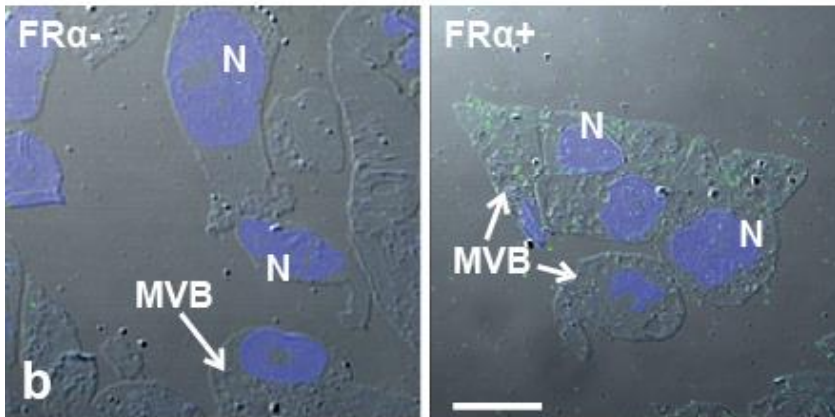
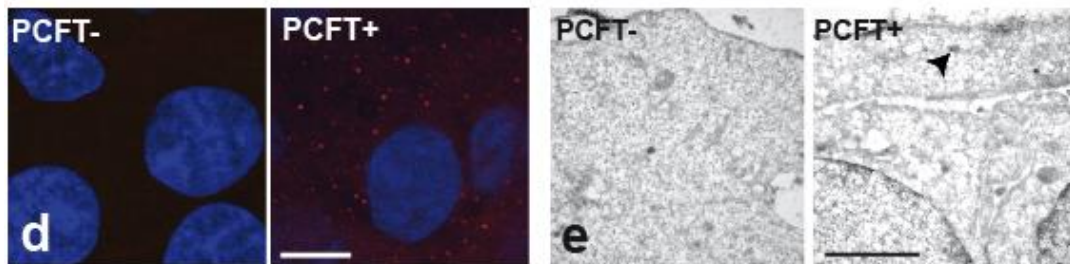
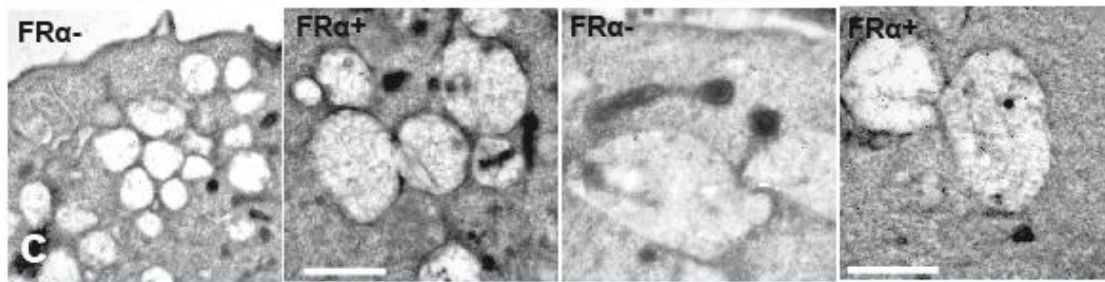


human choroid plexus



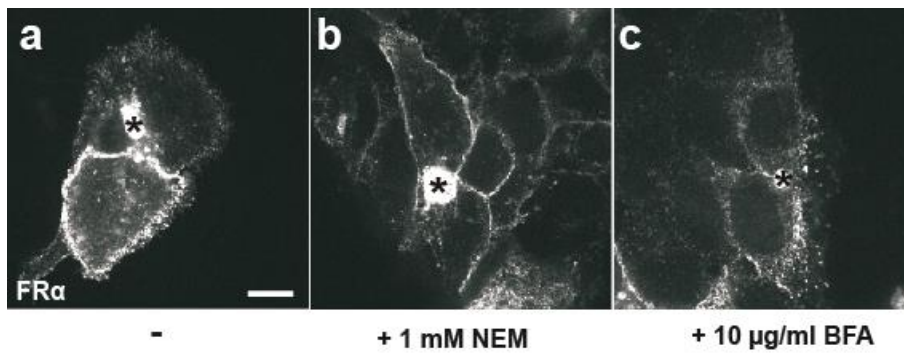
**Vector- or
FRα-transfected
Z310 cells**



FRα/PCFT-transfected Z310 cells

Supplementary Figure S1. Localization of the RFC in human choroid plexus and characterization of antibodies against human FRα and human PCFT in transfected rat choroid plexus Z310 cells.

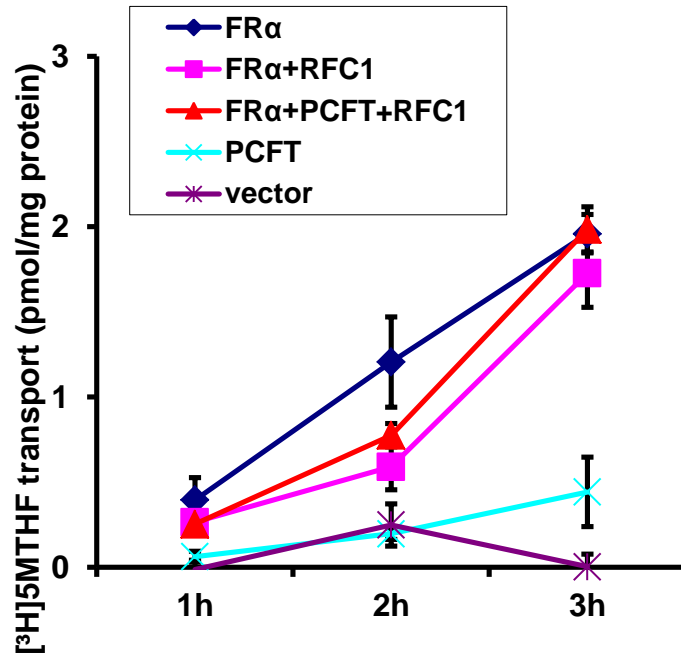
(a) Immunofluorescence microscopy of human choroid plexus specimen demonstrates the apical localization of the RFC that differs from the distribution of FRα. Scale bar 20 μm. **(b)** Combined fluorescent phase contrast microscopy of FRα- or vector-transfected Z310 cells reveals specific FRα+ (green) staining when compared with vector-transfected (FRα-) Z310 cells. FRα is detected at the cell surface and within MVBs. Nuclei (N) were counterstained with DAPI (blue). Scale bar 10 μm. **(c)** Immunogold staining of FRα- or vector-transfected Z310 cells demonstrates at least ten fold more gold particles in the FRα+ cells than in the FRα- ones. Left scale bar: 1 μm, right scale bar: 0.5 μm. **(d and e)** The specificity of the anti PCFT antibody is illustrated by immunofluorescence or immunogold electron microscopy using FRα-transfected Z310 cells (PCFT-) and FRα/PCFT-double-transfected Z310 cells (PCFT+). Nuclei were counterstained with DAPI (blue). Scale bar (d): 10 μm, scale bar (e): 500 nm. Very similar results were obtained for FRα/PCFT/RFC-triple-transfected Z310 cells.



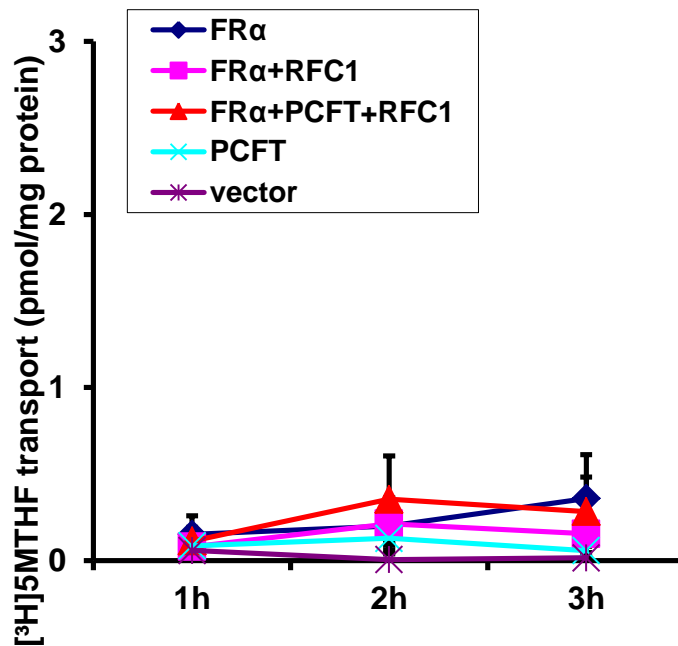
Supplementary Figure S2. Basolateral internalization of FR α in polarized hepatic HepG2 cells.

(a) HepG2 cells stably expressing FR α were grown on coverslips, fixed, permeabilized and stained with anti-human FR α antibody and secondary Cy3-conjugated antibody. Confocal microscopy shows a predominant localization of FR α at the apical bile canicular (BC) membrane (asterisk) and a minor staining at the basolateral membrane. **(b)** Cells were treated with the transcytosis and endocytosis inhibitor N-ethylmaleimide (NEM) for 90 s at 37 °C. Cells were then washed and incubated with fresh medium for further 45 min. The confocal images show that internalization of FR α at the basolateral membrane is blocked. **(c)** Cells were treated with brefeldin A (BFA), a fungal metabolite that inhibits Golgi-to-cell surface traffic of proteins. The punctate pattern at the basolateral membrane showing internalization of FR α intensifies, since no newly synthesized protein is delivered to the basolateral cell surface. The data suggest basolateral to apical transcytosis of FR α in HepG2 cells. Scale bar: 10 μ m.

PI-PLC dependent 5MTHF transport in Z310 cells (baso->api)

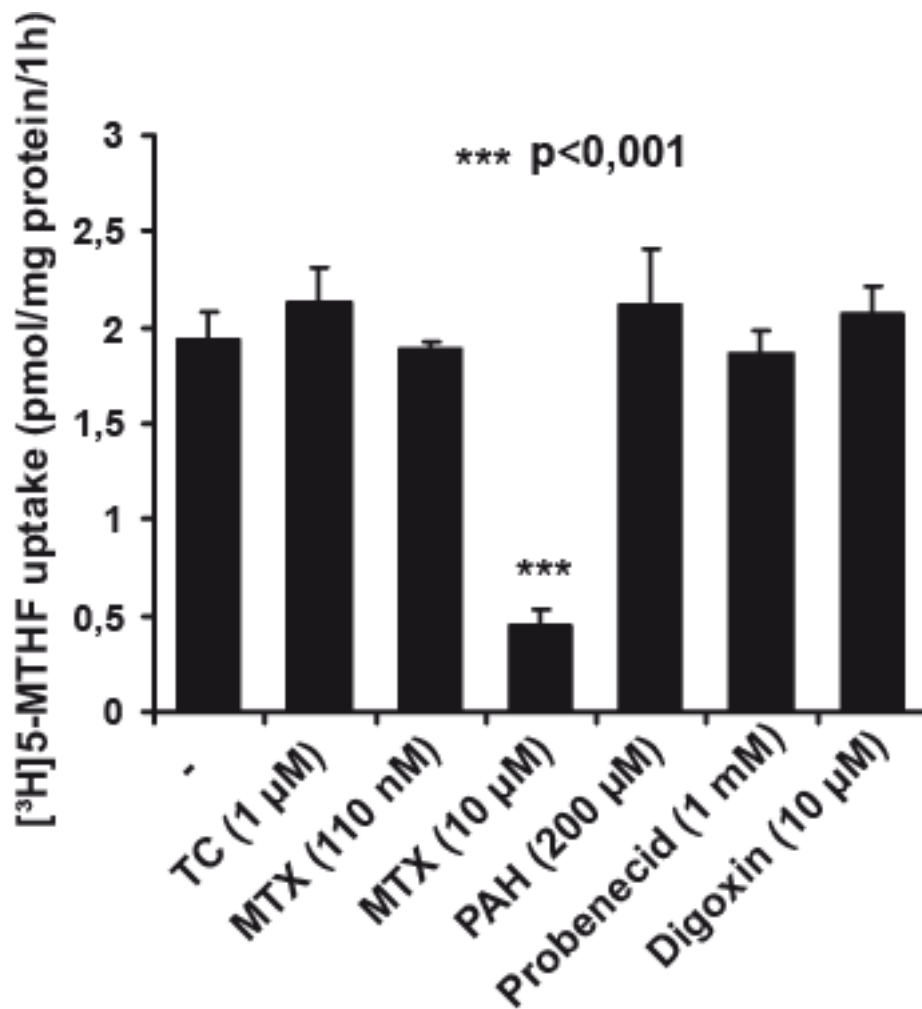


PI-PLC dependent 5MTHF transport in Z310 cells (api->baso)



Supplementary Figure S3: Directional 5MTHF transport in PI-PLC-treated Z310 cells

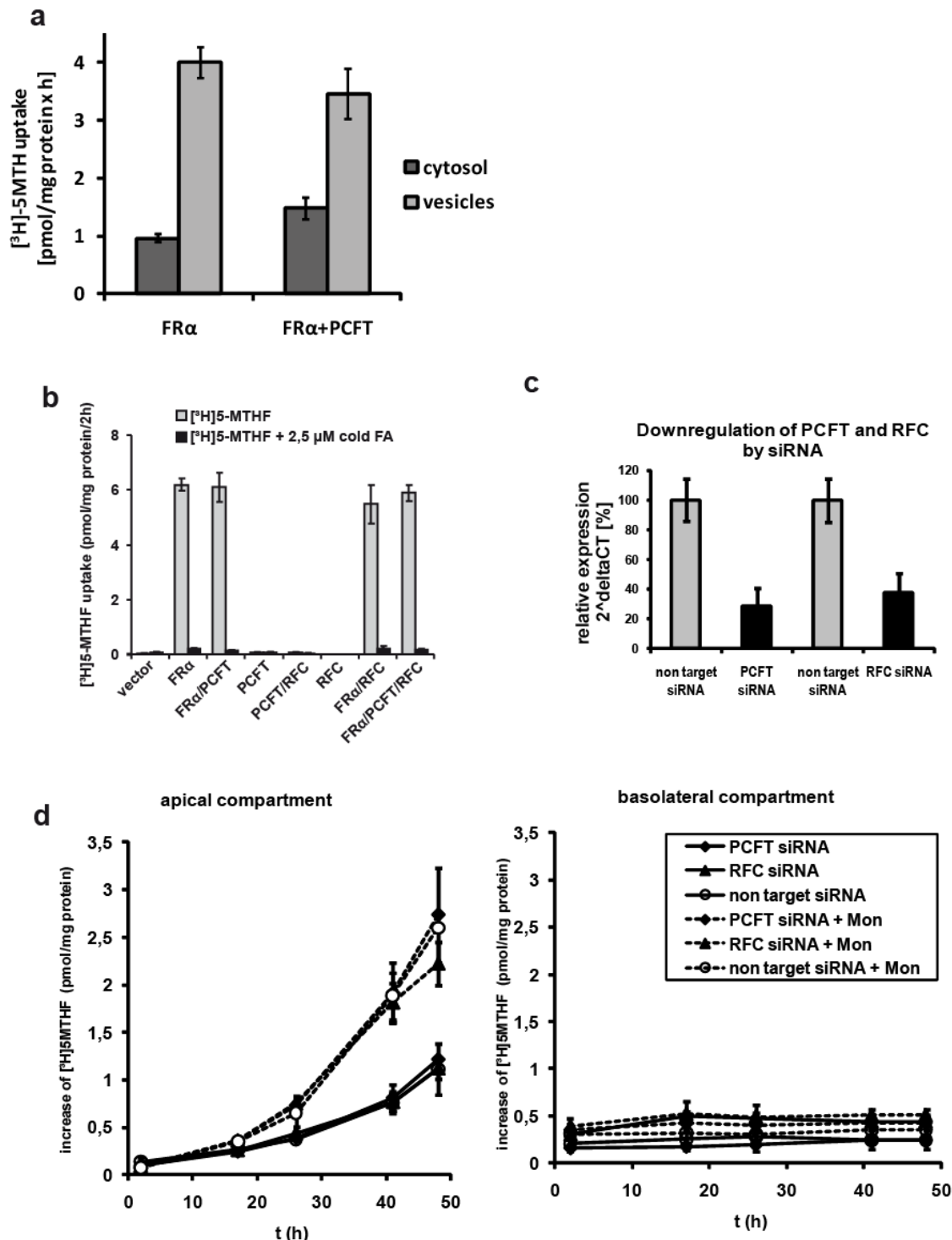
Transwell-grown Z310 cells stably expressing FR α , PCFT and/or RFC were incubated at 37°C with 12,5 nM ^3H -5MTHF and with PI-PLC in the opposite transwell chamber, respectively. 5MTHF continuously increased in the apical medium when added to the basolateral chamber. In contrast, no transport was measured when 5MTHF was added to the apical chamber and samples were collected from the basolateral chamber. PCFT and RFC did not contribute to FR α -mediated 5MTHF transport in Z310 cells. Data are means \pm s.e.m. of three independent experiments. Similar results were obtained with MDCK cells.



Supplementary Figure S4. Uptake of 5MTHF is not reduced by inhibition of organic anion transporters in FR α -expressing Z310 cells.

Z310 cells stably expressing FR α were incubated with 12.5 nM [³H]5MTHF alone or together with taurocholate (TC), methotrexate (MTX), p-aminohippuric acid (PAH), probenecid and digoxin for 1 hour at 37 °C, respectively. Subsequently, cells were lysed and intracellular radioactivity was measured. Only MTX at concentration of 10 μ M that is known to inhibit FR α binding significantly reduces 5MTHF uptake. Data are means \pm s.e.m. of three independent experiments, each done in duplicate (***, $p < 0.001$; Student's *t*-test).

At the indicated concentrations TC inhibits transport by Oat3 and Oatp3 (Ref. 46), PAH inhibits transport by Oat1, Oat3 (Ref. 47) and p-glycoprotein⁴⁸, Probenecid blocks Oat-mediated transport⁴⁶, additionally probenecid is a known inhibitor of MRP1 (Ref. 49) and MRP4 (Ref. 50). Digoxin is an inhibitor for Oatp2 (Ref. 46) and MTX at a concentration of 110 nM blocks RFC-mediated uptake of 5-MTHF.



Supplementary Figure S5. Contribution of PCFT and RFC on 5MTHF uptake and intracellular 5MTHF distribution in Z310 cells (a) The contribution of the PCFT on the intracellular distribution of 5MTHF was investigated with FR α -transfected and FR α /PCFT-cotransfected Z310 cells. Free (=cytosolic) and endosomal (=vesicular) 5MTHF was separated by the addition of hypotonic buffer (0.5 mM NaH₂PO₄, 1mM EDTA, pH7.0) and subsequent centrifugation as described before³⁰. In FR α -transfected cells the cytosolic 5MTHF fraction was 24% of the vesicular one. In FR α /PCFT-cotransfected cells the proportion of cytosolic 5MTHF increased to 43%. However, the amount of vesicular 5MTHF did not differ significantly between FR α -transfected and FR α /PCFT-cotransfected Z310 cells. Values are means \pm s.e.m. of three independent experiments. (b) Z310 cells stably transfected with FR α , PCFT and RFC either alone or in combination as indicated, were incubated with 12.5 nM [³H]5MTHF for 2 hours at 37 °C (gray bars). To block unspecific uptake, nonlabeled folic acid (FA) was added in an excess of 2.5 μ M (black bars). Subsequently, cells were lysed and intracellular radioactivity was measured. PCFT and RFC do not contribute to FR α -mediated 5MTHF uptake in Z310 cells. Data are means \pm s.e.m. of three independent experiments, each done in duplicate. (c and d) Z310 cells stably transfected with FR α were incubated with 25 nM of specific siRNA for 24h to downregulate the endogenous rat PCFT and rat RFC. Real-time PCR shows suppression of rat PCFT to 28% and rat RFC to 38% of the control cells that were incubated with non-targeted siRNA (c). Suppression of rat PCFT and RFC mRNA was related to rat GAPDH. Transport experiments that were performed as described in figure 5 were not altered by the downregulation of rat PCFT or rat RFC.

Supplementary Figure S6: Full gel scans of figures 2f, 2m, 3a, 3b, 4a, 4b, 4c, 5b and 5c

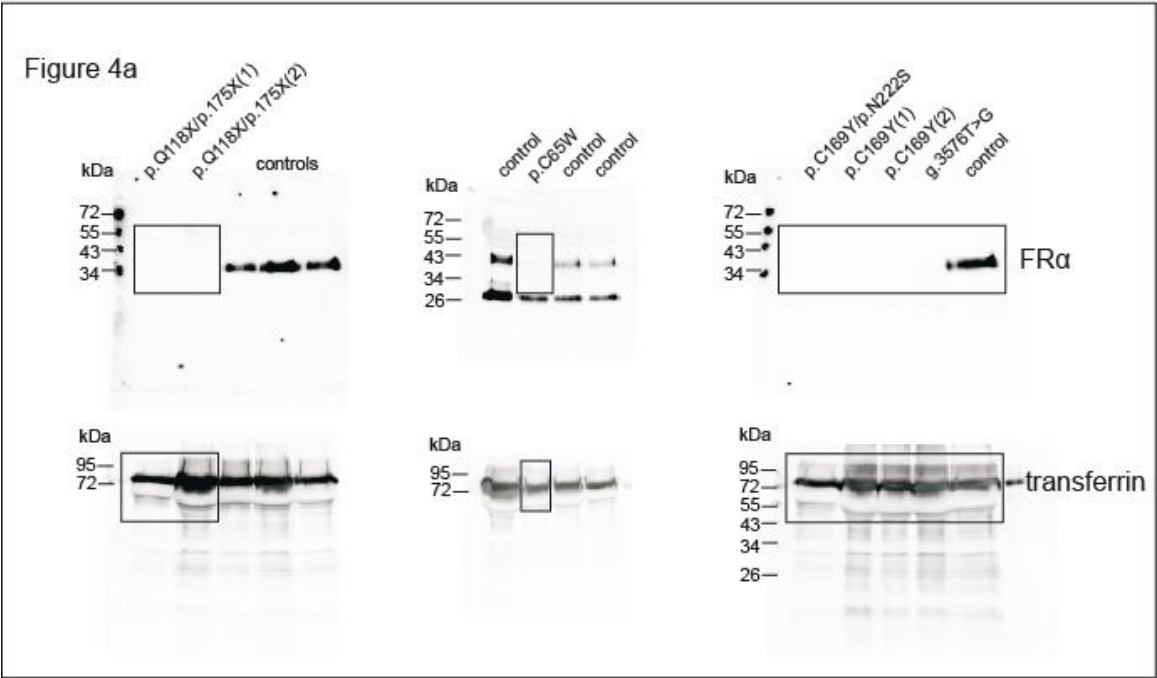
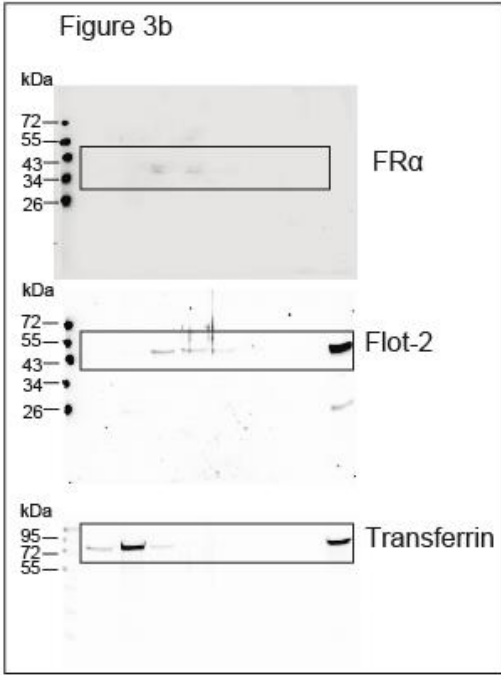
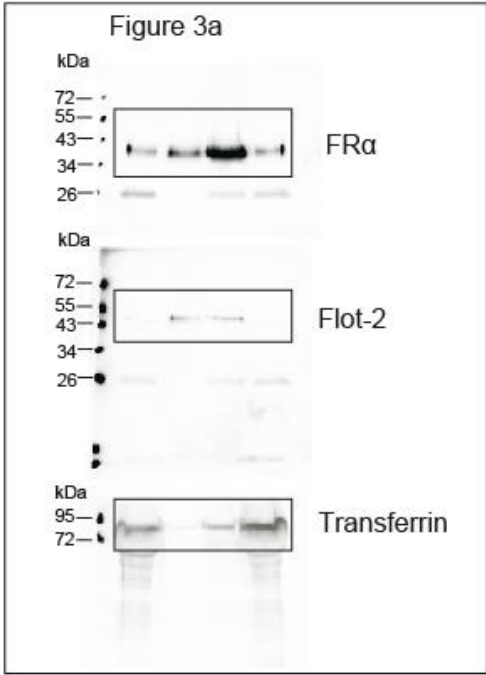
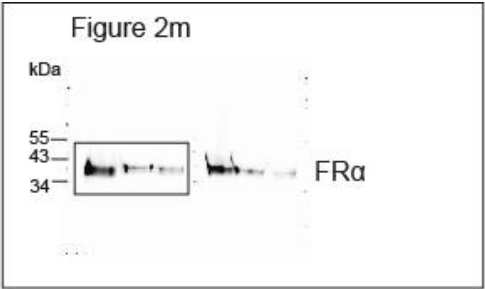
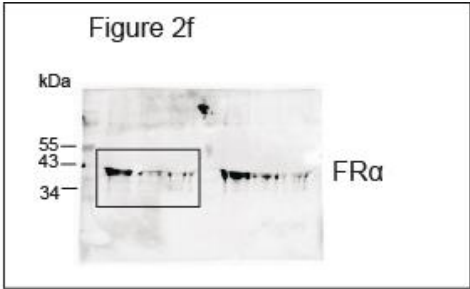


Figure 4b

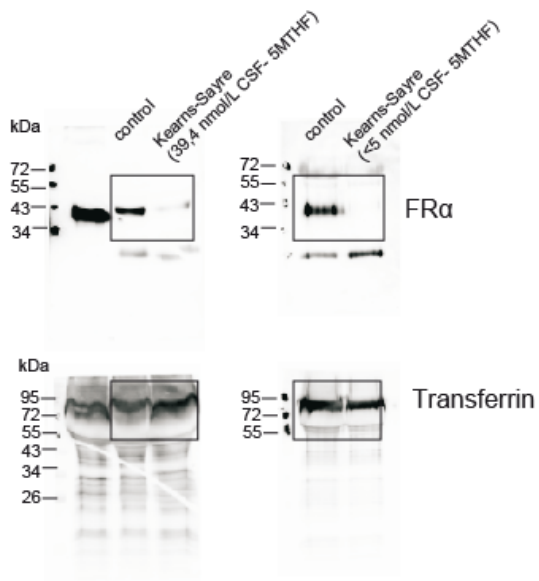


Figure 4c

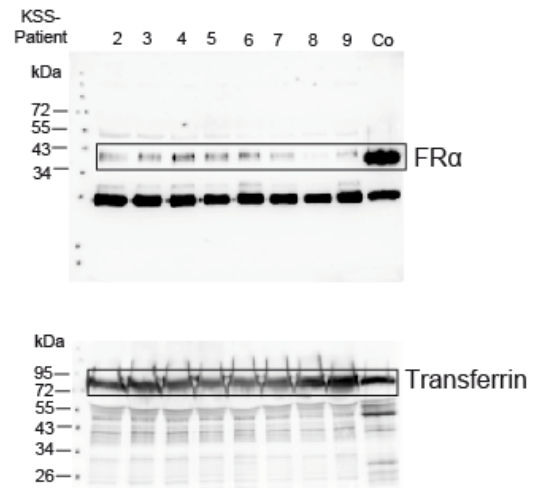


Figure 5b

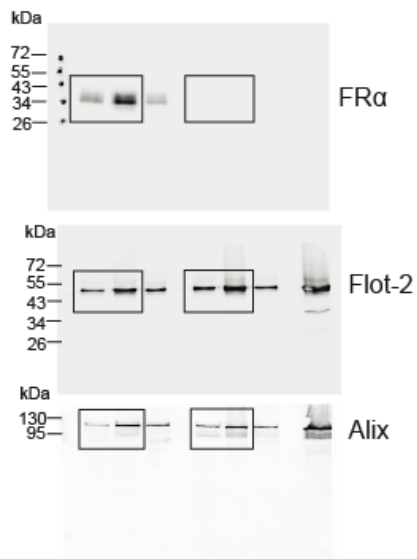
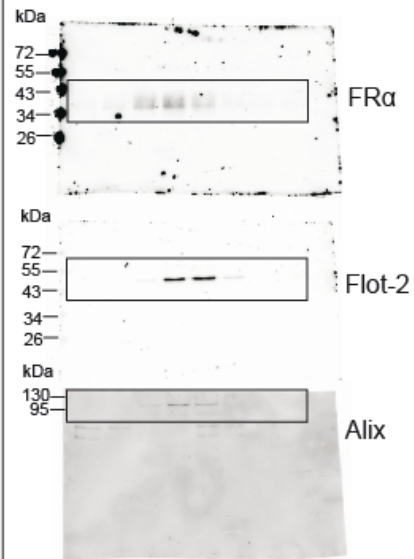


Figure 5c



Supplementary Table S1: Quantitative analysis of FR α and 5MTHF in exosome-related fractions.

	FRα in Z310 cells	[3H]5MTHF in Z310 cells
Supernatant before centrifugation	100%	100%
P10 fraction	8% (calculated)	6 +/-4%
P100 fraction	81% (calculated)	71 +/- 8%
Supernatant after centrifugation	11 +/- 2%	15 +/- 4%

The apical supernatants of transfected rat choroid plexus Z310 cells were separated by sequential centrifugation. The amount of FR α before and after the centrifugation steps was detected by Western blotting in the supernatant of Z310 cells. The P100 fraction contained on average 9.4 times more FR α signal than the P10 fraction. The amount of [3 H]5MTHF was determined for the supernatants and each fraction. Due to unspecific losses the total radioactivity was not recovered in the sum of radioactivity obtained from P10, P100 and remaining supernatant. Means \pm s.e.m are calculated from three independent experiments.

SUPPLEMENTARY METHODS

Cell culturing and cloning

Disassociated neonatal cortices from postnatal day 0-2 mice were cultured in DMEM supplemented with 10% fetal calf serum, 1% GlutaMAX (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin on laminin- and poly-L-lysine-coated coverslips at 37°C in 5% CO₂. Medium was changed every 3-4 days. At DIV 7-14, mixed cortical cultures consisting of oligodendrocytes, cortical neurons and microglia on an astrocyte monolayer were obtained.

Cloning of FR α cDNA has been described previously⁸. For expression in immortalized rat choroid plexus epithelial cells Z310¹⁶ FR α or PCFT cDNA was subcloned into pcDNA3.1 Hygro(+) (Invitrogen). For double transfectant FR α /PCFT Z310, PCFT cDNA was subcloned into pcDNA3.1 Zeo(+). RFC cDNA was subcloned into pEFIRES-P (Ref. 45).

PI-PLC dependent 5MTHF transport

Z310 cells were maintained in folate-free RPMI 1640 medium for at least 72h before the experiments. 12,5 nM 3H-5MTHF was added to either the basolateral or apical chamber of transwell-grown Z310 cells stably expressing FR α , PCFT and/or RFC. Transwell filters were incubated at 37°C with PI-PLC that was added to the non-radioactive apical or basolateral medium for the indicated time points. Radioactivity in medium samples was measured in a liquid scintillation spectrometer.

Assessment of intracellular 5MTHF distribution in FR α /PCFT-transfected Z310 cells

5MTHF uptake into cytosolic or vesicular compartments was determined as reported³⁰. Briefly, after 5MTHF uptake, cells were treated with 0.5 ml of hypotonic buffer (0.5 mM NaH₂PO₄, 1 mM EDTA, pH 7.0) containing 1µM nonlabeled folic acid for 10 min at 4 °C. The cells were then detached, resuspended and the suspension centrifuged at 14,000 rpm for 30 min at 4 °C. The Radioactivity in both the supernatant and the pellet, which was dissolved in 0.2 ml of 0.2 M NaOH, was determined.

Knock-down of folate transporters by siRNA

Rat PCFT and rat RFC were downregulated by specific siRNAs applied to FR α -transfected Z310 cells. 25 nM of four designed siRNAs targeting either rat PCFT, rat RFC, rat GAPDH or containing non-targeting siRNA (ON-TARGETplus SMARTpool, Thermo Scientific Dharmacon) were incubated with Z310 cells in the presence 0.2% of the DharmaFECT1 reagent. After 24h incubation time the FR α -mediated 5MTHF transport was measured as described above or cells were harvested to extract mRNA and synthesize cDNA. Downregulation of rat PCFT and rat RFC mRNA were detected by real-time PCR and were related to rat GAPDH. Means \pm s.e.m. were calculated from three experiments.

Statistical methods

Statistical analysis was done using Student's *t* test, where $p < 0.05$ was considered as statistically significant. Values are given as mean \pm s.e.m.

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