SUPPLEMENT

Materials and methods

Cell culture and reagents

Cell media and reagents were from Invitrogen unless otherwise indicated. Antibiotics and Tet-certified serum were from Clontech. In experiments with folate depletion folate-free RPMI 1640 media and dialyzed FBS have been used. Caspase inhibitor, zVAD-fmk and JNK inhibitor, SP600125 were from Calbiochem and Sigma, correspondently. Other chemicals were from Sigma.

Western blot and immunoprecipitation

Cells were lysed in RIPA buffer containing protease inhibitors cocktail (Sigma). Lysates were normalized by the level of total protein and analyzed by SDS-PAGE followed by immunoblot with corresponding antibodies: FDH-specific polyclonal (1:10000) (Krupenko and Oleinik, 2002); cofilin and phospho-cofilin (Ser3) polyclonal (1:500) from Sigma and Cell Signaling Technology, respectively; actin monoclonal (1:5000) from Calbiochem. For immunoprecipitation cell lysates (10^7 cells) were incubated with specific antibody (1-5 μg) for 1 h at 4 ^{o}C followed by overnight incubation with Protein G Sepharose 4 Fast Flow (50 μl of 50% slurry) (Amersham). Sepharose beads were washed three times (25 mM Tris-HCl, pH 7.5 containing 5 mM b-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$,), heated for 3 min in SDS-loading buffer and subjected to SDS-PAGE/Western blot.

Transwell migration and invasion assay

Cells were seeded into the upper chamber of a Transwell insert (BD Bioscience) in serum-free medium at a density of 50,000 cells/well. For migration assays inserts were pre-coated with 5 μg/ml fibronectin (Fisher Scientific). Medium containing 10% FBS was placed in the lower chamber as a chemoattractant, and cells were incubated for 24 h in CO_2 incubator. Non-migrating cells were removed from the upper chamber by scraping; the remaining cells were stained with Diff-Quick (Dade Behring, Inc.). Stained

cells were counted in 10 random microscopic fields in three independent inserts. Alternatively, stained cells were lysed with 10% acetic acid (100-200 μl/well) and quantified by colorimetry at 560 nm. Invasion assays were carried out in a similar manner using inserts pre-coated with extracellular matrix. For these experiments, Cell Invasion Assay Kit (Chemicon) has been used according to the manufacturer's protocol.

Migration track assay

Cell Motility HitKit (Cellomics) was used according to the manufacturer's manual. Cells were seeded at a low density (approximately 1000 cells/well) in four-chamber slide wells (pre-coated with 5 μg/ml fibronectin and overlaid with Blue Fluorescent Beads) and incubated for 24 h at 37 ^{o}C. The ability of cells to create phagokinetic non-fluorescent tracks was evaluated by fluorescence microscopy and quantified using NIH Image software.

Adhesion assay

Approximately 40,000 cells per well were seeded in 24-well plate pre-coated with increasing concentrations of fibronectin (0-8 μg/ml). After two-hour incubation non-adherent cells were removed by repeated washing. Adherent cells were fixed with 3% glutaraldehyde for 30 min at room temperature and stained with 0.1% crystal violet. After 30 min cells were washed and lysed with 10% acetic acid. OD was read at 560 nm.

Wound healing assay

Cells were grown to about 80% confluence in a six-well plate. After addition of doxycycline (2.5 μg/ml) to induce FDH, cells were allowed to grow for additional 16 h, then the medium was replaced with serum-free medium with or without calyculin (2.5 nM), and cells were left overnight before scrapping a wound down the center of the well with a pipette tip. Images of the denuded area were taken at 0, 24 and 48 h later using Axioscope 20 upright microscope (Carl Zeiss). zVad-fmk (50 μM) was added simultaneously with doxycycline to prevent FDH-induced apoptosis.

Culture synchronization and cell cycle analysis

Synchronization of cells at G0/G1 was achieved by maintaining the culture at confluence for 48 h. M/G2 arrest was induced by 16 h treatment with 0.4 μg/ml nocodazole. Cells were then fixed in 40% ethanol and stained in 38 mM sodium citrate buffer containing 50 mg/ml propidium iodide, 0.1% Triton X-100 and 7 Kunitz units/ml RNase A. Flow cytometry analysis was performed in the Hollings Cancer Center Core facility on a Becton Dickinson FACSCalibur using CellQuest and Mod Fit Software. Simultaneous induction of FDH in these experiments was achieved by the addition of doxycycline 48 h prior to the end-point of experiments.

Starvation experiments

These experiments were performed similar to the experiments with folate withdrawal. Cells have been incubated in DMEM media in the absense of the following components: (i) D-glucose and sodium pyruvate; (ii) L-methionine and L-cysteine (-Met, -Cys); (iii) FBS. Levels of phosphorylated cofilin were evaluated by Western blot 1-3 days after nutrient withdrawal.

Confocal microscopy

Cells were seeded in Lab-Tek II Chamber (Nulg Nunc International) and induced with Dox for FDH expression. At indicated time points cells were fixed with 3.7% of methanol-free formaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 10% pre-immune goat serum in PBS for 45 min slides were incubated with cofilin (1:100 dilution) and phospho-cofilin (clone 77G2, Cell Signaling Technology, 1:25 dilution) antibodies overnight at 4 ^{o}C. This was followed by incubation with secondary chicken anti-goat conjugated with Alexa Fluor 555 and/or goat anti-rabbit antibody labeled with Alexa Fluor 488 (Molecular Bioprobes) in dark chamber for 45 min at room temperature. Fluorescent staining of globular and filamentous actin was performed using fluorescent deoxyribonuclease I and phalloidin conjugates (Molecular Bioprobes), respectively, according to the manufacturer's manuals. Images were captured and processed using Leica TCS SP2 AOBS scanning confocal microscope.

Figure Legends

Figure S1

Effects of doxycycline and zVAD-fmk on motile characteristics of A549 cells and ATG10 clone (the parental clone of FDH-inducible A549/Tet-On cells, which has doxycycline-responsive element but lacks FDH expressing vector).

Figure S2

Top panel, time-dependence of cofilin knockdown after transfection of A549 cells with a siRNA Stealth duplex (see Materials and Methods of the manuscript for details). *Middle panel*, levels of cofilin in control and siRNA knockdown cells (72 h post-transfection), and levels of S3A and S3D cofilin mutants 48 h after transient transfection. The mutants were expressed as HA-tagged proteins and migrate slower on SDS-PAGE than cofilin (19 kDa protein). *Bottom panel*, expression of S3D cofilin mutants restores FDH-inhibited motility (assessed by transwell migration assays); four samples (each in triplicate) were analyzed for each cell type (average $\pm$SD is shown). Less than 100% restoration of motility could be associated with the efficiency of transfection with S3D mutant

Figure S3

Effects of nutrient deprivation on levels of phosphorylated cofilin. (**a**) Cell cycle analysis of A549 cells grown on different folate supplementation (ND, folate depleted media; NM, normal media containing 2.2 μM folic acid). (**b**) Levels of cofilin and phospho-cofilin in the same cells. (**c**) Levels of cofilin and its phosphorylated form in cells upon different nutrient deprivation. (**d**) Levels of phosphorylated cofilin in indicated cell lines at different times of media folate deprivation.

Folate-related dephosphorylation of cofilin was not associated with apoptotic response: no apoptosis was induced upon folate withdrawal as can be judged from the lack of sub-G0 peak (**a** and **b**). The robust dephosphorylation of cofilin appears to be specific to folate withdrawal and not to a general nutrient starvation: cells kept on FBS-free media or media deprived of either glucose plus pyruvate or methionine plus cysteine did not reduce levels of phospho-cofilin (**c**). Interestingly, dephosphorylation of cofilin in

response to folate depletion was observed in several other cell lines (**d**) indicating a universal nature of this phenomenon.

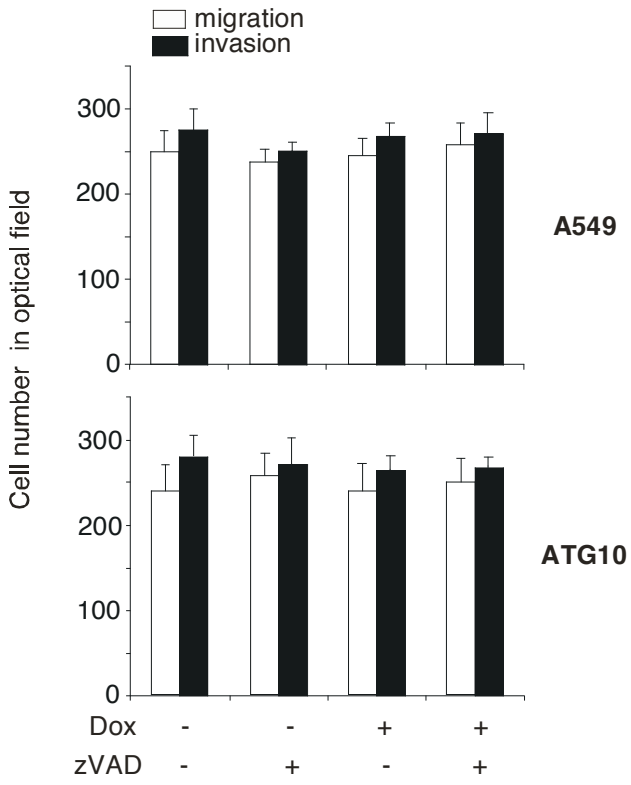
Figure S4

Motility of FDH-expressing A549 cells is restored by the addition of calyculin. A549 cells were subjected to transwell migration assays in the presence or in the absence of calyculin after FDH induction. In these experiments cells were grown in the presence of pan-caspase inhibitor zVAD-fmk to prevent FDH-associated apoptosis. Treatment with 2.5 nM calyculin restored motility in FDH expressing cells. The inhibitor itself did not produce any visible effects on the levels of phosphorylated cofilin or motility. Transwell migration assays were performed as described in Materials and Methods, this supplement.

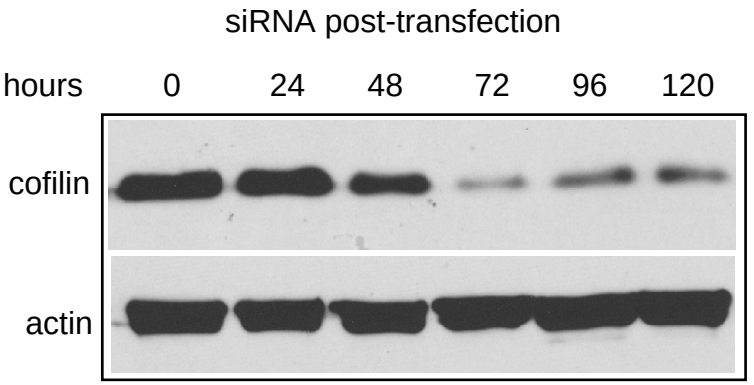
Figure S5

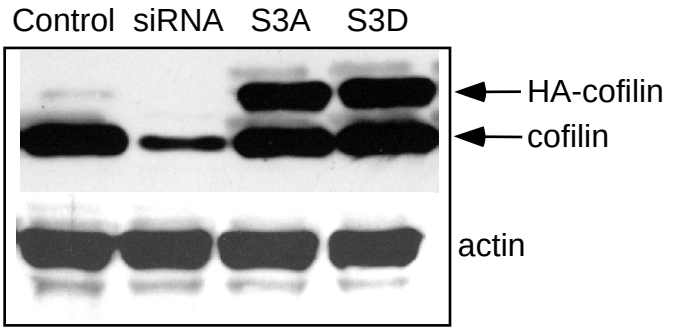
FDH does not increase co-accumulation of cofilin and G-actin in nuclei. Levels of cofilin (*green*) and G-actin (*red*) in FDH deficient (no FDH) and FDH expressing (+FDH) A549 cells imaged by confocal microscopy. Nuclei stained with TO-PRO-3 (Molecular Bioprobes) are shown in *blue*. *Right panels* show overlay of three colors. Co-localization of cofilin and G-actin can bee seen in nuclei of FDH-deficient cells as well as in FDH expressing cells. *Bar*, 20 μm.

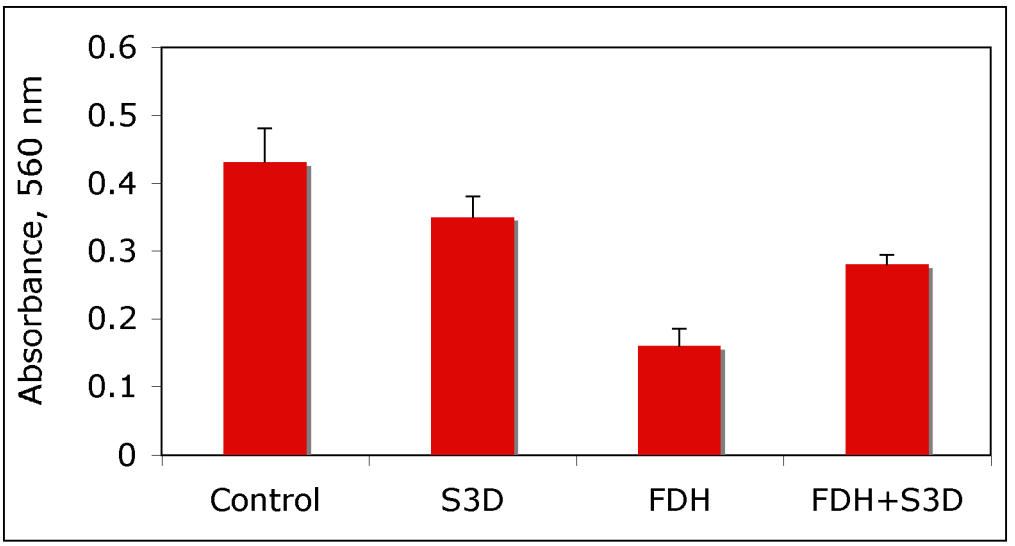
Oleinik N. Figure S1



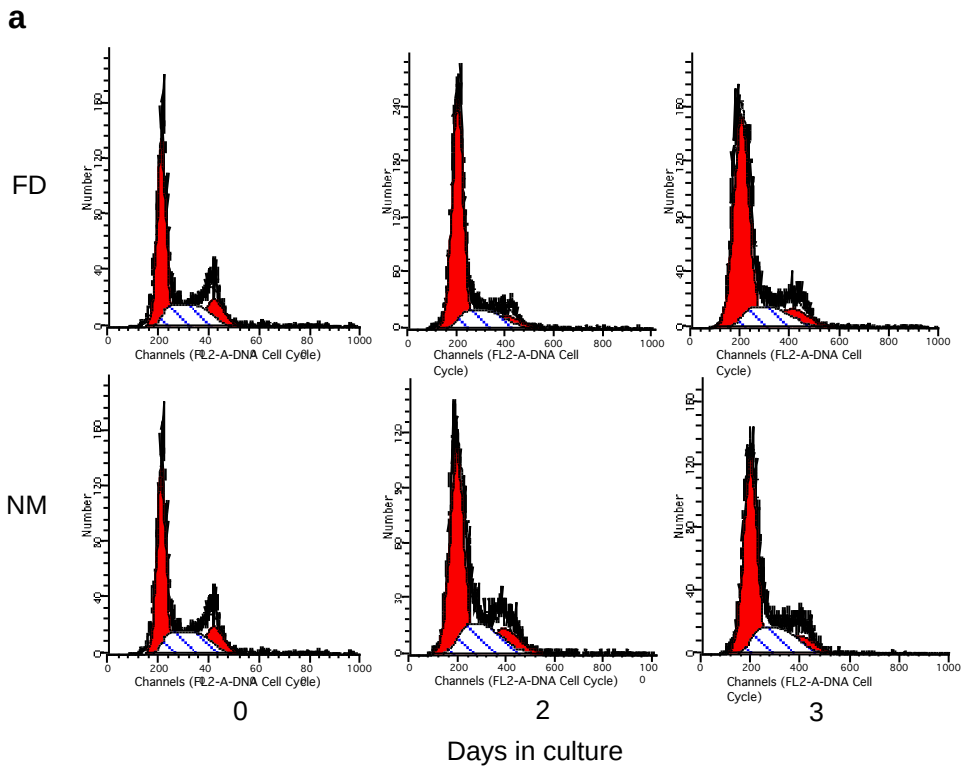
Oleinik N. Figure S2

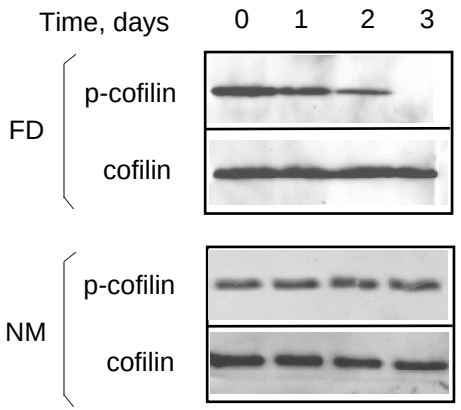


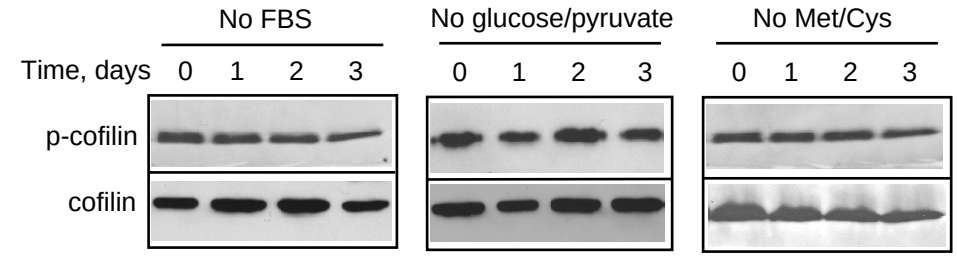


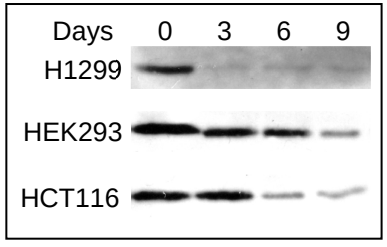


Oleinik N. Figure S3

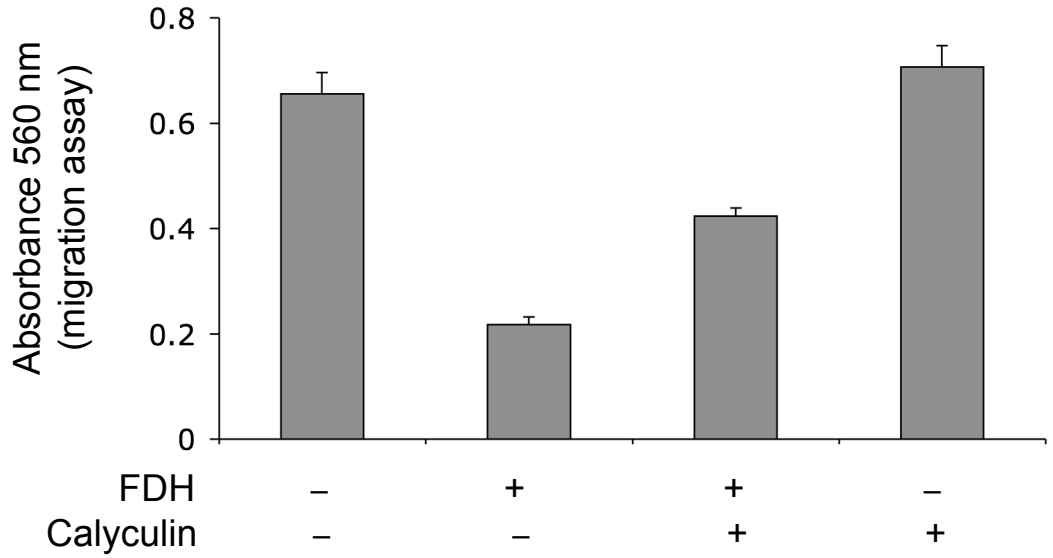








Oleinik N., Figure S4



Oleinik N., Figure S5