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The Ferritin-Heavy-Polypeptide-Like-17 (FTHL17) gene encodes a ferritin with low stability and no ferroxidase activity and with a partial nuclear localization

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ABSTRACT

Background: Three functional ferritin genes have been identified so far in mammals, and they encode the cytosolic Heavy (FTH) and Light chain (FTL) and the mitochondrial ferritin. The expression of a transcript by a fourth ferritin-like gene (Ferritin-Heavy-Polypeptide-Like-17, FTHL17) on the X chromosome was reported in mouse spermatogonia and in early embryonic cells.
Methods: The intronless human FTHL17 gene encodes a protein with 64% identity to human FTH with substitution of key residues of the ferroxidase center. The gene was cloned into vectors for expression in *Escherichia coli* and mammalian cells, linked to a flag-tag.
Results: The recombinant FTHL17 from *E. coli* purified as an assembled 24-mer ferritin devoid of ferroxidase activity and with a reduced physical stability. When transiently expressed in mammalian cells the flag-FTHL17 assembled in ferritin shells that showed reduced stability to denaturants compared with flag H and L ferritins. Immunocytochemistry with anti-flag antibody decorated the nuclei of flag-FTHL17 transfected COS cells, but not those of the cells transfected with flag-FTH or flag-FTL.
Conclusions: We concluded that FTHL17 encodes a ferritin-like protein without ferroxidase activity. Its restricted embryonic expression and partial nuclear localization suggest that this novel ferritin type may have functions other than iron storage.
General significance: The work confirms the presence of a fourth functional human ferritin gene with properties distinct from the canonical cytosolic ones.

1. Introduction

Ferritins are iron storage proteins, found in bacteria, archaea, plants and animals which are characterized by the assembly in almost spherical structures with a cavity for the accumulation of mineralized iron. They are typically highly stable and are composed of one or two subunit types [11]. Bacteria have up to four genes for different ferritin types that form distinct homopolymeric proteins composed of 24 or 12 subunits [3]. In mammals two ferritin genes with similar structure and 4 exons, FTH and FTL, cooperate for the formation of the cytosolic ferritins [12]. The human and mouse genomes contain more than 40 non-interrupted ferritin-like sequences, which are probably processed pseudogenes truncated of the 5′ end and with polyA sequence, which is typical of retrotransposition [34]. The mitochondrial ferritin gene (FtMt) is probably part of this group, is intronless with high homology with the FTH transcript and is highly conserved in mammals but not in other organisms [15]. The FtMt expression is tightly tissue/cell specific and almost exclusively found in the testis and spermatozoa, in the heart, brain and kidney [25]. It strongly differs from FTH and FTL, which are ubiquitous. Another functional ferritin-like gene may exist, it has been named FTHL17 (ferritin-heavy-polypeptide-like-17), is located on chromosome X and it was shown to be expressed. It was originally found to be part of a group of 25 genes specifically expressed in mouse spermatogonia but not in somatic cells [32]. Interestingly, 10 of the 25 genes identified in this systematic search were X-linked, and FTHL17 transcript was found only in the testis, but not in other tissues [32]. The work identified also the human ortholog on chromosome X, which also was specifically expressed in the testis. Work from another group confirmed the finding and added that FTHL17 can be expressed also in some forms of cancer, and thus it is part of the cancer-germline group of genes [19]. Moreover, they showed that FTHL17 expression can be induced in cancer cell lines by DNA demethylating agents such as 5-aza-2′-deoxycytidine. The finding that FTHL17 is specifically expressed in spermatogonia and in embryonic cells convinced

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Takahashi and Yamanaka to include this gene in the 24 candidate factors to induce pluripotency in somatic cells in their pioneering work on the induction of pluripotent stem cells from mouse cells [30]. FTHL17 shows evidence of gene conversion, and thus it was analyzed together with other 6 genes on chromosome X of human, chimp, mouse and rat to study the role of gene conversion in evolution [14]. The human FTHL17 gene was included in an epigenetic study of the X chromosome in the 46,XX-maleness sex chromosomal anomaly, which showed that the 24 methylation sites in the CpG islands of this gene were highly methylated both in males, females and in the patients tested [24]. The mouse FTHL17 is part of a large palindromic region of about 100 kb which includes 6 copies of the FTHL17 gene. The genes were highly expressed in testis and spermatogonia, with low expression in pachytene spermatocytes and round spermatids [23]. More recently, in a study to test sex differences in early stage embryos, the male and female blastocysts were separated and the gene expression pattern analyzed [13]. This showed that FTHL17 was predominantly expressed in female blastocysts. These genes are imprinted and expressed from the paternal X chromosome as early as the two-cell stage [13].

Altogether the data show that both human and mouse FTHL17 are expressed in embryonic germ cells, and perhaps in some cancer cells. The human FTHL17 seems to be a single gene located in chromosome Xp21.1, while mouse FTHL17 comprises a six-gene family in chromosome XqA1 [13,23]. So far the evidence for expression are limited to the identification of the transcript in spermatogonia and early embryos. This expression pattern is unusual for a storage protein and is typical for regulatory proteins. Thus, it is of interest to evaluate if the FTHL17 has ferritin-like properties, such as the capacity to assemble in a stable ferritin shell that can incorporate iron in the cavity, and which is its subcellular localization.

2. Experimental procedures

2.1. Plasmid construction

The full human FTHL17 gene was PCR amplified from human genomic DNA and subcloned into pET12a vector (Novagene) in the NdeI and BamHI sites. The constructs were verified by DNA sequencing, which showed that the sequence of human FTHL17 was correct. The constructs were used to transform *Escherichia coli* XL1 for plasmid amplification (Promega Kit) and in *E. coli* BL21-pLys for protein expression. The construct was used to produce and purify the human recombinant FTHL17 and compare its biochemical properties with those of the recombinant human FTH and FTL, already available in our laboratory. For cloning into pcDNA3.1 the human FTHL17 was PCR amplified by using primers carrying NheI-Flag at the 5′-terminus and XhoI at 3′-terminus. The DNA fragment containing the full coding sequence of FTHL17 (556 bp) was digested with NheI and XhoI and cloned into pcDNA3.1. The same procedure was used to clone the human flag-FTH in pcDNA3.1. Human flag-FTL cloned into pcDNA3.1 was a generous gift by Dr. Shapiro [28] and it differs for a longer flag-tag and a 7-residue linker between the flag-tag and the first amino acid of the ferritin, thus producing a peptide of 204 amino acids and 24 kDa. The constructs were verified by DNA sequencing and then used for the expression in mammalian cell lines.

2.2. Recombinant FTHL17

For expression in *E. coli*, the BL21-pLYS cells were transformed with pET12a-FTHL17, the positive colonies were picked and grown for 16 h in LB medium then they were diluted and grown until 0.5 OD. Then IPTG was added to 0.4 mM final, and the cells were grown for another 3 h. They were collected and analyzed in SDS-PAGE 12%. The expression of FTHL17 was verified by Coomassie blue stain or by immunoblotting with anti-human FTHL17 (Sigma-Aldrich) or with monoclonal antibodies for human FTH and FTL [21]. The protein purification followed the procedure described in [26], briefly the transformed and induced *E. coli* were disrupted by sonication, the supernatant was added of anti-protease agents (anti protease Cocktail, Sigma Aldrich) to avoid degradation, and heated at 65 °C for 10 min and the supernatant collected. It was treated with DNase, precipitated with ammonium sulfate (523 g/L), and dialyzed in 20 mM Tris HCl, pH 7.4. For further purification, the preparation was run on Superose 12 column, and the main peak after void volume collected. Ferritin ferroxidase activity was analyzed as in [20]: 0.1 μM apoferritin in 0.1 M Hepes, pH 6.5, was supplemented with 0.1 mM freshly made ferrous ammonium sulfate, and development of the amber color of Fe(III) was followed at 310 nm for 5 min. For iron incorporation capacity the ferritins (1 μM, 0.5 mg/mL) were incubated for 2 h at room temperature with 0.5–4.0 mM freshly made ferrous ammonium sulfate in 0.1 M Hepes buffer, pH 7.0. The samples were run on non-denaturing 7% polyacrylamide gels and stained for protein (Coomassie blue) or iron (Prussian blue). For the thermal stability experiments, the ferritins were heated at the indicated temperature (range 22–90 °C) for 10 min, then chilled, centrifuged and the supernatants run on non-denaturing PAGE.

2.3. Cellular studies

For expression in mammalian cells, HepG2 cells were grown in Minimun Essential Medium with Earle's salts (MEM/EBSS, Euroclone), 10% fetal bovine serum (FBS, Euroclone), 1 mM sodium pyruvate (Euroclone), 1 mM L-glutamine 1× (Euroclone), 0.04 mg/mL gentamicine (ITALFARMACO). COS7 cells were grown in DMEM (Euroclone) as above, but without Na pyruvate. Cells (3×10^5) were seeded in 12-well plates (Midi petri, NUNC) and transfected the following day with 0.5–1 μg of pcDNA3-flag-FTHL17, pcDNA3-flag-FTH or pcDNA3-flag-FTL using PolyJet reagent (SignaGene Laboratories) following the manufacturer's instructions. They were grown for 24–48 h and then harvested and analyzed. For immunological experiments the transfected cells were lysed in lysis buffer (40 mM KCl, 25 mM Tris/HCl pH 7.5, 2 mM sodium citrate, 0.6 mM $MnCl_2$, 1 mM DTT, 1% Triton-X), containing a mixture of protease inhibitors (Sigma-Aldrich) or with buffers for nuclear fractionation as described below. Samples of 30 μg of protein were loaded on 12% SDS-PAGE or 8% non-denaturing PAGE and then blotted with anti-flag, anti-TfR1 (Invitrogen), or anti-LAMIN A/C antibodies (Sigma Aldrich), using peroxidase-labeled secondary antibodies (anti-mouse immunoglobulin G, Dako or anti-rabbit immunoglobulin G, BioFX Laboratories). Bound activity was revealed by advance enhanced chemiluminescence (ECL) kit (GE) and detected with KODAK Image Station 440CF (Kodak). For nuclear and cytosolic fractionation, the transfected HepG2 or COS7 cells were grown as above, collected, resuspended in Buffer A (10 mM HEPES KOH pH 7.9; 1.5 mM $MgCl_2$; 0.5 mM DTT; 2 μM PMSF; 10 mM KCl; 0.2% NP-40) incubated for 15 min at 4 °C and centrifuged at 13,000 rpm for 1 min at 4 °C. The supernatant was considered the cytosolic fraction. The pellet was dissolved in Buffer C (20 mM HEPES KOH pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; 0.2 mM EDTA; 2 μM PMSF; 25% glycerol) incubated for 15 min at 4 °C to cause the lysis of nuclei. These extracts were centrifuged at 13,000 rpm for 1 min at 4 °C, the pellet was discarded and the supernatant considered the nuclear fraction. The two fractions were analyzed in Western Blotting for the content of flag-FTL, flag-FTH or flag-FTHL17, of TfR1 as non-nuclear cytosolic marker and of Lamin A/C as nuclear marker. For immunofluorescence analysis, COS7 or HepG2 cells were grown for one day on glass slide, and then transfected with 0.5 μg/mL of pcDNA3-flag-FTHL17, pcDNA3-flag-FTL or pcDNA3-flag-FTH, as described above. The cells, 24 h after transfection, were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The expression of Flag-FTL17, Flag-FTL or Flag-FTH were verified with anti-flag-antibody and mouse anti IgG-FITC Alexa, whereas nuclei stained with DAPI (stock solution 0.2 mg/mL, 1000×, for 10 min). Cells were observed using a Zeiss axiovert S100 microscope equipped with Zeiss MC 80DX Sensicam 12 bit cooled Imagine and analyzed with Image-ProPlus 4.5.1.

2.4. ES cell culture

C57BL/6 derived mouse ES cells [4] were adapted to grow without feeder cells and maintained onto 1.5% gelatin-coated dishes in DMEM supplemented with 15% fetal bovine serum (Hyclone), 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 0.1 mmol/L β-mercaptoethanol, 2 mmol/L L-glutamine, and 1000 U/mL LIF (ESGRO, Chemicon). Then, $5 * 10^5$ ES cells were seeded on gelatin-coated 60 mm cell culture dishes and maintained in undifferentiated state in LIF-containing medium (day 0) or deprived of LIF to induce monolayer differentiation up to 7 days. ES cells were harvested at the indicated days (1, 2, 4, 5, and 7 days) to allow further analysis.

2.5. RNA extraction and RT-PCR analysis

Total RNA was extracted from mouse ES cells using TRI Reagent (Sigma-Aldrich), according to the manufacturer's instructions. Contaminating DNA was digested using DNAse, following indications reported in RNeasy® Micro Handbook (Qiagen). Two micrograms of total RNA was retro-transcribed with Improm reverse transcriptase (Promega) using oligodT primer in a final 20 μL volume. For semi-quantitative PCR, 2 μL of the retrotranscribed RNA were subjected to polymerase chain reaction (PCR) using ExtraTaq and specific Buffer XTaq (GeneSpine). PCR conditions for HPRT1: 95 °C 5 min; 94 °C 30 s, 62 °C 30 s, 72 °C 40 s (for 35 cycles); 70 °C 7 min and 4 °C. PCR conditions for FTHL17: 95 °C 5 min; 94 °C 30 s, 56 °C 30 s, 72 °C 40 s (for 35 cycles); 70 °C 7 min and 4 °C. The RT-PCR specific primers (final concentration 0.2 μM) were: FTHL17 forward 5′-GGAATCTCAT ATGGCCGAAGCGCCCTCTCGAG-3′; FTHL17 reverse 5′-CGCGGATCCCTT TGCTTCAAAACCAAGATGAACATC-3′; HPRT forward: 5′-CTGGTTAAGC AGTACAGCCCCAA-3′; HPRT1 reverse: 5′-CAGGAGGTCCTTTTCACCAGC-3′; FtL forward: 5′-GTCTCTGGGCGAGTATCT-3′; FtL reverse: 5′- CTAG TGGCTTGAGAGGTTCA-3′; FtH forward: 5′- TAAAGAACTGGGTGACCA CGTGAC; FtH reverse: 5′- AAGTCAGCTTAGCTCTCATCACCG.

3. Results

3.1. Predicted structure of FTHL17

Fig. 1 shows the alignment of the predicted amino acid sequence of human FTHL17 with those human FTH, FTL and FtMt chains. Compared with human H-chain the length of 182 residues is conserved, the homology is 81% with no gaps, and identity is 64%. The identity with the human L chain is slightly lower, 46%. An important feature of FTHL17 is the substitution of the critical Glu62 into Lys that was shown to be sufficient to abolish ferroxidase activity in human ferritin [16]. Also Glu61 of the center is substituted with an Asp, while the other residues of the ferroxidase center are conserved. FTHL17 has a high number of cysteines: 5, compared with 3 and 1 in the FTH and FTL, respectively. Cys 130 is conserved in all of them. Altogether the sequence indicates that FTHL17 should have a structure analogous to that of the H and L chains and be devoid of ferroxidase activity.

3.2. Recombinant FTHL17 expression

We cloned the human FTHL17 in the pET12a vector for expression in *E. coli*. A polypeptide with the expected molecular weight of 20 kDa was found in the cells induced with IPTG (Fig. 2A). The cells contained a component with slow mobility in non-denaturing PAGE similar to that of assembled human FTL and FTH, which we attributed to the assembled FTHL17 (not shown). This was enriched with the protocol used for the other recombinant human ferritins, which included heating

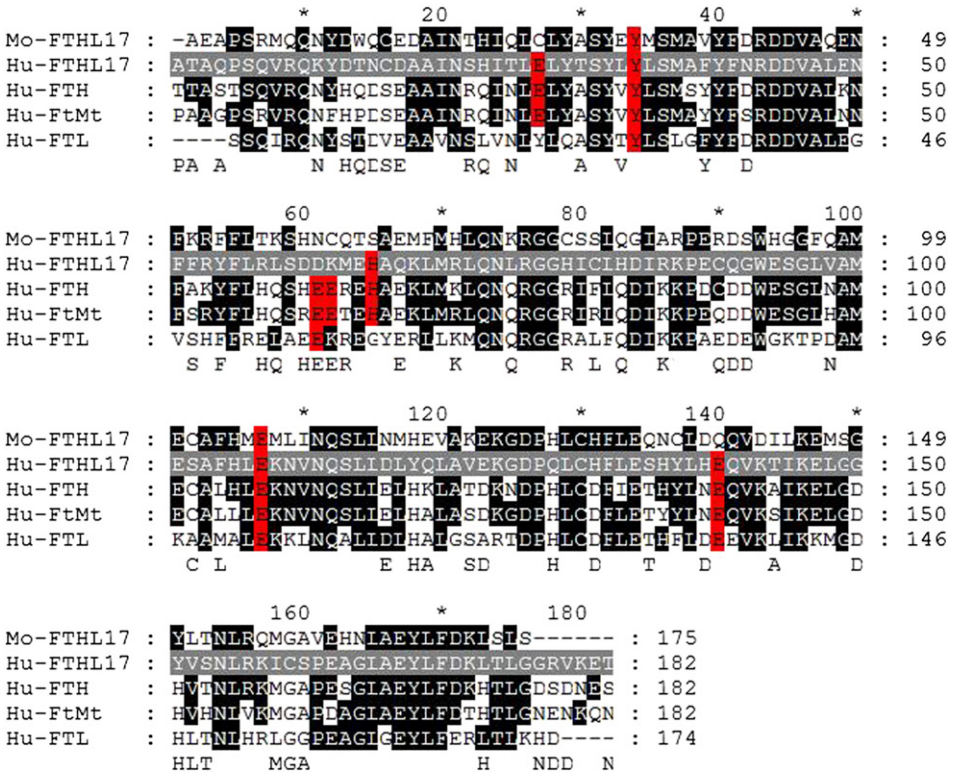

Fig. 1. Alignment of the amino acid sequences of the predicted mature ferritin subunits. The residues identical to those of human FTHL17 are in black background. In red the residues that form the ferroxidase center in the human ferritin H chain (Hu-FTH). The N-terminal extension of mitochondrial ferritin (Hu-FtMt) was removed. The identity (and homology) percentage are: human FTHL17 vs mouse FTHL17 = 56% (69%); vs Hu-FTH = 64% (81%); vs Hu-FtMt = 64% (79%); vs Hu-FTL = 46% (67%).

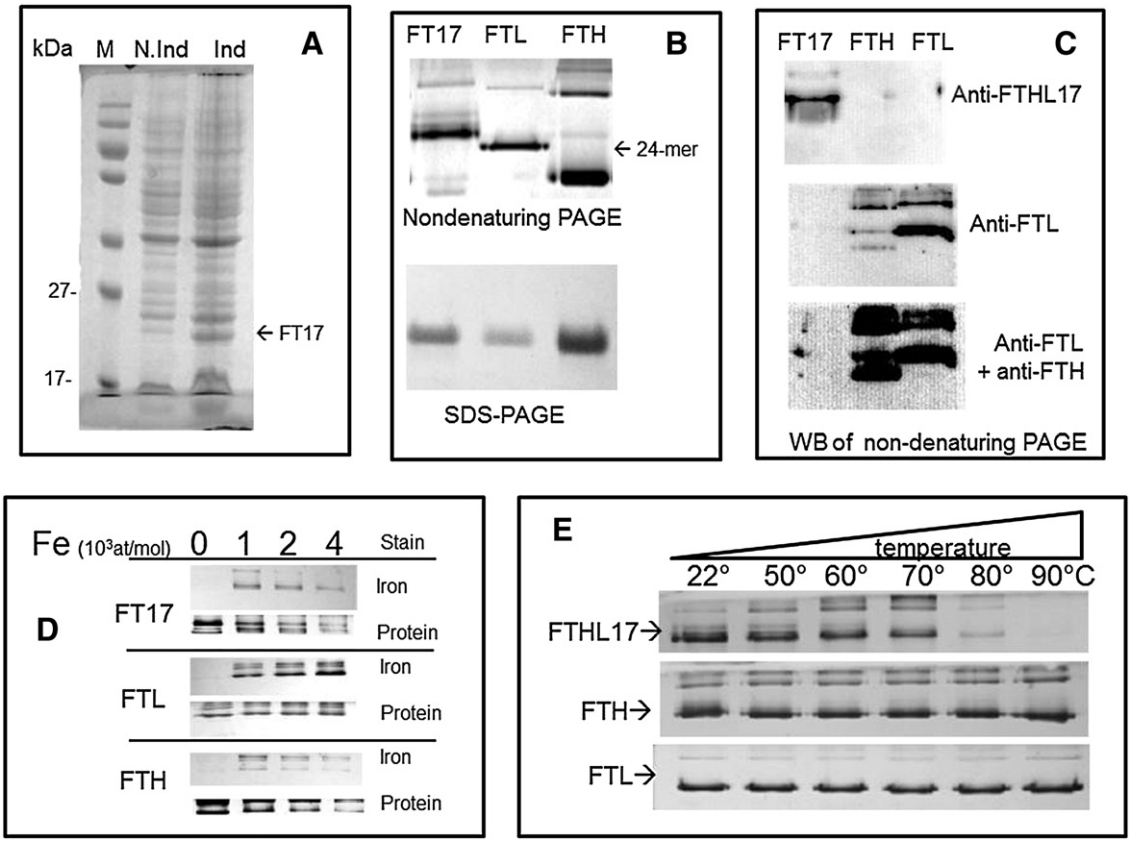

Fig. 2. Expression and characteristics of recombinant human FTHL17. (A) Expression gel of the *E. coli* transformed with pET-FTHL17 vector stained with Coomassie blue. A band of the expected molecular weight of 20 kDa is evident only in the induced (Ind) cells, but not in the non-induced ones (N.Ind). M is for molecular markers. (B) Analysis of the purified recombinant FTHL17 (FT17) in comparison with recombinant FTH and FTL, the upper panel shows non-denaturing PAGE indicating that FTHL17 assembles in ferritin shells, and the lower panel is the corresponding SDS-PAGE, both stained with Coomassie Blue. (C) Western Blotting of the non-denaturing PAGE using a polyclonal anti-human FTHL17, a monoclonal anti-human FTL and the same overlaid with a monoclonal anti-human FTH. (D) The purified preparations in Hepes pH 7.0 were added of the indicated Fe(II) increments (indicated as 1000 Fe/mol of ferritin), separated on non-denaturing PAGE and then stained for iron with Prussian Blue or for protein with Coomassie Blue. (E) The purified ferritins were incubated at the indicated temperature for 10 min, chilled and centrifuged and the supernatant loaded on non-denaturing PAGE and the gels were stained with Coomassie blue.

the homogenates at 65 °C and separation on gel filtration on Superose 12 column [26]. The preparation contained a single band of about 20 kD, with the same mobility of the human H and L ferritin subunits (Fig. 2B bottom) and in non-denaturing PAGE showed a major band with a mobility slightly slower than that of human FTL, traces of the oligomers typical of ferritin, and minor band with faster mobility, possibly a degradation product or contamination with the endogenous bacterial ferritin (Fig. 2B, top). The assembled FTHL17 was not recognized by a monoclonal antibody specific for human FTL and FTH in immunoblotting, while a commercial antibody for FTHL17 was specific for the antigen with no apparent cross reactivity with the other human ferritins (Fig. 2C). The ferroxidase activity was analyzed by adding the protein in Hepes pH 6.5 with Fe(II) and monitoring the formation of the amber-color Fe(III) at 310 nm. FTHL17 did not show activity and behaved similarly to FTL (not shown). To assess the iron incorporation capacity, the purified ferritins in Hepes pH 7.0 were added with various increments of Fe(II), then separated on non-denaturing PAGE and stained with Prussian blue. Under these conditions FTHL17 incorporated iron similarly to FTH and FTL (Fig. 2D). Finally to evaluate protein thermal stability, FTHL17 was incubated for 10 min in water bath with various temperatures, then chilled, centrifuged and separated on non-denaturing PAGE. At temperature above 70 °C FTHL17 fell out of solution, while FTH and FTL remained soluble and assembled (Fig. 2E). Altogether the data showed that FTHL17 is a ferritin devoid of ferroxidase activity that assembles in a ferritin shell that can incorporate iron in vitro. It differs from FTH and FTL for a remarkable lower thermo stability, and also for a much lower recovery of the purified recombinant protein.

3.3. FTHL17 expression in mammalian cells

The FTHL17 antibody was specific and recognized the recombinant FTHL17 expressed from *E. coli*, but its binding affinity was not sufficient to detect the low levels of FTHL17 expressed in mammalian cells. Thus, to facilitate the detection of the recombinant protein, we fused the flag-tag at the 5′ of the human FTHL17 in pcDNA3.1 vector. As a control we attached the same tag to the human FTH cloned in pcDNA3.1 and also we used a flag-tagged-FTL vector that was a kind gift of Dr. Shapiro [28]. This has a linker between the flag and ferritin sequence that makes a product of 24 kDa, in comparison with the 22 kDa of flag-FTHL17 and flag-FTH. The constructs were transiently transfected in HepG2 cells, the cells were harvested after 24 h and the homogenate analyzed. The cells transfected with pcDNA3-flag-FTHL17 and pcDNA3-flag-FTH expressed a peptide of 22 kDa detectable with anti-flag antibody. Also the cells transfected with the pcDNA3-flag-FTL expressed at similar level a peptide recognized by the anti-flag antibody with the expected molecular weight of 24 kDa (Fig. 3A). The flag-tag on the N-terminus of the subunit is exposed on the outer surface of the ferritin shell and not expected to interfere with ferritin assembly [22]. Thus the tag can be used to recognize both the assembled ferritin shells and the denatured subunits. We analyzed on non-denaturing PAGE the homogenates of the HepG2 cells transfected with the three flag-ferritins. They all showed a flag-positive band of slow mobility, similar to that of the fully assembled FTL and FTH (Fig. 3B). Human H and L ferritins are known to be resistant to incubation with 1% SDS at room temperature [27], and in fact flag-FTL and flag-FTH were not affected by this treatment. In contrast this caused the complete disappearance of the

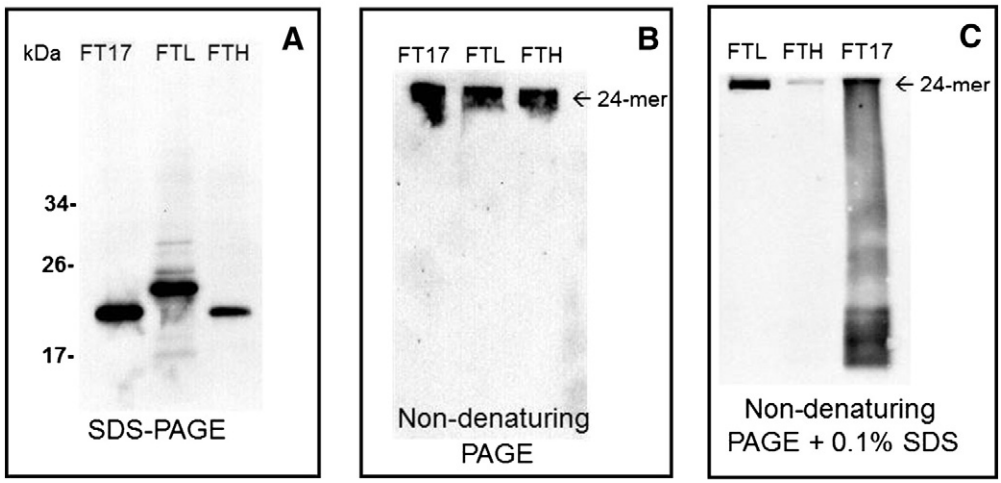

Fig. 3. Expression of flag-FTHL17 in mammalian cells. The cDNA for flag-FTHL17 was cloned in pcDNA3.1 vector for expression in mammalian cells, flag-FTH and flag-FTL were used as controls. HepG2 cells transfected with pcDNA3-flag FTHL17, with pcDNA3-flag FTH or pCDNA3-flag-FTL were grown for 24 h and then the homogenates analyzed on SDS PAGE (A) or non-denaturing PAGE (B) and blotted with anti-flag antibody. On non-denaturing PAGE the three ferritins showed similar mobility, while in SDS-PAGE they showed the expected molecular weight of 24 kDa for flag-FTL and of 21 kDa for flag FTHL17 and flag-FTH. (C) The homogenates of HepG2 cells transfected with flag-FTHL17 (FT17) or with flag-FTL (FTL) or flag-FTH were incubated for 10 min in 0.1% SDS at 21 °C, run on non-denaturing PAGE, and then blotted with anti-flag antibody. FTHL17 shell dissociated in the presence of cold 0.1% SDS, while FTL and FTH did not.

assembled flag-FTHL17 molecule, with the formation of a subassembly species (Fig. 3C). Thus, FTHL17 ferritin shell differs from FTL and FTH shells for not being stable in 0.1% SDS at room temperature.

3.4. FTHL17 is found in the nucleus of transfected cells

To study the subcellular localization of FTHL17 we performed immunofluorescence analysis of COS7 transfected and permeabilized cells using fluorescein-anti-flag antibodies. We obtained similar results using HepG2 cells (not shown) (Fig. 4). In flag-FTH transfected cells the fluorescent signal was mostly confined to the cytoplasm, in flag-FTL transfected cells the signal was uniformly distributed throughout the cell, while in flag-FTHL17 transfected cells the antibody strongly decorated the nuclei together with the cytoplasm (Fig. 4A). The homogenates of the transfected cells were separated into the cytosolic and nuclear fractions by sequential centrifugation, and blotting experiments showed that most ferritins were in the cytosolic TfR1 positive fraction. The nuclear, lamin-A positive fraction did not contain detectable FTH, traces of FTL and an evident band of flag-FTHL17 (Fig. 4B).

3.5. Expression of mouse FTHL17 in ES differentiation

Most studies analyzed mouse FTHL17, whose gene is also in the X chromosome and is expected to perform the same function of the human counterpart, despite an identity as low as 56%. We analyzed FTHL17 transcript by RT-PCR in various mouse tissues, and confirmed it to be expressed in the testis, but not in other adult tissues (not shown). We found it also in mouse ES cells. To verify that it is specific for germ cells, we allowed these cells to differentiate in monolayer in the absence of LIF for up to 7 days. FTHL17 transcript slightly increased in the first 2 days then it steadily decreased to disappear at day 7 (Fig. 5). The transcripts for FTH and FTL did not vary in this period. This finding supports the hypothesis that FTHL17 expression is restricted to embryonic cells.

4. Discussion

The recombinant human FTHL17 readily formed 24-mer ferritin shells when expressed in *E. coli* and was stable enough to be purified with the normal procedure for used mammalian ferritins, which takes advantage of the stability at temperature above 65 °C of these proteins. The protein behaved similarly to L ferritin and incorporated iron in vitro at pH 7.0, under conditions of spontaneous iron autoxidation. At lower pH values (pH 6.5), where the ferroxidase activity is needed to oxidize and incorporate iron, FTHL17 was inactive as expected for the substitution of a key residue of the ferroxidase center. A peculiarity of this ferritin was a reduced stability compared to the well characterized cytosolic H and L ferritins, and also mitochondrial ferritin. The protein disassembled at temperature >70 °C, while the cytosolic ferritins are fully stable above 80 °C heating. We observed also that FTHL17 was much more difficult to express and purify. It was cloned in the same vector used for FTH, it was recovered in the soluble fraction of the homogenate but at lower levels than FTH, and a large part of it was lost in the gel filtration purification step; we did not examined further if this was due to insolubility or denaturation/disassembly. In different purifications we obtained some 2–3 mg per liter of culture of assembled FTHL17, to be compared with the 20–80 mg of purified FTH per liter of cell culture we normally obtain. Altogether this study indicates that the recombinant FTHL17 behaves like a normal ferritin devoid of ferroxidase activity with some major stability problems. More interesting was the study of its behavior in mammalian cells. The N-terminal tagged FTHL17 assembled during expression in HepG2 cells, and behaved similarly to the flag-tagged FTH and FTL. However, the FTHL17 shell disassembled in the presence of 1% SDS, while FTH and FTL were fully stable under these conditions. This may suggest that FTHL17 shell is in an easier equilibrium with its subunits. The other interesting finding was that FTHL17 accumulated, at least in part, within the nuclei, at variance with FTH and FTL which are mostly confined in the cytosol. Analysis by pSort software did not indicate any nuclear localization signal in FTHL17, thus the mechanism of its transport to the nucleus is unclear.

The presence of ferritins within the nucleus has been observed in various cellular systems and studied with attention in canine corneal epithelial cells [2,5,7]. They lack a nuclear localization signal, and they have been proposed to act primarily as an iron scavenging molecule that protects DNA from iron-dependent oxidative damage [29,31]. The same role has been attributed to the ferritin-like bacterial Dps, that have ferroxidase activity and can bind to DNA [8,9]. However FTHL17 does not have a ferroxidase activity, thus it should not have a major role in protecting against oxidative damage. For example, the ferritin L chain is also devoid of ferroxidase activity and has no evident antioxidant activity [10,17]. Interestingly the translocation of FTH into the nucleus has been also observed in neurons upon CXCL12 stimulation [18]

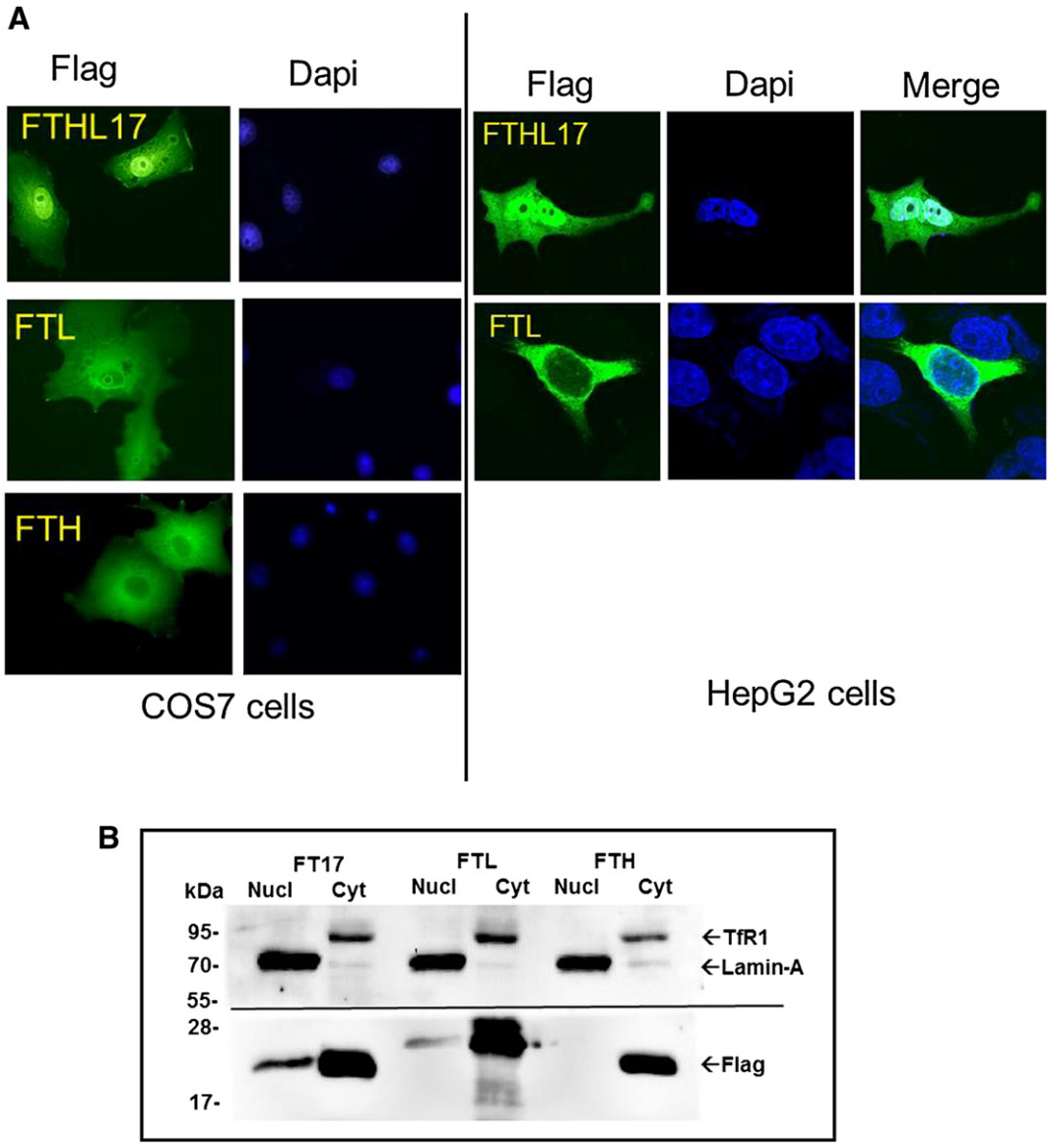

Fig. 4. Flag-FTHL17 preferentially accumulates in the nuclei of transfected COS7 cells. COS7 and HepG2 cells transiently transfected with the flag-tagged ferritins were grown for 24 h and analyzed. (A) Analysis of COS7 (left) and of HepG2 cells with optical and confocal microscopy, respectively. The staining with the anti-flag antibody shows an evident colocalization with the nuclear dye DAPI in the cells overexpressing the flag-tagged FTHL17, while t the staining of the epitope-tagged FTH and FTL is preferentially confined to the cytosol. (B) The homogenates of the cells transfected with the different expression plasmids were fractionated in nuclear (Nucl) and cytosolic fractions (Cyt) and analyzed for the epitope/tagged ferritins, and for cytosolic (TfR1) and nuclear (Lamin A) protein markers with specific antibodies.

as part of a process involved in the regulation of CXCR4 signaling; this function seems to be independent of the FTL chain and of the ferroxidase activity [1], thus suggesting possible iron-independent roles for the ferritin peptides.

The few papers that studied the expression of human and mouse FTHL17 genes showed that they are transcribed in spermatogonia and in germ cells and in some tumor types but not in normal adult somatic cells [19,32]. These genes are intronless, and this may complicate the RT-PCR determination of transcripts expressed at low level. To verify the restricted expression of FTHL17 we performed RT-PCR in various mouse tissues and in mouse ES cells. We detected the expected RT-PCR band only in the testis (not shown) and in ES cells, but not in other tissues, in agreement with previous data. Its level in ES cells decreased during differentiation in monolayer in the absence of LIF to disappear at day 7, while the transcript of cytosolic H and L ferritins were unchanged during this period (Fig. 5). This expression pattern indicates that FTHL17 has roles and functions that differ from those of cytosolic H and L chains, and probably unrelated to iron storage. It was indicated that nuclear ferritins can bind to specific elements and regulate gene expression [6]. This might be the case for FTHL17, an activity that may be facilitated by the lower stability of the assembled shell that may be in equilibrium with free subunits. Recently it has been shown that a FTH peptide, but not the fully assembled FTH, binds Survivin and acts as an inhibitor [33]. Such a function as a regulator of gene expression would be more consistent with the transient expression in ES cells that we have observed, and also with the observation that its expression in the early model for iPS cell induction modified cell differentiation [30].

In conclusion, present data show the existence of a fourth functional ferritin gene, FTHL17, which encodes a protein with the capacity to form ferritin shells that incorporate iron in vitro. However its lack of ferroxidase activity and low stability suggest that it does not have iron storage function, but possibly regulatory functions, a hypothesis supported by

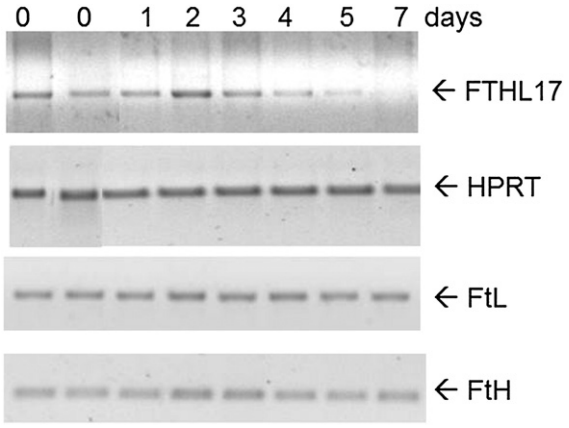

Fig. 5. FTHL17 expression in differentiating mouse ES cells. Mouse ES cells were maintained in undifferentiated state in LIF (day 0) or deprived of LIF to induce differentiation, then were harvested at the indicated days. The RNA was extracted and transcripts analyzed by semi-quantitative RT-PCR with specific primers. The PCR's products are HPRT1 (117 bp), FTH (119 bp), FTL (96 bp) and FTHL17 (630 bp).

its capacity to localize in the nuclei and by its specific expression in germ cells.

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Role in the work

PR and MA planned and performed most experiments, SM performed and analyzed the IHC experiments, EC directed the experiments on mouse ES cells, FM, MR and MG assisted in most experiments, and DF and PA evaluated the data and MP elaborated the study and wrote the work.

Transparency document

The Transparency document associated with this article can be found in the online version.

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