Lipid raft-dependent endocytosis: a new route for hepcidin-mediated regulation of ferroportin in macrophages

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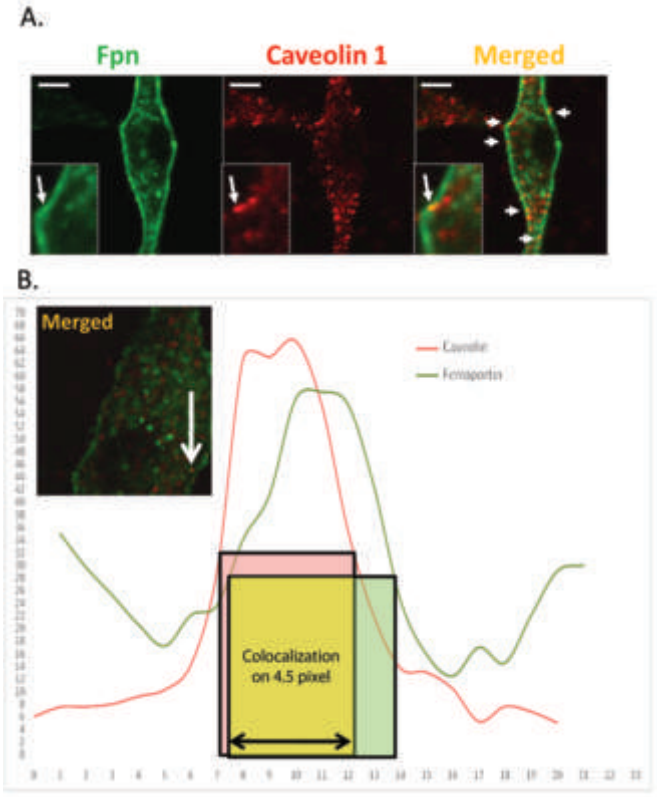
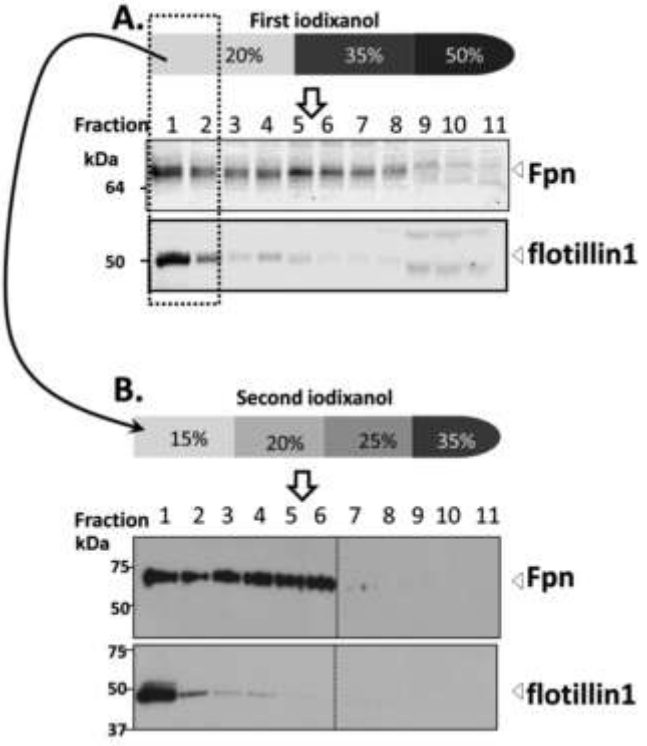
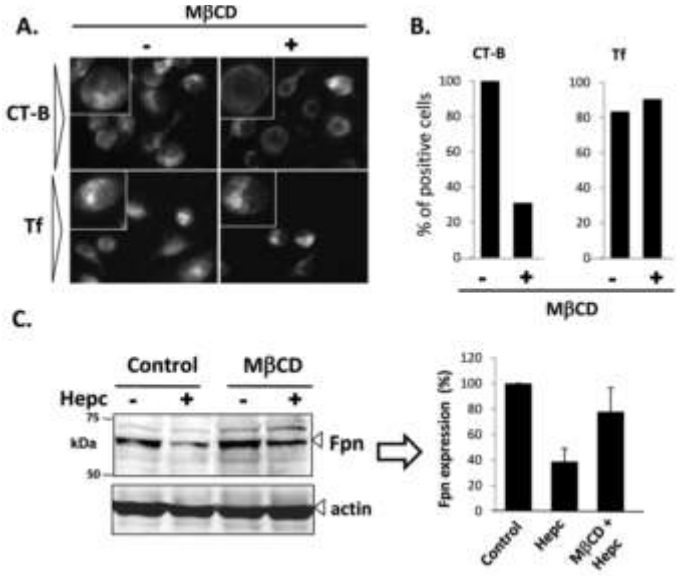


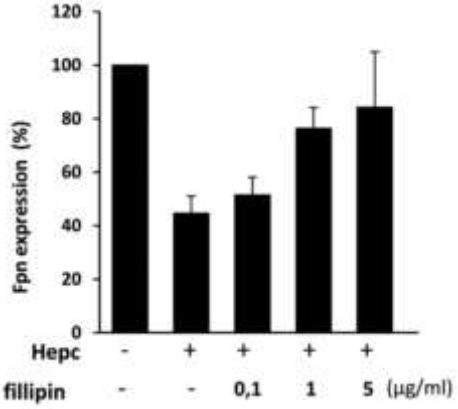
Online Supplementary Figure S1. Confocal analysis of ferroportin and caveolin 1 staining in iron treated BMDM. After overnight incubation with Fe-NTA, BMDM were fixed in methanol, processed for Fpn and Caveolin 1 immunofluorescence staining and analyzed by confocal microscopy. Single confocal images of isolated BMDM cells (A). In merged images and insets, arrows indicate partial co-localization of Fpn with caveolin 1. White bar in upper left coin of each panel represents 5 µm. (B) Partial colocalization of Fpn with caveolin 1 in some vesicular compartments was controlled by calculations of the red and green pixel areas in a single section as described previously.[1] Single image overlays were opened in the software Image J. Pixel areas on yellow/orange dots (white arrow) which were then measured using the plot profile function and exported to Excel (Microsoft). In this example, the partial colocalization of the two proteins was observed on 4.5 pixel = 270 nm (1 pixel = 60nm), a distance longer than the microscope resolution (150 nm).



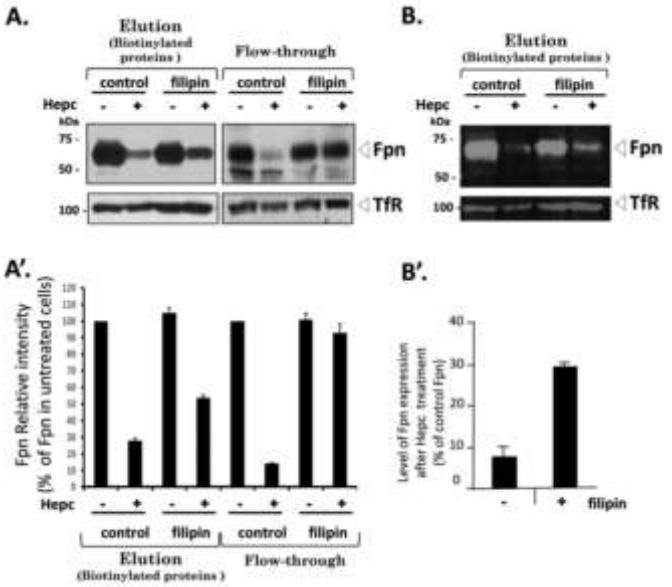
Online Supplementary Figure S2. Sequential separation of detergent resistant membranes containing Fpn in J774 treated with iron. After a first iodixanol gradient (A) the lightest fraction 1 &2 containing Fpn were processed on a second density gradient (B). The density fractions obtained were analyzed by Western blot for the presence of Fpn and flotillin 1. In such an experiment, the raft markers flotilin was still enriched in the lightest density fraction (N. 1) whereas Fpn was distributed in other more dense fractions (until N. 6), suggesting some heterogeneity of the detergent resistant membranes containing Fpn.



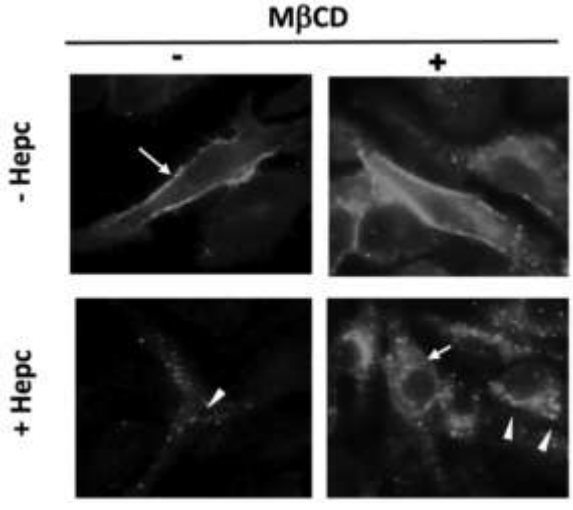
Online Supplementary Figure S3. Effects of cholesterol depletion on Fpn degradation by Hepc in BMDM macrophages. (A) Subcellular localization of Fpn in Fe-NTA treated BMDM and treated or not with methyl-β-cyclodextrin (MβCD) and Hepc. (B) Typical histogram representation of the number of positive cells for intracellular staining of cholera-toxin B (CT-B) and transferrin (Tf) after MβCD treatment. MβCD blocks raft-dependent endocytosis of CT-B but not clathrin–dependent endocytosis of Tf. (C) BMDM were treated or not with MβCD in the presence or in the absence of human Hepc (300nM, 3 h). Total extracts were then analyzed by Western blot using anti-Fpn (Fpn) or actin (loading control) antibodies and LI-COR IRDye™ 800 or 700-labeled secondary antibodies, respectively. Histogram on the right represents quantification of the Fpn band detected using the Odyssey Infrared Imaging System. Cells pre-treated with MβCD prior Hepc show more Fpn level than the cells only treated with Hepc.



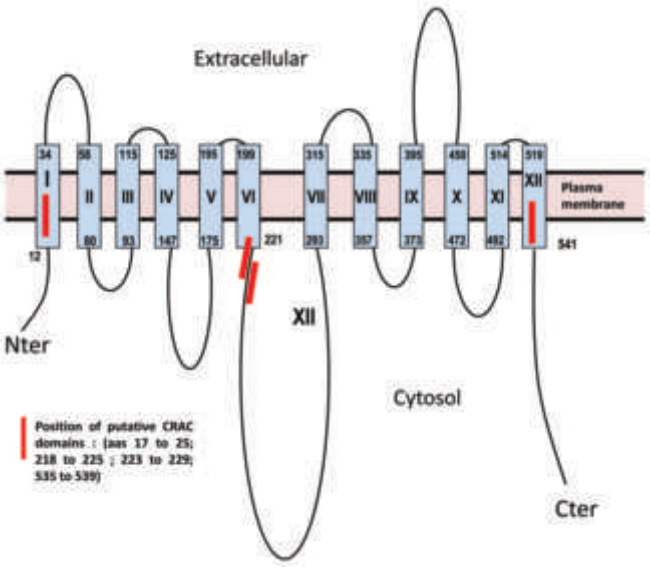
Online Supplementary Figure S4. In-cell-Western detection and quantification of Fpn expression after Hepc treatment on cells pre-treated with increasing concentration of filipin. (***P<0.001, Student's t-test).



Online Supplementary Figure S5. Filipin affects both hepcidin–mediated endocytosis and degradation of ferroportin. Fe-NTA treated J774a1 were incubated or not with filipin prior treatment with Hepc (300 nM; 1 h). After biotinylation labeling, cell surface (biotinylated proteins) and intracellular (flow-through) extracts were then analyzed by Western blotting using anti-Fpn or anti-TfR1 (control) antibodies. The position and size in kilodaltons (kDa) of molecular mass markers are indicated on the left. Protein species were revealed either using ECL (A) or using specific fluorescent secondary antibodies and the Odyssey infrared imaging system (LI–COR Biosciences; B) . For both detection, quantification of specific signals in each sample was represented in A' (densitometry analysis of the autoradiogramms) and B' (fluorescence quantification), respectively. In B', the values obtained after Hepc treatment are expressed as a percentage of the basal Fpn expression (untreated with Hepc). After 1 h of hepc treatment, up to four times more Fpn were present at the plasma membrane in cells pretreated with filipin when compared to untreated cells.



Online Supplementary Figure S6. Immunofluorescence staining of Fpn in iron treated BMDM incubated with or without MβCD prior Hepc treatment. Arrows and arrowheads indicate cell surface staining and intracellular detection of the iron transporter, respectively. As observed with filipin, pre-treatment of the cells with MβCD prevent degradation of Fpn by Hepc.



Online Supplementary Figure S7. Schematic representation of Fpn in cell membrane with positions of putative CRAC domains along the backbone of the iron transporter. CRAC is a common cholesterol recognition and interaction amino acid consensus pattern. It is defined as a sequence pattern –L/V-(X) (1-5)-Y-(X) (1-5)–R/K- where (X) (1-5)- is a sequence of one to five random amino acids. In the sequence of Fpn, there are four putative CRAC motifs (red boxes). According to the recent topology model of Fpn proposed by Rice A. et al.,[2] two are present in two predictive transmembrane domains (I and XII; aas 17-25 and 535-539) and two (aas 218-225; 223-229) are near the membrane interface of transmembrane domain VI of the transporter. These CRAC motifs could play a role in sequestering Fpn into a cholesterol-rich membrane domain.

References

1. Bolte S, Brown S, Satiat-Jeunemaitre B. The N-myristoylated Rab-GTPase m-Rabmc is involved in post-Golgi trafficking events to the lytic vacuole in plant cells. J Cell Sci. 2004;117(Pt 6):943-54.
2. Rice AE, Mendez MJ, Hokanson CA, Rees DC, Bjorkman PJ. Investigation of the biophysical and cell biological properties of ferroportin, a multipass integral membrane protein iron exporter. J Mol Biol. 2009; 386(3):717-32.