) (17,25,27) were used to predict the 5′ end of the FHv transcript.

## RESULTS

### *Identification and Expression of* FHv *in Human Tissues*

Evidence for a variant *FH* sequence came from The AceView gene database (http://ncbi.nih.gov/IEB/Research/Acembly/index.html). A 414-bp sequence (GenBank accession No. AI971779.1) containing exon 2, a part of exon 3, and an upstream sequence (exon 1b) that mapped to the intronic region between exons 1 and 2 was derived from ovarian tissue (Fig. 1). In the transcribed sequence, exon 1b replaces exon 1, which encodes a mitochondrial signal peptide and targets the protein to the mitochondria. The *FHv* transcript including exon 1b and exons 2–10 was amplified using the cloning primers (see above). In silico studies showed no evidence for 5′ extension of exon 1b, CpG island, core promoter, evolutionary conserved transcription factor binding sites such as heat or hypoxia response elements, or nuclear localization signal in the *FHv* sequence. A transcript including exon 1b was not found in other species, but a similar kind of variant lacking the first exon encoding the mitochondrial signal peptide was found in another TCAC-related tumor suppressor gene, *SDHB* (http://vega.sanger.ac.uk/index.html).

The mRNA expression of *FHv* in different tissues was studied by quantitative real-time PCR. *FHv* was widely expressed in fetal and adult human tissues (Table 2, Fig. 2), and in several cancer cell lines. The expression of *FHv* was clearly lower compared to mitochondrial *FH*, with relative expression on average 200- and 400-fold that of *FHv* in fetal and adult tissues, respectively (Table 2).

TABLE 2
RELATIVE EXPRESSION OF *FHv* AND *FH* IN HUMAN TISSUES

| Tissue | *FHv* (SD) | *FH* (SD) | *FH*/*FHv* Ratio |
|---|---|---|---|
| Fetal | | | |
| Skeletal muscle | 3.37 (0.5) | 644 (38) | 191 |
| Lung | 1.62 (0.1) | 201 (0.0) | 124 |
| Brain | 7.53 (2.8) | 418 (27) | 55.5 |
| Kidney | 2.04 (0.5) | 276 (34) | 136 |
| Liver | 2.59 (1.2) | 697 (24) | 269 |
| Heart | 1.59 (0.1) | 443 (22) | 279 |
| Spleen | 1.33 (0.2) | 221 (23) | 166 |
| Thymus | 1.03 (0.1) | 170 (4.2) | 164 |
| **Average (median)** | **2.64** | **384** | **173 (165)** |
| Adult | | | |
| Skeletal muscle | 19.40 (5.5) | 1920 (777) | 99.2 |
| Lung | 12.60 (1.6) | 972 (199) | 77.1 |
| Brain | 18.00 (11) | 1350 (666) | 75.2 |
| Kidney | 8.89 (2.2) | 1579 (604) | 178 |
| Liver | 28.40 (2.9) | 9546 (3344) | 336 |
| Heart | 1.43 (0.6) | 3295 (178) | 2299 |
| Placenta | 17.50 (8.5) | 1226 (286) | 70.3 |
| Pancreas | 19.30 (7.9) | 2906 (1724) | 150 |
| **Average (median)** | **15.7** | **2849** | **411 (125)** |

Relative expression of *FHv* and *FH* within each sample was derived by proportioning the values to expression of the housekeeping gene *PGK*.

### FHv *Expression Is Induced in Heat Shock and Prolonged Hypoxia*

The effect of different stress conditions on the expression of *FHv* was studied in HEK293 and HeLa cells. Glucose and serum deprivation, $H_2O_2$ treatment, or hypoxia (1% $O_2$) for up to 48 h had no detectable effect on endogenous *FHv* or *FH* mRNA expression levels (data not shown). However, a trend in the induction of the *FHv* expression in more prolonged (72 and 96 h) hypoxia was seen. Subsequently, 13 cell lines were analyzed in prolonged hypoxia. The hypoxia-treated HTB115 cells (uterine leiomyosarcoma cell line) had fourfold increased *FHv* mRNA levels compared to the untreated control cells (Fig. 3a). A similar trend was also observed in approximately half of the other cell lines studied (6 out of 12; data not shown). Subsequently, HTB115 cells were used to analyze *FHv* response to heat shock. Incubating the cells at 42°C resulted in a gradual increase in *FHv* expression. At 0.5, 1, and 2 h *FHv* was induced up to three-, six, and ninefold, respectively (Fig. 3b). The same effect (with twofold induction in 2 h) was detected in HEK293 cells, the other cell line analyzed (data not shown). No changes were seen in the *FH* mRNA levels after hypoxia or heat shock (Fig. 3).

### *Translation of FHv*

Translation of FHv was studied by Western blots of proteins from HEK293 cells transfected with FHv-

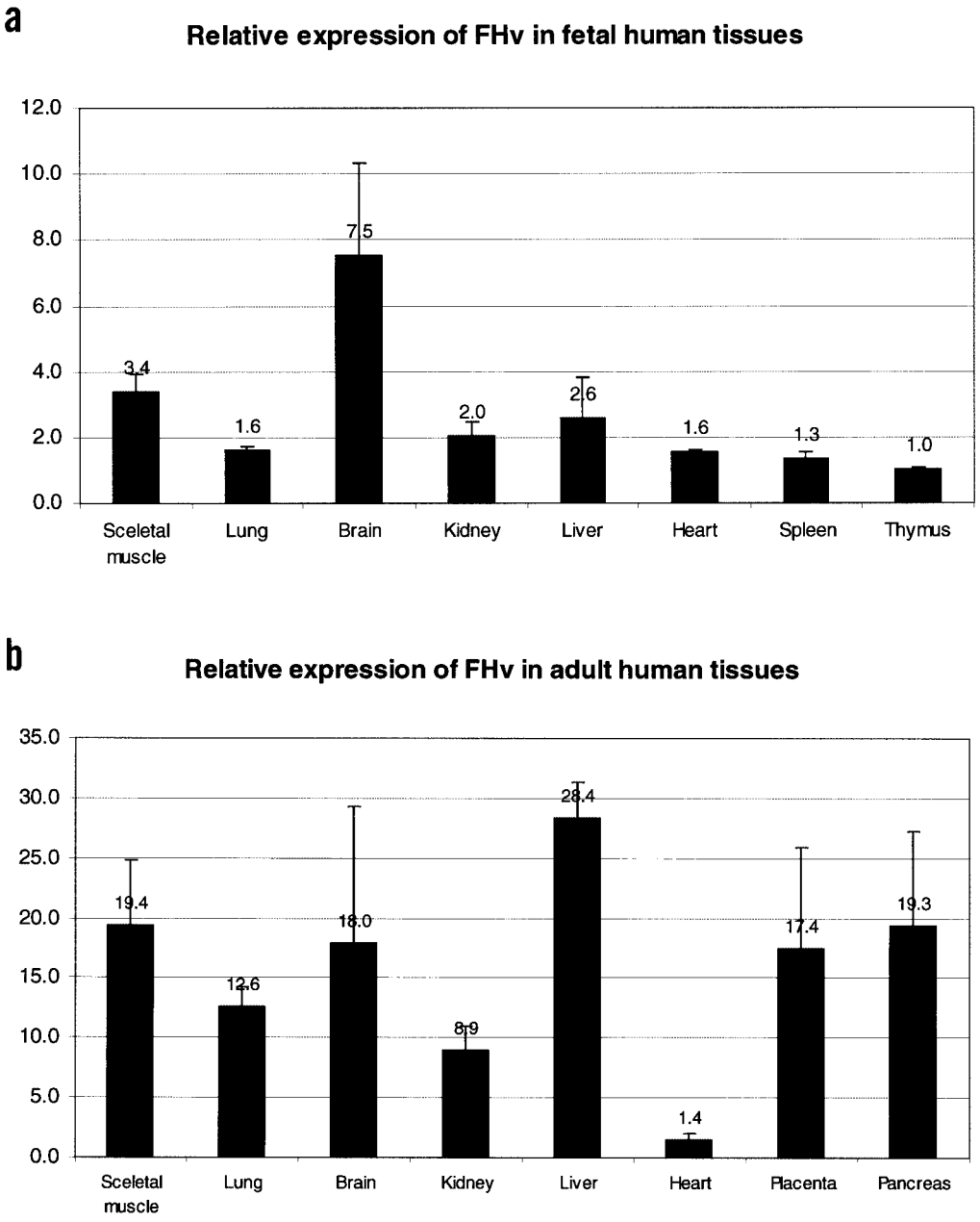


Figure 2. Relative expression of *FHv* mRNA in (a) fetal and (b) adult human tissues. The columns illustrate the relative expression of FHv in different tissues so that the value in the tissue with lowest expression was set as 1. The housekeeping gene *PGK* was used for normalization of FHv expression.

GFP cDNA constructs. FHv-GFP resulted in three protein products, of which the middle one was very faint. The novel exon 1b does not contain an ATG to function as a translation initiation codon (Fig. 1). Therefore, five different in-frame codons were considered to be potential translation initiation sites for FHv: two CTGs in exon 1b and three ATGs in exons 2 and 3. To define the correct site, cDNA constructs enabling translation from the different putative codons were created (Fig. 4a). To evaluate the CTGs in exon 1b as initiation sites, they were individually mutated to CCC to inhibit translation, and to ATG to enhance it. Because none of the three bands vanished after mutation of the CTGs to CCC (constructs $FHv_{ctg1ccc}$-GFP and $FHv_{ctg2ccc}$-GFP), the possibility of translation of FHv from the CTGs was excluded. This result was confirmed by $FHv_{ctg1atg}$-GFP and $FHv_{ctg2atg}$-GFP constructs (the CTGs mutated to ATG), which resulted in an additional larger peptide (translated from the enhanced CTG site) and the three protein

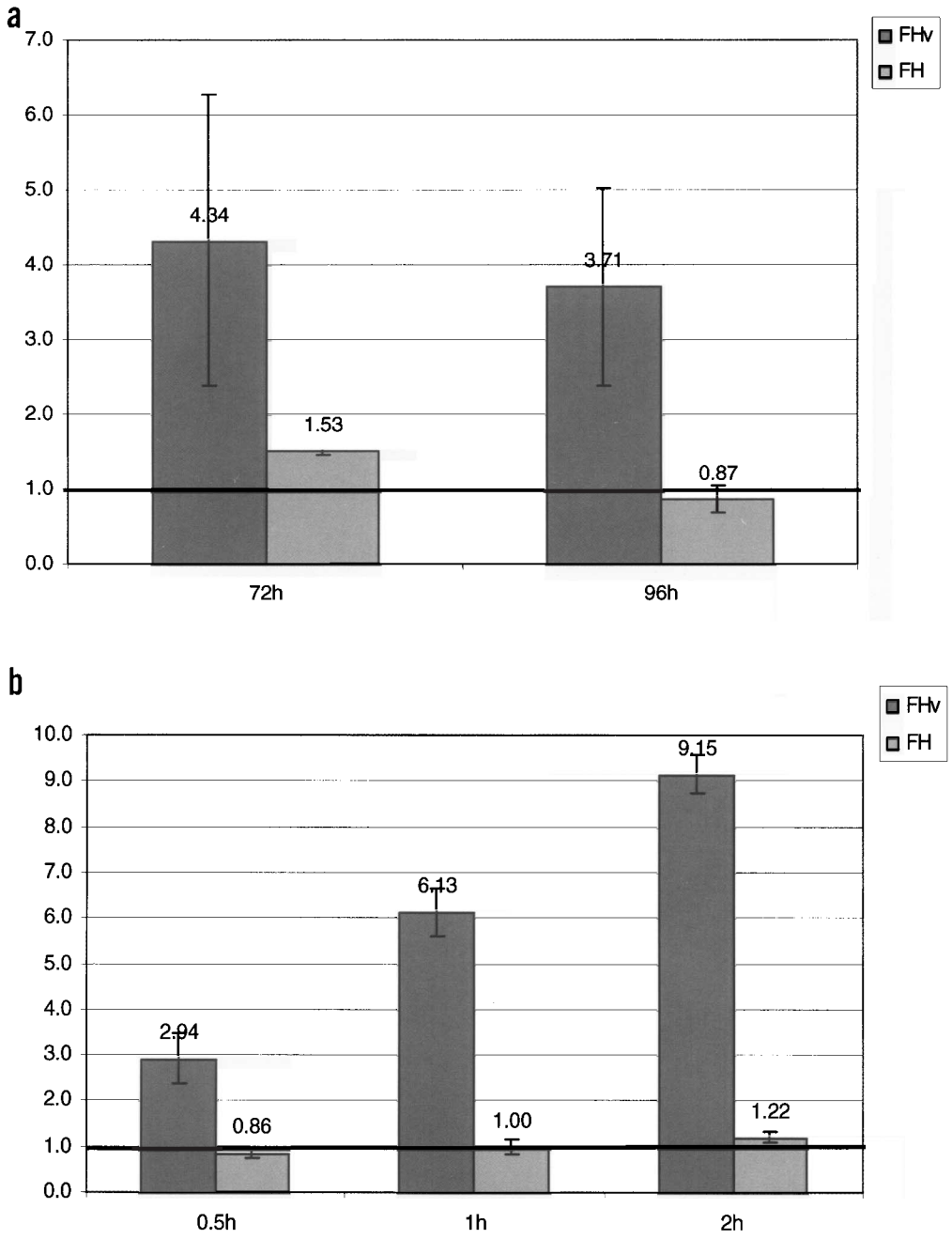


Figure 3. Relative mRNA expression of *FHv* and *FH* in HTB115 cells after hypoxia (1% $O_2$) and heat shock (42°C). (a) *FHv* mRNA expression increased fourfold in hypoxia when compared to the control cells during 72–96-h exposure. (b) In heat stress, *FHv* expression increased gradually during 2-h exposure up to ninefold. No changes were observed in the expression of *FH* in either condition. The results are shown as a ratio of the expression of stress-treated cells and nontreated control cells (the ratio of 1 is obtained if there is no difference between treated and control samples). Because expression level of *FHv* in general is much lower than of *FH* (in HTB115 cells over 100 times), the expression levels of *FHv* and *FH* were analyzed individually and are not comparable with each other. The housekeeping gene β-actin was used for normalization of *FHv* and *FH* expression. The error bars indicate the SD.

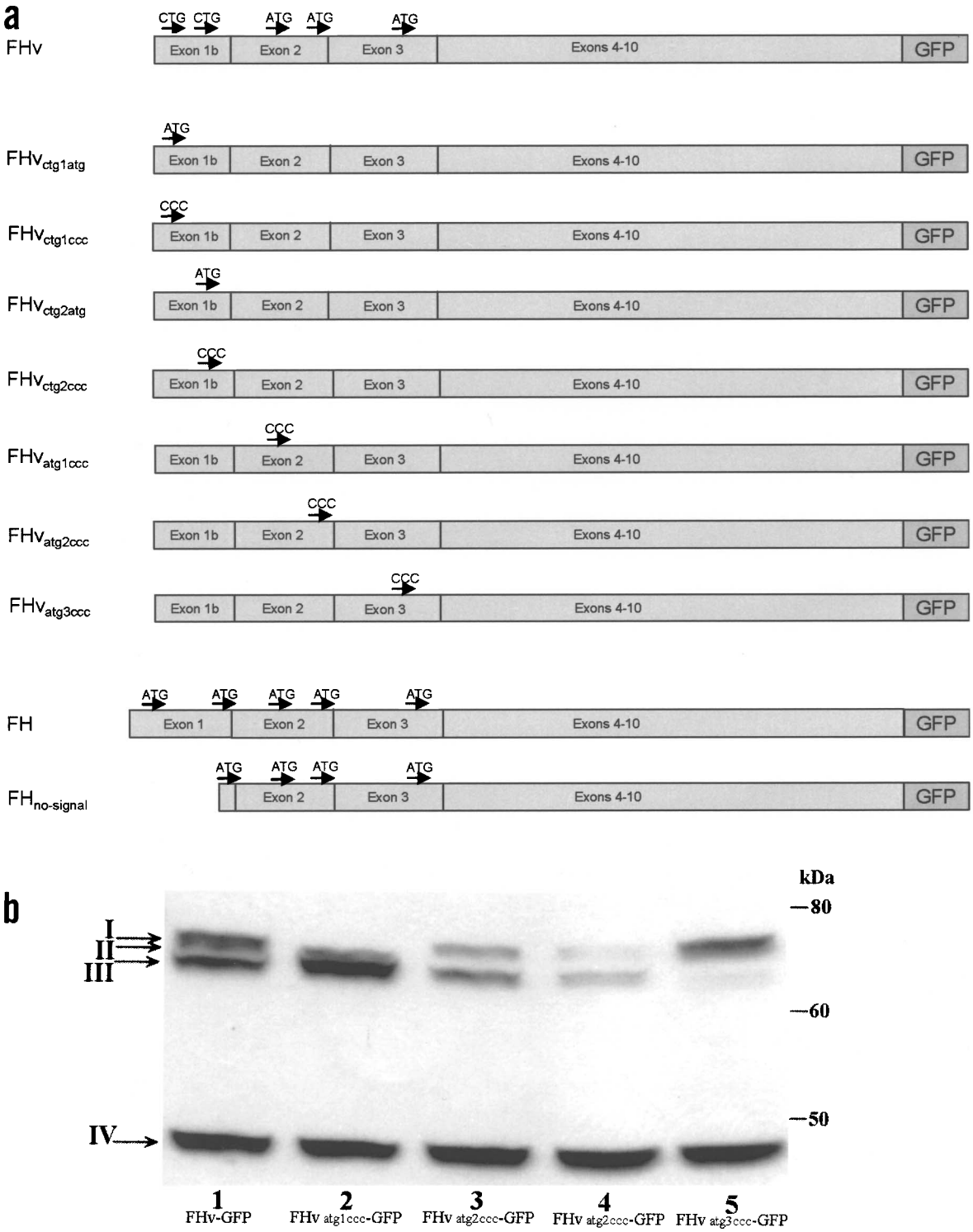


Figure 4. FHv-GFP and FH-GFP constructs and analysis of translation initiation of FHv by Western blot. (a) The putative translation initiation codons in exons 1b, 2, and 3 are illustrated. To define the translation initiation site of FHv, the function of the putative codons were either amplified (CTG → ATG) or inhibited (ATG → CCC). In the mutated FHv-GFP constructs only the altered codons are presented. Translation of the mitochondrial FH initiates from the first ATG of exon 1, which encodes the mitochondrial targeting signal. The $FH_{no\text{-}signal}$ construct represents the processed form of FH after removal of the mitochondrial targeting signal. (b) In lane 1, FHv-GFP shows three translation products (I, II, and III). When the first ATG in exon 2 was mutated to CCC ($FHv_{atg1ccc}$-GFP), the largest of the three bands (I) vanished, as seen in lane 2. Similarly, when the other ATGs in exon 2 ($FHv_{atg2ccc}$-GFP) and 3 ($FHv_{atg3ccc}$-GFP) were mutated, translation was inhibited and no corresponding bands (II and III) were seen (lanes 3–4, replicates, and lane 5, respectively). Band IV represents endogenous FH (lanes 1–5).

products identical to that of FHv-GFP (data not shown). Subsequently, the next ATGs (two in exon 2 and one in exon 3) were analyzed by mutating them to CCCs ($FHv_{atg1ccc}$-GFP, $FHv_{atg2ccc}$-GFP, and $FHv_{atg3ccc}$-GFP). Translation of FHv initiated in vitro from all three ATGs studied, with the resulting peptides approximately 4, 5, and 8 kDa smaller in size than FH. These equate with the predicted sizes (46, 45, and 42 kDa) of peptides generated from the three ATGs (Fig. 4b). Consequently, these results suggest that exon 1b is a 5′ UTR of FHv.

*Subcellular Localization of FHv*

The cellular localization of FHv was analyzed by overexpressing the FHv-GFP construct in HEK293 cells. Confocal immunofluorescence microscopy showed that the FHv-GFP fusion protein localized in the nucleus and in the cytosol (Fig. 5A). Proteins translated from the three GFP constructs with mutated ATGs ($FHv_{atg1ccc}$-GFP, $FHv_{atg2ccc}$-GFP, and $FHv_{atg3ccc}$-GFP) localized similarly to the FHv-GFP protein (data not shown). For comparison, the construct lacking exon 1 ($FH_{no\text{-}signal}$) localized to the cytoplasm (Fig. 5B). Endogenous FH (Fig. 5C) as well as FH-GFP (Fig. 5D) localized to mitochondria. In addition, a subset of cells expressing FH-GFP showed cytosolic fluorescence.

*FH Activity*

An FH enzyme activity assay was used to quantify the activity of FHv. The activity of the nontransfected cells (HEK293) was 36 nmol/min/mg of protein, representing the endogenous enzyme activity of the cell line. The cells transfected with FH and FHv showed FH activity of 390 and 36 nmol/min/mg of protein. Overexpression of FH increased the activity 10-fold, whereas in the FHv transfected cells the value remained at the basal level, signifying that the FHv protein has no FH enzyme activity. The activities of the controls were also previously published by Ylisaukkooja et al. (31).

*Mutation Analysis of FH Variant Exon 1b*

*FH* exon 1b was screened for mutations from a total of 139 human DNA samples. Mutation analysis revealed no mutations in exon 1b and its flanking sequences.

## DISCUSSION

Fumarate hydratase (FH) is known to be an essential component of cellular metabolism. Recently, FH has also been described to be involved in tumor susceptibility, but the underlying molecular mechanism leading to tumorigenesis is still not fully understood. Here we have identified and characterized a novel human fumarate hydratase variant (*FHv*). In the *FHv* transcript, exon 1, which encodes the mitochondrial signal peptide of FH, is replaced with an alternative exon 1b (Fig. 1). These two *FH* transcripts might be generated by separate promoters as described earlier for several genes with alternative first exons (1,10). Removal of interaction or localization domains by alternative transcription start sites, translation start sites, or alternative splicing are known to be common mechanisms regulating protein localization and function (14,18).

*FHv* mRNA was found to be ubiquitously expressed in human tissues, although at relatively low levels. Because stress-related induction in the expression of splice variants has earlier been reported in several other genes, such as *VEGF* and *COX1* (7,20, 29), the expression of *FHv* was also studied under several stress conditions. Interestingly, significant induction of *FHv* mRNA expression was observed in the HTB115 cell line after heat shock treatment and prolonged (72–96 h) hypoxia. Many of the other cell lines studied showed similar induction, but these uterine leiomyosarcoma cells responded most strongly. Several conditions, such as elevated temperature, causing acute or chronic stress in cells can induce an event called the heat shock response (2,22,24). In this response, heat shock factors (HSFs) induce the transcription and synthesis of proteins of the heat shock family (HSPs), as well as several other proteins (20,24). HSPs are involved in the folding of proteins, regulation of the cellular redox state, and in protein turnover. As a result of stress, HSPs either promote the survival of the cell or, if the stress is too severe, induce apoptosis (24). In addition to heat, hypoxia is also known to result in the heat shock response and, interestingly, chronic hypoxia in particular induces the HSP70 and downstream pathways in a *Lymnaea stagnalis* snail model (8). Hypoxic conditions can also trigger specific hypoxia responses, including upregulation of hypoxia-activated transcription factors, of which HIF1 is the most important one (6). Recently, hypoxia and heat shock response were linked in a novel manner in a study in which HIF1 was shown to regulate HSF and activate the heat shock pathway in hypoxia (2). These results suggest that FHv could be regulated by stress-induced factors and play a role in the adaptation of a cell to a disadvantageous environment or to be related to the stress-induced apoptosis. Other hypothetical functions of FHv could be involvement in the processing of cytoplasmic fumarate or in the shifting of energy metabolism from oxidative phosphorylation toward the gly-

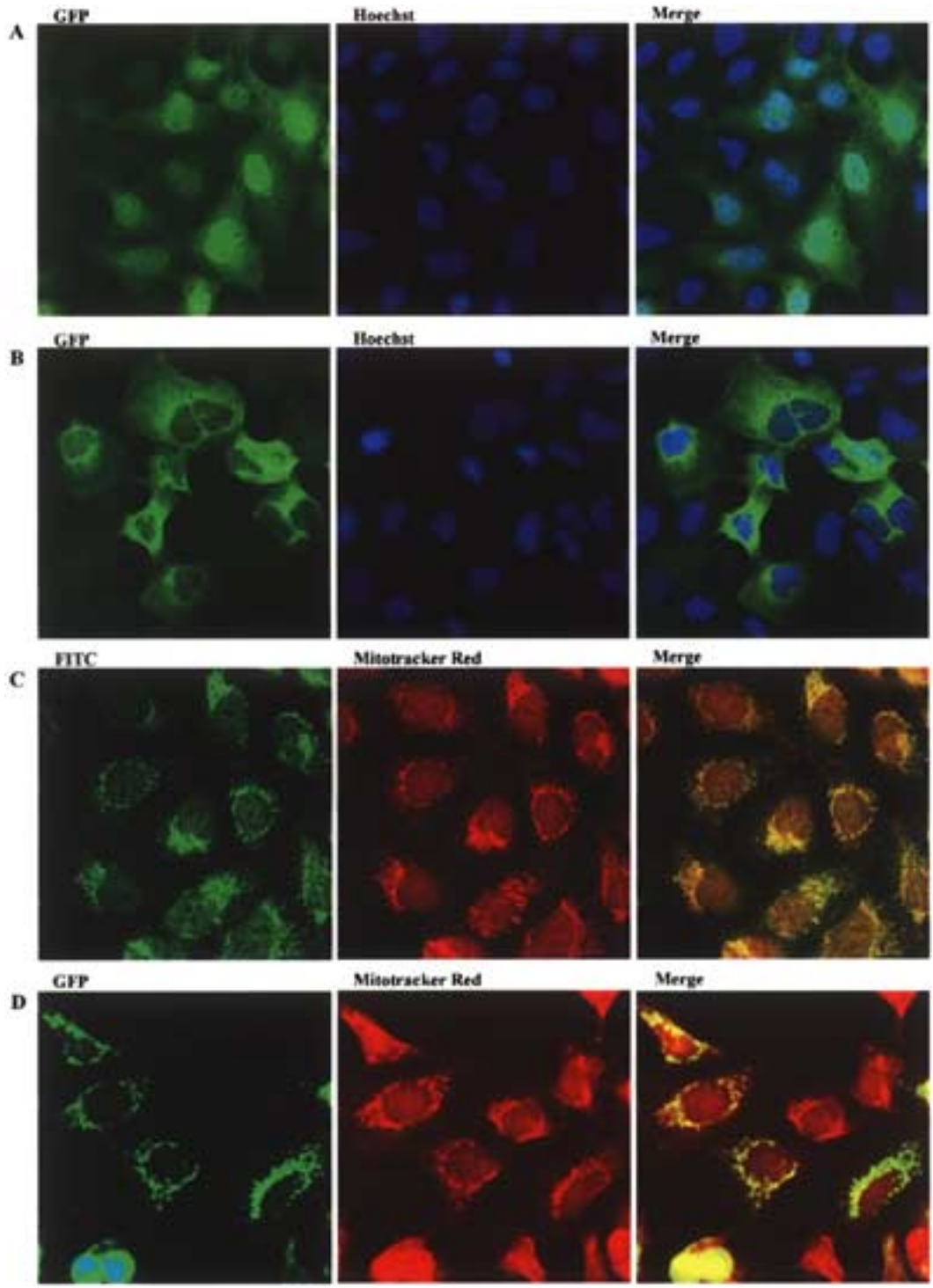


Figure 5. Cellular localization of FH and FHv in HEK293 cells. The localization of the proteins was compared to nuclear staining (Hoechst 33258) and a mitochondrial marker (MitoTracker Red). Yellow fluorescence generated in the merged images indicates colocalization of FH and the mitochondrial marker. (A) FHv-GFP localized to the cytosol as well as the nuclei. (B) The FH construct without mitochondrial targeting signal ($FH_{no\text{-}signal}$-GFP) localized as expected to the cytosol. The cell membrane and the filopodia were also highly fluorescent. (C) Endogenous FH localized to the mitochondria. FH was visualized by polyclonal FH antibody and FITC-conjugated secondary antibody. (D) FH-GFP fusion protein localized to the mitochondria similarly as endogenous FH.

colytic pathway. However, these hypotheses remain to be verified.

The endogenous FHv protein could not be detected probably due to low expression. Our overexpression studies suggested that translation of FHv is initiated from three ATGs located in exons 2 and 3, and that exon 1b seems to be an untranslated 5′ sequence. These initiation sites generate proteins of 32, 43, or 77 amino acids shorter (initiated from the first, second, or third ATG, respectively) than the processed FH protein without the signal peptide (467 amino acids). According to the sequences flanking the ATGs, translation from the first and the third codons are supported most strongly (13). The absence of some of the N-terminal amino acids might have an effect on the formation of the homotetramer (the active form of FH) and thus on the catalytic activity of the protein. This may result in an inactive form of the protein and explain the observed absence of enzyme activity. However, FHv might be unrelated to fumarate metabolism, and therefore the lack of enzyme activity could be inconsequential to the function of FHv.

Although no nuclear localization signal was found by in silico modeling, FHv-GFP was found to localize into the nucleus and the cytosol. Localization of FHv-GFP was also studied with mutated constructs ($FHv_{atg1ccc}$-GFP and $FHv_{atg3ccc}$-GFP) in order to detect any differences between the peptides produced from the two most likely initiation sites. However, no additional data were obtained because the peptides produced either from the first or the third ATG localized similarly. Thus, whether FHv is truly translated as various peptides remains unclear. As expected, FH-GFP localized to the mitochondria, and a subset of cells also displayed cytosolic expression. Of note, with more sensitive methods (immunogold labeling and transmission electron microscopy) endogenous FH has been previously detected in the nucleus, as well as in zymogen granules and endoplasmic reticulum, of rat tissues (4). Several other examples describing extramitochondrial localization of primarily mitochondrial proteins have also been published (4, 18). Therefore, we searched in silico for similar kinds of variants existing in three other nuclear encoded mitochondrial proteins (SDHB, SDHC, and SDHD; three subunits of succinate dehydrogenase), which are known to act as tumor suppressors (9). An alternative transcript lacking the first exon encoding the mitochondrial signal peptide was found in the *SDHB* gene. Both FH and SDH operate in the TCAC, and same pathways leading to the tumorigenesis are activated due to the defects in FH and SDH function (9). It could be hypothesized that the regulation mechanism of *FH* and *SDHB* alternative transcripts would also be similar.

In summary, we identified and characterized a novel variant form of *FH*. *FHv* was found to be expressed ubiquitously in human fetal and adult tissues, but the expression levels were clearly lower when compared to the mitochondrial form of *FH*. FHv localized to both the cytosol and nucleus and it had no FH enzyme activity. *FHv* mRNA expression was significantly induced in stress conditions. FHv was in vitro translated from three alternative ATGs but endogenous expression of the FHv protein was not detected reliably, probably due to low expression levels. These findings provide evidence of an alternative function for FH, but the physiological significance of *FHv* remains to be verified.

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