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Ferritin is secreted via two distinct non-classical vesicular pathways

Ferritin Trafficking and Secretion

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Key Points

- Iron-loaded ferritin is secreted via both the non-classical secretory-autophagy and the multivesicular-body-exosome pathways.
- A motif on both ferritin subunits is involved in the regulation of ferritin secretion.

Abstract

Ferritin turnover plays a major role in tissue iron homeostasis and ferritin misfunction is associated with impaired iron homeostasis and neurodegenerative diseases. In most eukaryotes, ferritin is considered an intracellular protein that stores iron in a non-toxic and bio-available form. In insects, ferritin is a classically secreted protein and plays a major role in systemic iron distribution. Mammalian ferritin lacks the signal peptide for classical ER-Golgi secretion but is found in serum and is secreted via a non-classical lysosomal secretion pathway. This study applied bioinformatics and biochemical tools, alongside a protein trafficking mouse models, to characterize the mechanisms of ferritin secretion. Ferritin trafficking via the classical secretion pathway was ruled out and a 2:1 distribution of intracellular ferritin between membrane-bound compartments and the cytosol was observed, suggesting a role for ferritin in the vesicular compartments of the cell. Focusing on non-classical secretion, we analyzed mouse models of impaired endo-lysosomal trafficking and found that ferritin secretion was decreased by a BLOC-1 mutation but increased by BLOC-2, -3 and Rab27A mutations of the cellular trafficking machinery, suggesting multiple export routes. A 13 amino-acid motif unique to ferritins that lack the secretion signal peptide was identified on the BC-loop of both subunits and plays a role in the regulation of ferritin secretion. Finally, we provide evidence that secretion of iron-rich ferritin was mediated via the multivesicular body-exosome pathway. These results enhance our understanding of the mechanism of ferritin secretion, which is an important piece in the puzzle of tissue iron homeostasis.

Introduction

Iron plays a central role in many metabolic pathways, such as oxygen transport, energy production and erythropoiesis, which require strict regulation to avoid the toxic effects of excess iron.[1,2] In mammalians, the amounts of dietary iron uptake and daily losses are small compared to the amount of iron recycled internally and regulated iron recycling is achieved by regulation of cellular iron uptake and release.[3] The dominant iron uptake system varies between cells, and can involve the Transferrin-Transferrin receptor system, erythrophagocytosis, the divalent metal transporter 1 or other systems.[4] Iron is predominantly released from cells by ferroportin, a ferrous iron transporter,[5,6] however, heme export and ferritin secretion are alternative routes for cellular iron release.[7,8]

Ferritin is known in mammals as an iron storage protein that stores about 2,000 ferric iron atoms in a soluble and non-toxic, but bio-available form.[9–11] Ferritin is composed of two subunits, H and L, that assemble to a 24-subunit multimer.[12] The H-subunit bears ferroxidase activity, which converts Fe^{2+} to Fe^{3+} during iron entry into the ferritin shell, while the L-subunit facilitates nucleation of the iron core.[13] Ferritin synthesis and degradation are both controlled by the cellular iron status; when iron levels are low, ferritin synthesis is low due to translational repression.[14] Ferritin entry into lysosomes for degradation, mediated by the selective autophagy receptor NCOA4, is elevated when the cell is in need of iron for cellular usage. Conversely, when iron levels are high, ferritin entry to the lysosome is decreased.[15,16] It has been generally accepted that ferritin is predominantly cytosolic,[10,17,18] but ferritin also demonstrates punctate distribution[8,19–22] and has been associated with vesicular location.[23,24]

Small amounts of ferritin are found in serum and other extracellular fluids, with macrophages being the main cellular source of serum ferritin in mice.[8,25–27] Serum ferritin levels serve in the differential diagnosis of anemia and as an indicator for numerous conditions, including inflammatory, neurodegenerative and malignant diseases.[28–30] Yet, still little is known about the mechanisms underlying ferritin intracellular trafficking and secretion. Most secreted proteins in eukaryotes utilize the classical ER-Golgi secretion pathway and are characterized by a signal-peptide (SP) which is a leader sequence that mediates protein entry to the ER.[31,32] Despite the absence of a leader sequence, it was suggested that ferritin is secreted through the ER-Golgi route.[33] However, we showed evidence that ferritin is secreted through the non-classical lysosomal pathway, specifically through secretory lysosomes.[8] Ferritin may enter the lysosome via NCOA4. Yet, NCOA4 is elevated when cellular iron is low, while serum ferritin is elevated when systemic iron is high.[34,35] In addition, knockdown of NCOA4 increased ferritin secretion from monocytic cells, implying that other secretion routes may be involved. Furthermore, secretion through a NCOA4-independent "secretory-autophagy" pathway was described for ferritin and interleukin 1β.[36]

Vesicle trafficking to specialized lysosome-related organelles (LROs) is regulated in mice by at least 15 genes. Mutants in these genes serve as models for the inherited Hermansky-Pudlak Syndrome (HPS), which is caused by abnormalities in the synthesis and/or trafficking of LROs, such as melanosomes. *HPS* genes control a wide range of physiological processes including pigmentation, lysosome secretion and neuronal functions. Most of the HPS proteins are components of three multi-subunit complexes named biogenesis of lysosome-related organelles complexes (BLOCs), which orchestrate the biogenesis and trafficking of LROs.[37–39] Vesicle trafficking is also regulated by Rab GTPases such as Rab27A.[40] Mutation in the *RAB27A* gene results in Griscelli syndrome, an autosomal disorder characterized by hypo-pigmentation and immunodeficiency.[41,42] BLOC-1 functions at an early stage of endo-lysosomal trafficking,[43] while BLOC-2, BLOC-3 and Rab27a are involved at later stages.[43,44]

Monoubiquitination was shown on ferritin,[45] suggesting that the multivesicular body (MVB)-exosome pathway may be relevant for ferritin secretion as well. Furthermore, ferritin was detected in urinary exosomes as part of proteomic screen.[46,47] MVBs are generated by invagination of endosomal membranes into large cisternae, creating intra-endosomal vesicles. MVBs then either fuse with lysosomes, or fuse with the cell membrane, followed by their release as "exosomes" into the extracellular space.[48,49]

In the current work, we describe novel pathways for ferritin secretion from mouse macrophages. We show that intracellular ferritin did not colocalize with Golgi apparatus and early endosome markers and that ferritin expression and secretion was not affected by an agent that disrupts the Golgi structure. Ferritin was located in punctate structures that expressed markers of the late endo-lysosomal compartment; cellular fractionation demonstrated the distribution of ferritin between the cytosolic and lysosomal fractions. The role of the endo-lysosomal pathway in ferritin secretion was further characterized, using mice with impaired endo-lysosomal trafficking in which serum ferritin levels were greatly affected. Finally, we identified a unique motif that is important for non-classical secretory pathways and provide evidence that ferritin-bound iron is secreted through exosomes.

Methods

Cell cultures and DNA transfections

Primary bone marrow-derived macrophages were isolated and cultured as previously described.[50] RAW264.7 cells were grown in DMEM (Sigma, Israel) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 units/mL Penicillin and 100 μg/mL Streptomycin (Biological Industries, Israel). When indicated, cells

were treated with 100µg/mL ferric ammonium citrate (FAC) for 24 h, to increase cellular ferritin content. Transfection of the GFP-NCOA4 construct (a kind gift from Prof. Alec Kimmelman, Harvard Medical School, Boston, USA) was carried out using the Lipofectamine 2000 reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. For transfection of the ferritin constructs, CalFectin (SignaGen Laboratories, MD, USA) was used.

Immunofluorescence and iron stain

Immunofluorescence staining was performed as previously described.[8,19] Iron staining of gels was performed using the Perls-DAB stain, as previously described.[19]

Subcellular fractionation and immunoblotting

Differential detergent fractionation, to obtain a fraction enriched for membrane-defined vesicles in general, and subcellular fractionation, to obtain lysosome-enriched fractions, were performed as previously described.[51,52] Fractions were lysed, subjected to SDS-PAGE and immunoblot with the indicated antibodies.

Isolation of exosomes by ultracentrifugation, sample preparation for transmission electron microscopy (TEM) and analysis

Exosomes were isolated from cell culture medium, as previously described.[53] Ferritin cores were determined by TEM using a JEOL (JEM-2100) electron microscope. Negative-stain TEM and cryo-TEM experiments were performed using a Tecnai T12 G2 TEM, and images were digitally captured using a Gatan Ultrascan 1000 camera. Cryo-TEM vitrified samples were examined using imaging procedures as previously described.[54]

Animal studies

All mice were on a C57BL/6J background, except for Rab27A$^{-/-}$ mice, which were on a C3H background. All mouse experiments were performed under ethics approval for animal experiments, granted to Esther Meyron-Holtz, Wei Li and Huifang Lou (Ethics numbers: IL-042-03-11 and IL-091-08-15, Technion ; KYD-2006-002, IGDB). A detailed description of mouse mutations and phenotypes is provided in the supplemental section. Blood samples were tested by a routine veterinary laboratory in a blinded manner for complete blood count, iron and inflammatory markers.

Additional methods

All other methods, including ELISA, metabolic labeling and pulse-chase analysis, ferrozine iron assay, computational tools, targeted deletion of human ferritin H- and L-subunits by CRISPR/Cas9 and murine ferritin H-subunit mutagenesis, are described in the supplemental section.

Results

Intracellular ferritin is found in membrane-bound vesicle-fractions, specifically in the late endo-lysosomal compartment

To further characterize ferritin secretory pathways, we visualized ferritin subcellular distribution in murine bone marrow-derived macrophages (BMDM), in the presence of iron (100 µg/mL FAC). A punctate distribution of intracellular ferritin was observed (Figure 1), in accordance with multiple images published in the literature.[19–22,8] When testing for ferritin colocalization with several major organelle markers, we found that

ferritin co-localized with a late endo-lysosomal marker (Figure 1C), but not with cis-Golgi and early endosome markers (Figure 1A-B). We also observed that ferritin partially colocalized with NCOA4 (Figure S1), a cargo receptor mediating ferritinophagy.[55] These results suggest, that ferritin is found in vesicular compartments of the cell also in iron replete conditions.

Two subcellular fractionation approaches were employed to further characterize vesicular ferritin. Ferritin was found located in both cytosolic and membrane-bound fractions (Figure 2A). Normalization and quantification of the subcellular distribution showed 2-fold more ferritin in the membrane-bound vesicle fraction compared to the cytosol and the S-subunit, which was previously observed,[8,56] was found predominantly in the membrane-fraction (Figure 2B). When specifically isolating a lysosome-enriched fraction, the S/L ferritin subunit ratio was approximately 11-fold ($p<0.01$) and 7-fold ($p<0.05$) higher in the lysosomal fraction compared to whole-cell-lysate, with and without the addition of iron, respectively (Figure 2D). Using electron microscopy, we also found that ferritin in all fractions contained iron (Figure 2E, confirmed by EDS analysis - Figure S2).

Ferritin is secreted through non-classical pathways

To further study the role of the endo-lysosomal trafficking machinery in ferritin secretion, we examined mice with defects in this pathway. We used serum ferritin levels in these mice as indicator for ferritin secretion and found large differences. Three genotypes showed different degrees of elevated serum ferritin concentrations compared to wild type mice, while BLOC-1$^{-/-}$ mice showed a 3-fold decrease in serum ferritin levels (Figure 3A). Serum iron and transferrin saturation were normal in all mice (Figures 3B-C). Inflammation markers were also normal (Figure S3). Liver iron showed a ~2-fold increase in BLOC-1$^{-/-}$, BLOC-2$^{-/-}$ and Rab27A$^{-/-}$ and was unchanged in BLOC-3$^{-/-}$ mice, as compared to wild type (Figure 3D), indicating that there is no correlation between serum ferritin levels and liver iron in these mice. Moreover, liver transferrin receptor levels were unchanged in all mice (Figure 3E). Thus, the serum ferritin changes in these mice were caused neither by systemic iron fluctuations nor by inflammation, strengthening the notion that defects in endo-lysosomal trafficking machinery impact the levels of secreted ferritin.

Although ferritin did not co-localize with the Golgi apparatus (Figure 1A), we tested if ferritin is secreted through the classical ER-Golgi pathway. To this end brefeldin A (BFA), an agent which disrupts the Golgi structure (Figure 4B),[57] was administered to murine BMDM, and the medium was later subjected to a pulse chase analysis (Figure 4A). BFA did not induce accumulation of intracellular ferritin and did not inhibit ferritin secretion over time, thereby excluding the classical ER-Golgi route as a pathway for ferritin secretion.

To better understand the evolution of the classical and non-classical pathways for ferritin secretion, we used phyloT[58] and Dendroscope[59] bioinformatics tools, to create a phylogenetic tree of ferritins from eukaryotic organisms, containing or lacking the classical ER targeting sequence (Figure 5A, based on sequences listed in S8). The unique pattern received in the phylogenetic tree reinforced previously known distribution of signal-peptide (SP) in ferritin. Specifically, classical SP sequences were found in many insects, but were absent in most other eukaryotes, including mammals (red and black respectively, Figure 5A and Figure S7).[60] We reasoned that the ferritins that lack the SP may contain a sequence needed for non-classical pathways and thus looked for motifs that are unique to these ferritins. A 13-amino acid motif (residues 74-86 on the mouse H-subunit and residues 70-82 on the mouse L-subunit) that was highly enriched (94%) in the SP-negative group and less enriched (48%) in the SP-positive group was identified (Figure 5B and motif 4 in Figure S4). The motif was further visualized on the mouse H-chain ferritin crystal structure (PDB: 3wnw) and located on an unstructured elongated loop between the B and C helices termed BC-loop.[61] The motif is oriented almost

entirely towards the interface between two neighboring ferritin subunits, thus forming a continuum zipper-like structure (Figure 5C) which functions as a dimerization motif. Application of the Optimal Docking Area (ODA)[62] software to compute the probabilities of amino acids engagement in protein-protein interactions, predicted that most motif residues participate in putative protein-protein interactions (Figures 5D-F, amino acids with high probability of interaction are shown in red). Strikingly, R79, F81 and Q83 of the motif are exposed to the surface of the assembled 24-mer (Figure 5F-G) and thus are excellent candidates for interaction with other proteins. Thus, site-directed mutagenesis efforts focused on these three residues. To this end, ferritin secretion from human ferritin H- and L-subunit knockout HeLa cells, transfected with plasmid encoding the wild type murine H-subunit (FTH^{wt}) or plasmids containing a point mutation in R79, F81 or Q83, was analyzed. FTH^{F81A} and FTH^{Q83A} caused marked increase in ferritin secretion and thus demonstrated an inhibitory role in ferritin secretion (Figure 5H). Both mutations did not impact basic ferritin functions, such as protein assembly to a 24-mer or iron incorporation (Figure S5).

These data suggest that ferritin is secreted via the endo-lysosomal system, where ferritin can take more than one exit route from this system. Due to the reported mono-ubiquitination and appearance in urinary exosomes of ferritin[45–47], we examined the MVB-exosome pathway. Both ferritin as well as the exosomal marker TSG101 were detected in exosomes purified from the medium of RAW264.7 macrophages (Figure 6A). Negative staining (TEM) demonstrated the typical cup-shaped morphology of these nano-vesicles, which were ~100 nm in size[48,49] (Figure 6B). However, since the exosome isolation protocol includes ultracentrifugation steps, free ferritin released through the secretory-autophagy route may co-precipitate with exosomes due to its heavy iron cores (Figure 6B red circles and Figure S6). Thus, we further analyzed these TSG-101-positive exosomal fractions by cryo-TEM without any staining and found typical ferritin-iron cores within exosomal structures (Figure 6C, white arrows). Size analysis of approximately 100 iron cores, demonstrated an average core size of 3.8nm±0.9 versus 4.9nm±0.8, inside and outside the exosomes, respectively ($p<0.0001$). Taken together, we propose that mouse ferritin is secreted not only by the non-classical secretory-autophagy route, but also through the MVB-exosome pathway.

Discussion

Intracellular ferritin trafficking

Ferritin is the major intracellular iron storage protein in many organisms.[10,63,61] Although it has been generally accepted that ferritin is predominantly cytosolic,[10,17,18] it also displays punctate, uneven distribution[19–22,8] that has been associated with vesicular location.[23,24] Ferritin entrance to the endo-lysosomal system has been shown to be dependent on the autophagy receptor NCOA4, and recently, the specialized secretory-autophagy receptor TRIM16 was ascribed a role in ferritin entry to secretory autophagosomes.[36] Focusing on macrophage cell models, we found ferritin in the endo-lysosomal system, but not in the Golgi apparatus (Figure 1A). The classical ER-Golgi inhibitor BFA, did not affect cellular ferritin localization (Figure 4B) or secretion (Figure 4A), which strongly implied, in contrast to the general consensus, that ferritin does not traffic through the classical ER-Golgi secretion pathway.[33] Further, most ferritin colocalized with a late-endo-lysosomal marker, supporting the notion that iron-loaded ferritin is trafficked and secreted via a non-classical vesicular route. Yet, some punctate ferritin entities did not colocalize with this marker (red arrows in Figure 1D), suggesting the presence of membrane-bound fractions of ferritin that are not lysosomal.

Bioinformatics of ferritin secretion

In insects, ferritin is secreted via the classical ER-Golgi route and contains an ER-targeting SP.[60] Analysis of the distribution of eukaryotic ferritins on a taxonomy-based phylogenetic tree (Figure 5A and S7), showed that

most of the tested eukaryotic organisms do not have a classical SP on their ferritins. We identified a motif with a high likelihood of engaging in protein-protein interactions, that was highly enriched in all ferritins lacking the SP, but appeared in only 48% of classically secreted ferritins (Figures 5B and S4 - motif 4). This suggested that classically secreted ferritins had no need to conserve this motif, indicating that it may serve a function in the non-classical secretion pathway. Furthermore, the amino acids colored red in Figures 5D-F are mostly exposed to the solvent and very likely to participate in protein-protein interaction (Figure 5F). While most amino acids in this patch likely interact with neighboring ferritin subunits, R22 (colored orange in Figure 5G) interacts with NCOA4 and is the key arginine required for delivery of ferritin to degrading lysosomes.[16] Mutation of residues F81 and Q83, led to highly increased ferritin secretion (Figure 5H), suggesting they have an inhibitory role in ferritin secretion presumably by preventing ferritin from entering the secretion route.

Analysis of serum ferritin in mice with endo-lysosomal trafficking defects suggests two alternative non-classical routes for ferritin secretion

We previously proposed that serum ferritin, which is the most accessible secreted ferritin in a whole organism, is an actively secreted ferritin, containing L- and H- subunits and iron, although its iron content and H/L-subunit ratio are much lower than in any known intracellular ferritin. In contrast, ferritin secreted from cell lines or primary BMDMs is similar to intracellular ferritin in its iron content and subunit composition, suggesting that cytosolic ferritin enters a secretory pathway and is secreted without much processing. The discrepancy between secreted ferritin and serum ferritin is not well understood, nevertheless, serum ferritin levels are in good correlation with macrophage iron loading[8] (and Figure 4A). To further understand the reported pathway of secretory-autophagy, described for interleukin-1β and ferritin,[8,36] we focused on mice with defects in trafficking via LROs (secretory-lysosomes or secretory-autophagy). Serum ferritin levels in BLOC-1-deficient mice were significantly reduced, but were significantly elevated in BLOC-2, BLOC-3 and Rab27A-deficient mice (Figure 3A), suggesting that specific defects in early or later stages of vesicle trafficking to LROs have opposite effects on ferritin secretion. Studies have shown intracellular accumulation of pre-lysosomes in the cortical neurons of the BLOC-1-component snapin-KO mice[64] and of endo-lysosomes of MEFs from three different BLOC-1 subunit null mice (BLOS1-KO, BLOS2-KO, or pallid).[65,66] Thus, ferritin may be trapped in these prelysosomes or endo-lysosomes and targeted for degradation in BLOC-1-deficient mice. In contrast, in BLOC-2, BLOC-3 or Rab27A-deficient mice, lysosomal function or secretion was blocked, causing ferritin to accumulate and exit the cell through the alternative MVB-route via exosomes. This notion is supported by the fact that the number of MVBs was increased, while fewer lysosomes were observed in BLOC-2-deficient cells.[67] Interestingly, the size of MVBs was also strongly increased by Rab27A silencing.[68] While ferritin enters the protein-degrading compartment of the endo-lysosomal system through the autophagy receptor NCOA4, a recent study suggested that NCOA4 does not take part in ferritin secretion.[36] NCOA4-null mice showed elevated serum-ferritin levels, further demonstrating that ferritin secretion increases when ferritin accumulates due to decreased degradation. Finally, BLOC-1-deficient mice exhibited impaired MVB maturation,[69] which further explains the lower ferritin secretion in these mice. Taken together, these observations suggest that ferritin is secreted via two non-classical routes.

Ferritin secretion through the MVB-exosome pathway

Exosomes are secreted upon fusion of MVBs with the plasma membrane. Exosomes in body fluids play an important role in exchanging information between cells and can act in a paracrine or even endocrine manner to modify gene expression, signaling and overall function of adjacent or distant cells.[70] Recently, it was reported that the iron carrier molecules, transferrin and lactoferrin, are found in exosomes and can be delivered into mammalian cells via this route.[71] Ferritin was also found in human urinary exosomes in a large-scale

proteomic analysis.[46,47] However, neither local ferritin secretion via exosomes nor its iron content have been reported. Here, we present evidence that ferritin-bound iron is secreted via exosomes (Figure 6). This ability of exosomes to transfer iron carrier molecules between cells may play an important role in inter-cellular communication and in the maintenance of iron homeostasis under physiological conditions.

Possible functions of secreted ferritin

Ferroportin is the only known ferrous iron exporter. However, we and others suggested that secreted ferritin may function as an iron exporter of ferric iron;[19,8,72] experimental evidence was provided by both Sibille et al. and Leimberg et al., who showed that ferritin secreted by hepatocytes and macrophages, functioned as an iron-donor protein.[73,74] This suggests that ferritin secreted by macrophages, which specialize in iron acquisition, can be taken up by cell types specialized in iron storage, such as hepatocytes, or high-iron consumers, such as developing erythroid cells. The existence of ferritin receptors such as murine TIM2, human TfR1 and SCARA5, on these cells,[75–77] strengthens our hypothesis, while exosome-mediated ferritin transport is independent of receptor-mediated ferritin uptake. However, there remains a missing link between the secreted ferritin, that is iron-bound and bears the subunit composition of its intracellular counterpart, and the serum ferritin that contains very little iron and H-subunits only. We believe that ferritins containing iron and at least one H-subunit are locally delivered to cells via vesicular structures, such as exosomes, and via receptor-mediated internalization, and thus never reach the serum. Only iron-poor L-subunit ferritins, that are barely taken up by the H-specific ferritin receptors, reach the serum. Vesicular location and secretion of ferritin was also found in additional cell types, such as Sertoli cells,[19] HepG2 and Caco-2 epithelial cells (unpublished) and thus, the described machineries for regulated ferritin secretion may not be unique to macrophages. It will be interesting to further investigate the ferritin secretion pathways in both hepatocytes and in epithelial barrier cells. Lastly, since many of the major neurodegenerative diseases are associated with iron and ferritin misdistribution in the brain, defects in ferritin trafficking and secretion may play a central role in the development of these diseases. Taken together, our findings and evidence that exosomes pass the blood-brain barrier[78] may also shed light on iron entry to the adult brain and on neurodegenerative diseases accompanied by impaired iron-distribution in the brain.

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Authorship Contributions

M.T.R., L.A.C, S.B.M., L.L., T.A.R. and E.G.M.H. designed the research

M.T.R., L.S. and E.G.M.H. designed and performed *in-vitro* experiments

M.T.R., J.M., W.L., H.L., K.L., H.L and E.G.M.H. designed and performed *in-vivo* and all BLOCs- and Rab27A-related analyses

M.T.R., L.G., E.K., I.A.I., D.D. and E.G.M.H. designed and performed electron microscopy experiments

M.T.R., D.B., L.S., F.G. and E.G.M.H. designed and performed bioinformatics

M.R. and M.P. designed and produced HeLa FTH-FTL knockout cells by CRISPR/Cas9

M.T.R., T.A.R and E.G.M.H. wrote and edited the manuscript

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Ferritin co-localizes with the late endo-lysosomal marker Cathepsin D (CatD), but not with Golgi or early endosomal markers. Representative confocal images of ferritin (red) and subcellular compartments (green): (A.) Golgi apparatus, stained for GM-130 (B.) Early endosomes, stained for EEA1 and (C.) Late endo-lysosomes, stained for CatD, in murine bone marrow-derived macrophages grown in the presence of 100 μg/mL ferric ammonium citrate (FAC) for 24 h. Scale bar: 10 μm. Enlargement of the ferritin-CatD co-staining is shown in (D.), with red arrows indicating ferritin, green arrows indicating late endo-lysosomes and yellow arrows indicating colocalization of the two. Negative controls were treated with secondary antibodies only and with one primary antibody followed by both secondary antibodies (data not shown). Sample visualization was performed with a LSM 700 (Zeiss) laser scanning inverted confocal microscope, equipped with a Plan-Apochromat X63 /1.4 NA oil DIC objective.

Figure 2. Iron-loaded ferritin is located in membrane-bound vesicles, specifically in lysosome-enriched fractions. (A.) Control and iron-treated (100 μg/mL FAC for 24 h) bone marrow-derived macrophages were fractionated using a differential subcellular fractionation method. Total cell lysates and cytosol and membrane-bound vesicle-enriched subfractions (Membranes) were separated on SDS-PAGE (40 μg protein/lane) and analyzed by Western blot, with anti-L-ferritin, LAMP1 (lysosomal marker) and anti-tubulin (cytosolic marker) antibodies. The results of one out of three representative experiments is shown. (B.) Intensity of L- and S-ferritin bands of control (Ctrl) samples were quantified using Adobe Photoshop software. Mean band intensities were normalized to whole-cell protein by volume ratios. The results of one out of three representative experiments is shown. (C.) Control and iron-treated (100 μg/mL FAC for 24 h) RAW264.7 macrophages were fractionated using a lysosomal enrichment method. Total cell lysates and lysosomal fractions were separated on SDS-PAGE and analyzed by Western blot with anti-L-ferritin and anti-LAMP1 antibodies. (D.) Intensity of L- and S-ferritin bands on immunoblot was quantified; each bar represents mean ± SD, n=4, * $p<0.05$, ** $p<0.01$ compared to control samples). (E.) A drop of each fraction was mounted on a carbon-coated copper grid at room temperature. Iron cores (white arrows) were determined using a JEOL (JEM-2100) electron microscope operated at 200 KeV. Scale bar: 100 nm.

Figure 3. Serum ferritin levels are affected in mice with trafficking defects of the endo-lysosomal pathways. (A.) Serum ferritin levels were estimated by ELISA. (B.) Serum iron and all other blood tests were performed in a by trained staff in a veterinary laboratory. (C.) Transferrin saturation percentage was calculated by dividing serum iron by total iron binding capacity (TIBC) and multiplying by 100. (D.) Liver iron was evaluated using a colorimetric ferrozine-based assay. (E.) Transferrin receptor 1 (TfR1) Western-blot: Liver lysates were separated by SDS-PAGE (35 μg protein/lane) and subjected to Western blotting with anti-transferrin receptor 1 (TfR1) and anti-actin antibodies. Representative result of livers from 2/4 mice are shown. Measurements of BLOC-$1^{-/-}$,-$2^{-/-}$ and-$3^{-/-}$ mice were compared to WT C57BL/6J mice. Rab$27A^{-/-}$ mice were on a C3H background and were compared to their WT counterparts. Each bar represents mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Statistical significance was evaluated by an unpaired t-test, in the GraphPad software.

Figure 4. Ferritin is not secreted through the classical ER-Golgi secretion pathway. (A.) Murine bone marrow derived macrophages were metabolically labeled with ^{35}S in the presence of 100 μg/mL FAC and in presence or absence of 5 μg/mL BFA. Cells and media were collected at 0, 2 and 4 h. Ferritin was immunoprecipitated from lysates and media with an anti L-ferritin antibody and separated on SDS-PAGE. L- and H-ferritin subunit band intensity was quantified using the Adobe Photoshop software (each bar represents mean ± SD, n=2). (B.) Representative confocal images of ferritin (green) and Golgi marker GM-130 (red) in

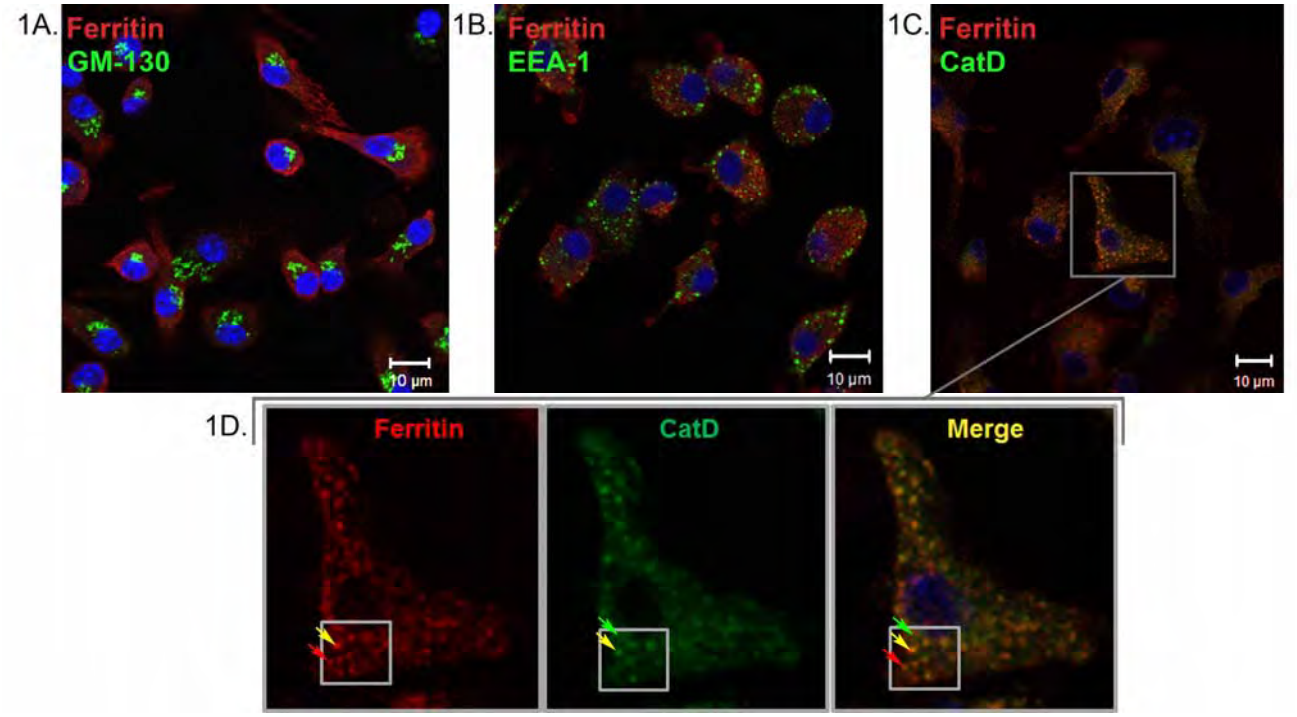
murine macrophages. Upper panel: Control, non-treated macrophages; Lower panel: BFA-treated macrophages. Negative controls were done with secondary antibodies only (insert top panel) and with one primary antibody followed by both secondary antibodies, to exclude channel leakage (data not shown). Scale bar: 10 μm. Image visualization was performed on a LSM 700 (Zeiss) laser scanning inverted confocal microscope with a Plan-Apochromat X63 /1.4 NA oil DIC objective.

Figure 5. Identification of a motif enriched in ferritins that do not have a classical secretion signal. (A.) A taxonomy-based phylogenetic tree of ferritins containing or lacking the classical ER SP targeting sequence. The organisms marked red are SP-positive organisms. The organisms marked black are SP-negative organisms. The pink area is a dominantly SP-positive cluster. The green area is a dominantly SP-negative cluster. Mus-musculus and Homo-sapiens locations are marked in yellow and shown in the enlargement. (B.) A 13-amino acid motif detected by the MEME[79] and FIMO[79] tools. The motif includes residues 74-86 of the H-subunit and residues 70-82 of the L-subunit (C.) The motif is situated along an unstructured loop of the ferritin subunits, and sits at the interface between two subunits (colored in cyan and gold), creating a continuous long zipper-like structure (i.e. a motif dimer) with its neighbor motif. (D.) R79, F81 and Q83 of the motif received high Optimal Docking Area (ODA) scores for protein-protein interaction (high score is labeled in red, low score in blue). (E.) Visualization of protein-protein interaction patch, calculated by ODA score. (F.) Visualization of protein-protein interaction patches calculated by ODA score shown in the context of the whole ferritin multimer. Our motif, R22 and an additional unidentified group of residues are marked in red. (G.) Motif distribution and location throughout the entire ferritin 24-mer. The motif forms a long dimer (colored in green) at the interface of two subunits. R79 is marked in red and R22 is marked in orange. (H) Human ferritin H- and L-subunit knockout HeLa cells were transfected with plasmids coding for either wild type murine H-ferritin (FTH^{WT}) or for FTH^{R79A}, FTH^{F81A} or FTH^{Q83A}. Non-transfected Hela-cells (NT) were analyzed as negative control. WT RAW264.7 (non-transfected) cells were analyzed as positive control for both L- and H-ferritin subunits (lane 12 in 'Cell lysates' gel and lane 17 in 'Media' gel). 24-hours after transfection, cells were metabolically labeled with ^{35}S (2 h pulse) and chased for 0, 4, and 18 h. Cell lysates were collected after 4 and 18 h (lanes 2-6 and 7-11 in 'Cell lysates' gel, respectively) while media samples were collected after 0, 4 and 18 h (lanes 2-6, 7-11 and 12-16 in 'Media' gel, respectively). Ferritin was immunoprecipitated with an anti-murine H-ferritin antibody. All samples were pre-cleared (PC) by incubation with protein-A sepharose beads alone to clear samples from non-specific binding to the beads. Two of these samples, 4 h of WT cell lysates and 18 h of WT medium, where most non-specific binding was expected, were analyzed on the gels (lanes 1). All samples were separated by SDS-PAGE and visualized by phosphor-imaging.

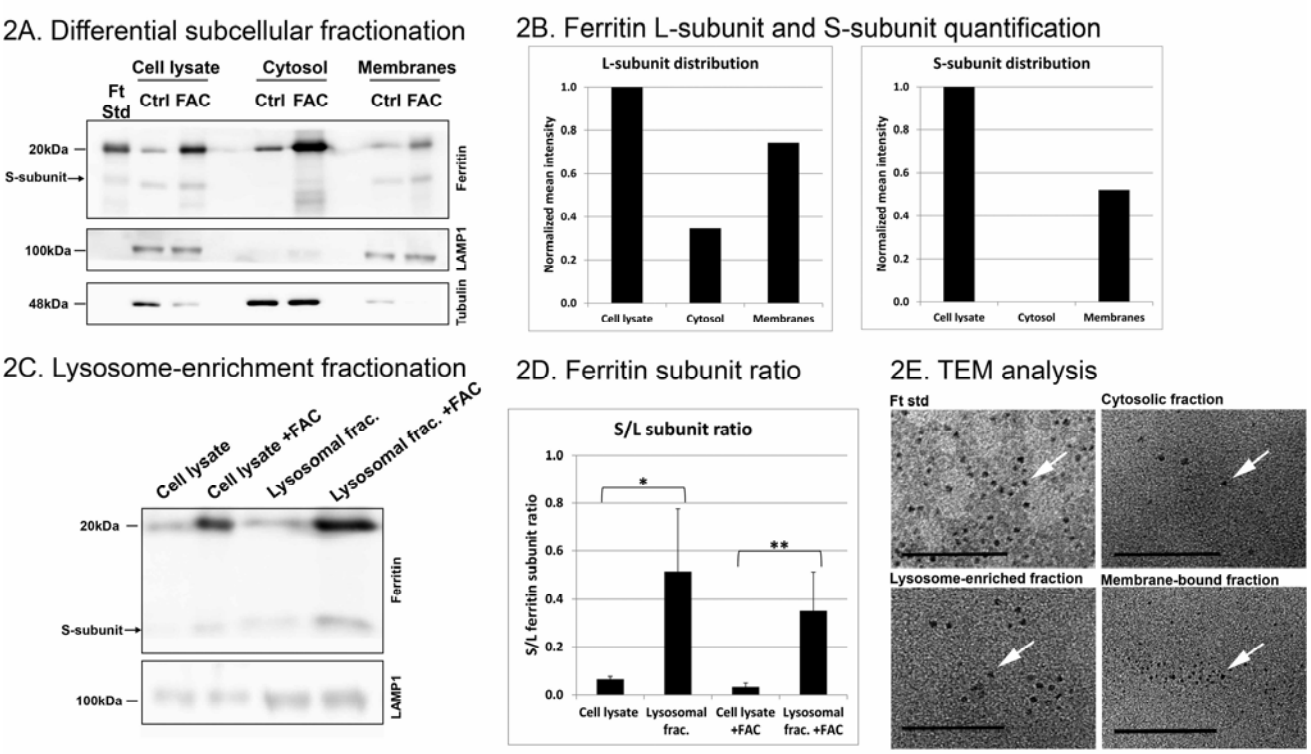
Figure 6. Ferritin-iron cores are present in exosomes. (A.) RAW264.7 macrophages were incubated for 24 h in a mixture of OptiMEM I medium and DMEM (1:1 volume ratio) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 units/mL Penicillin and 100 μg/mL Streptomycin 1mg/L BSA, 20mM β-mercaptoethanol and 100 ug/mL FAC. To precipitate exosomes, cells were harvested and medium was collected and centrifuged at 100,000 x *g* for 1.5 h. Samples were then separated by SDS-PAGE and analyzed by Western-blot with anti-ferritin L-subunit and anti-TSG101 (serving as exosomal marker) antibodies. The results of one out of four experiments are shown. (B.) Exosomal samples were re-suspended in 0.1% glutaraldehyde and a drop was mounted on an ion-coated copper grid supported by a carbon-coated film. The sample was stained with 1% uranyl acetate and visualized by TEM. Scale bar: 100 nm. (C) Exosomal samples were captured by cryo-TEM. Vitrified unstained specimens were loaded to a Tecnai T12 G2, operating at 120 kV, and examined at a low dose to minimize radiation damage. The white arrows point to iron cores. Scale bar: 100 nm.

Figures

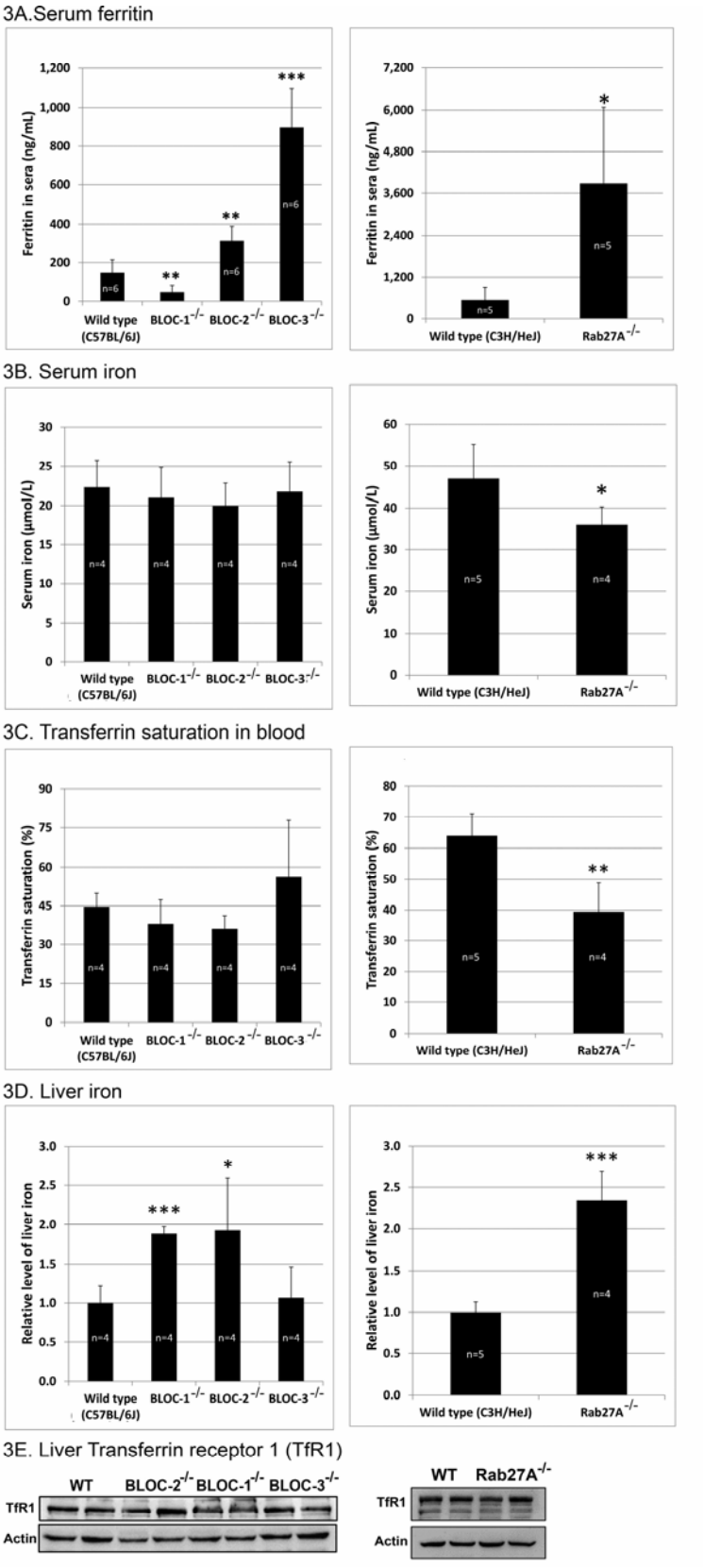
----------FIGURE 1----------



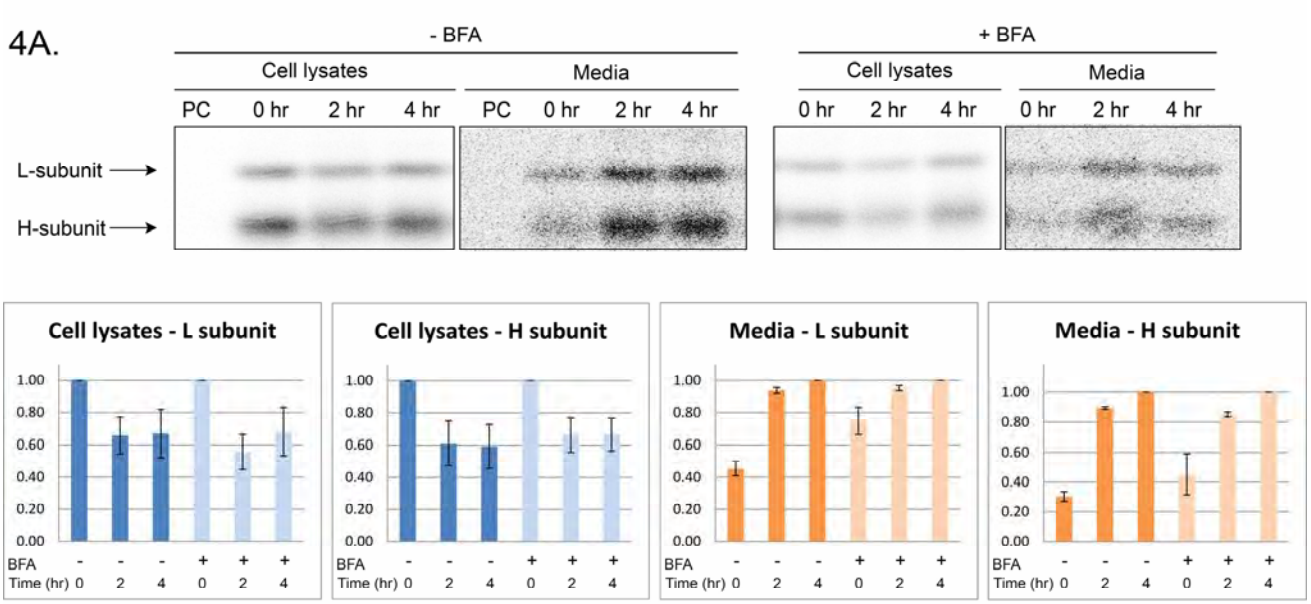
----------FIGURE 2----------

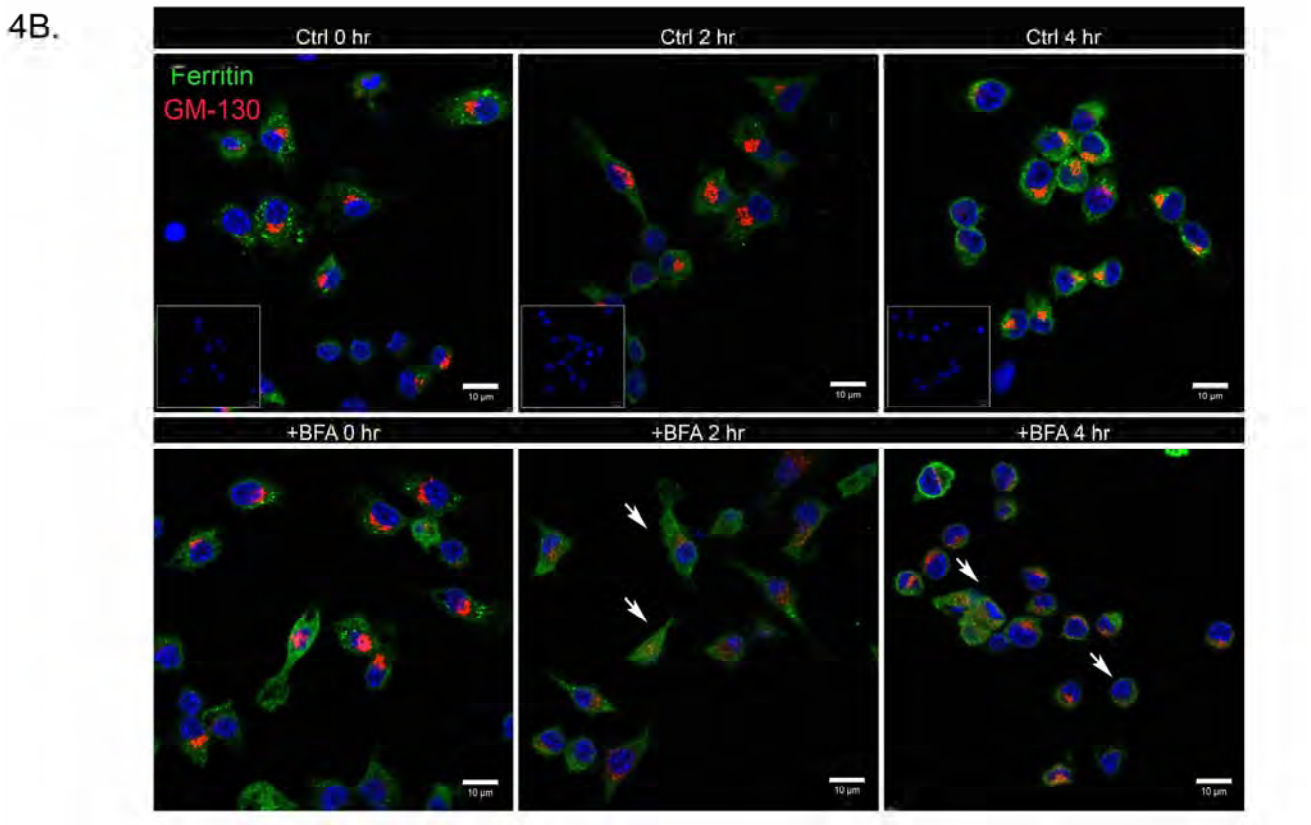


----------FIGURE 3----------

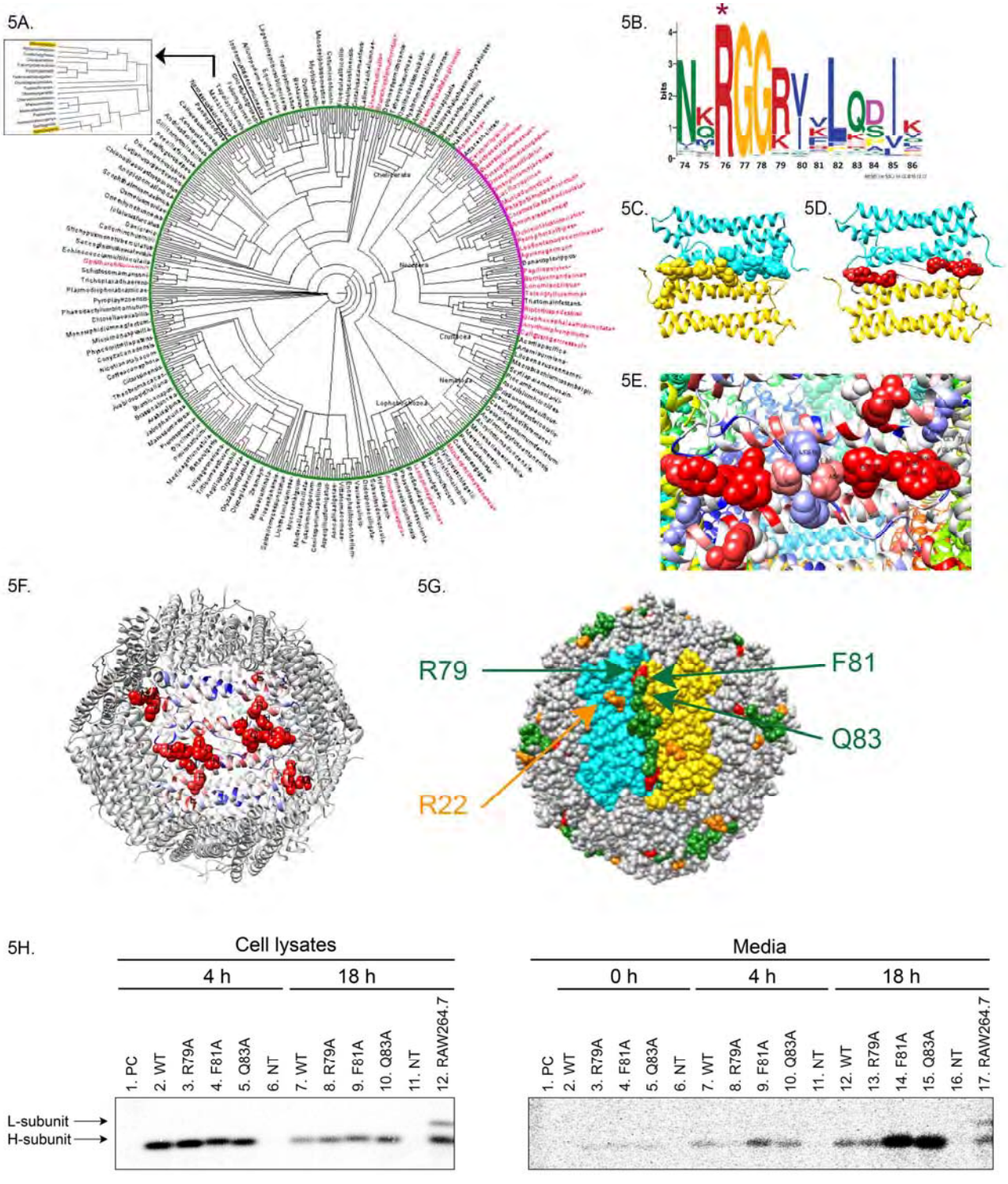


----------**FIGURE 4**----------





----------FIGURE 5----------



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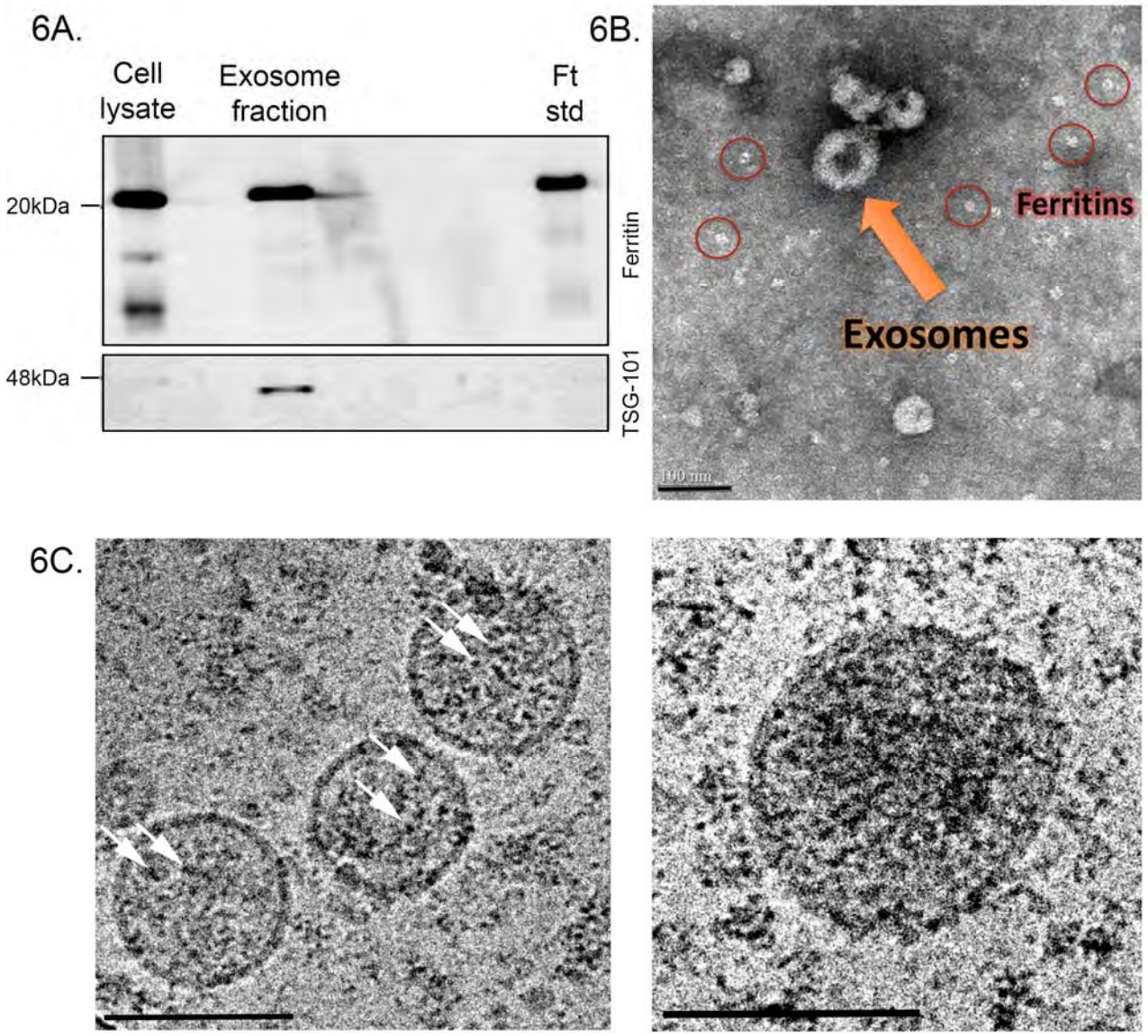

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Ferritin is secreted via two distinct non-classical vesicular pathways

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