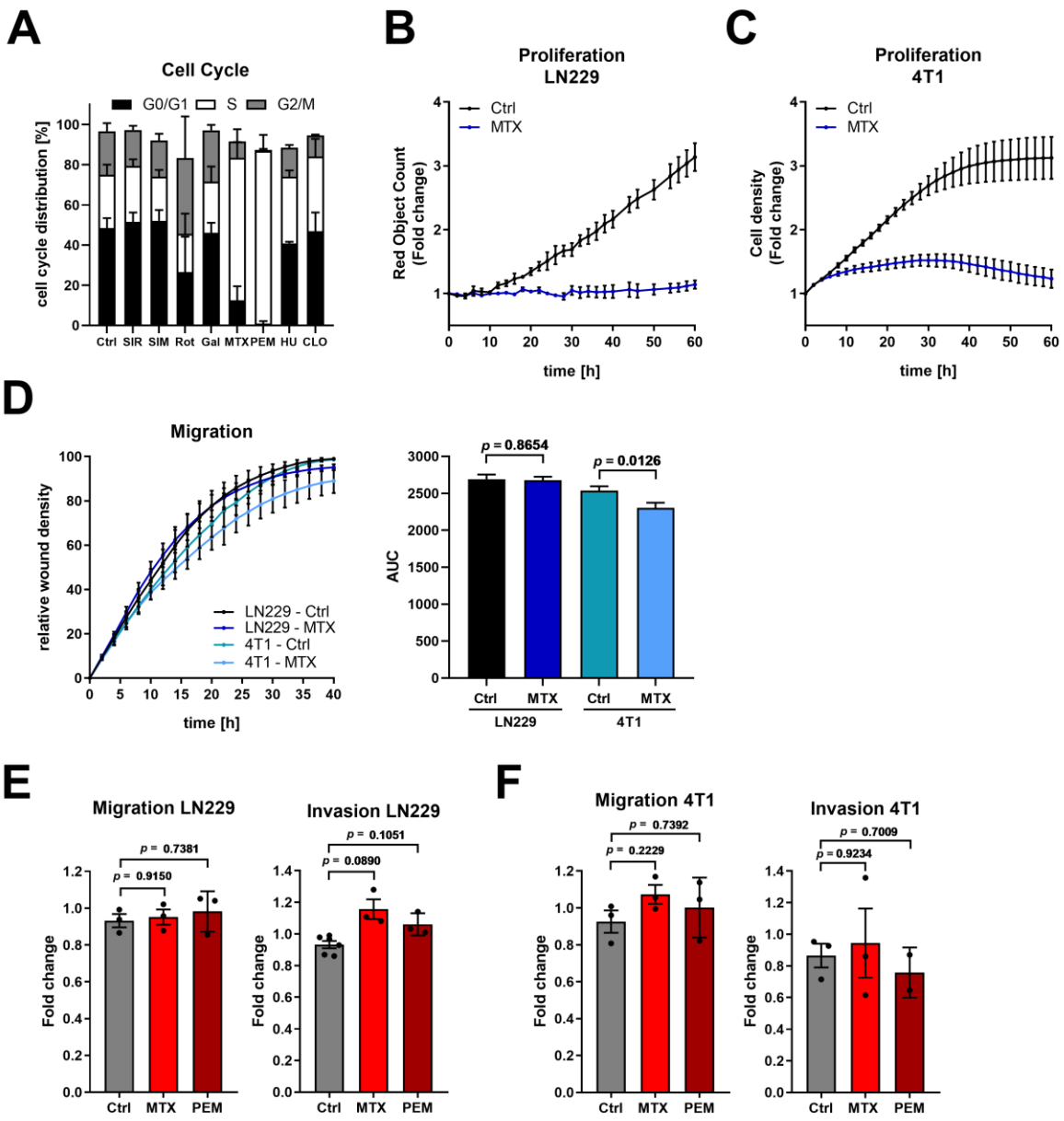
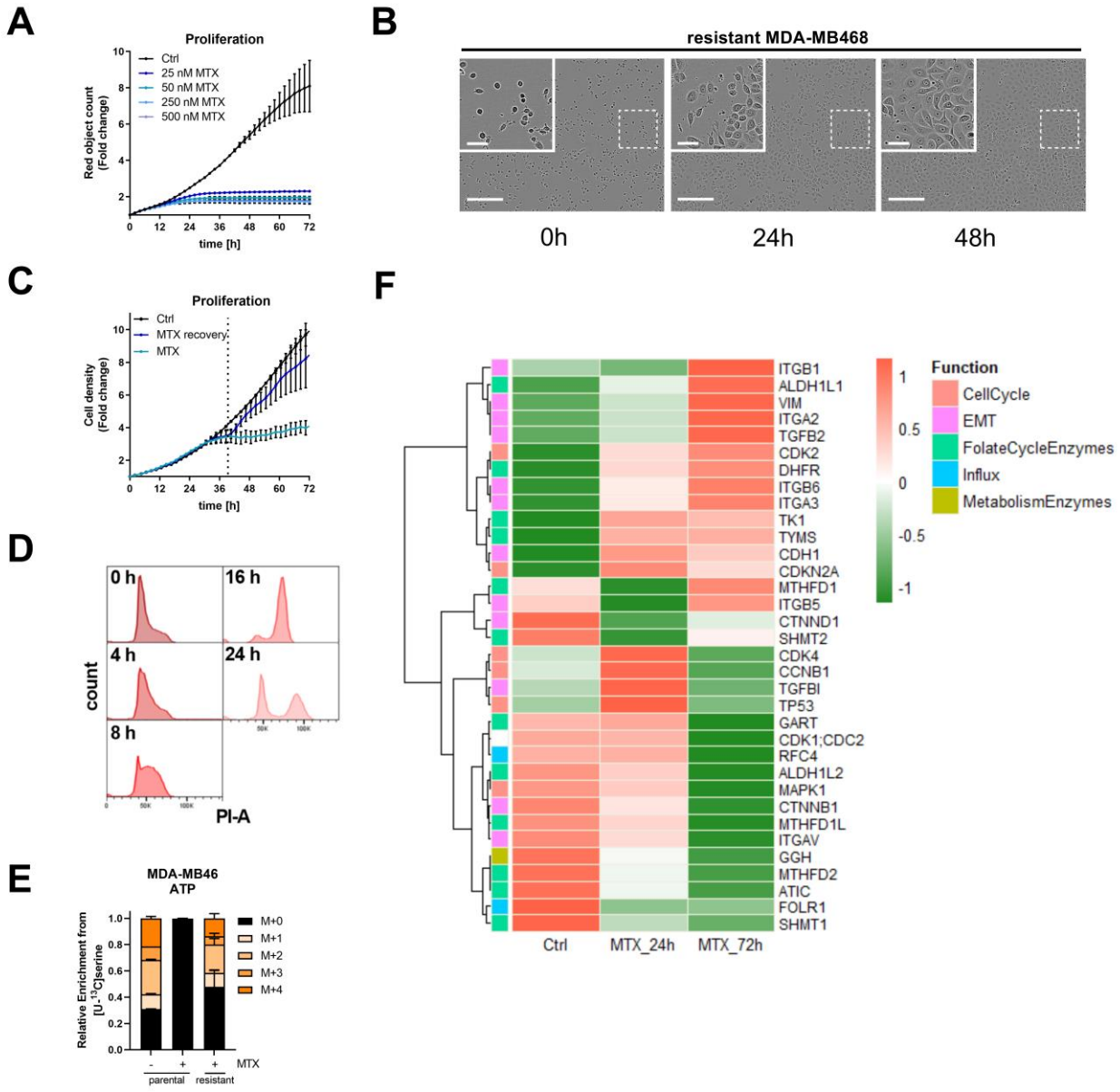
Supplementary Figure 1

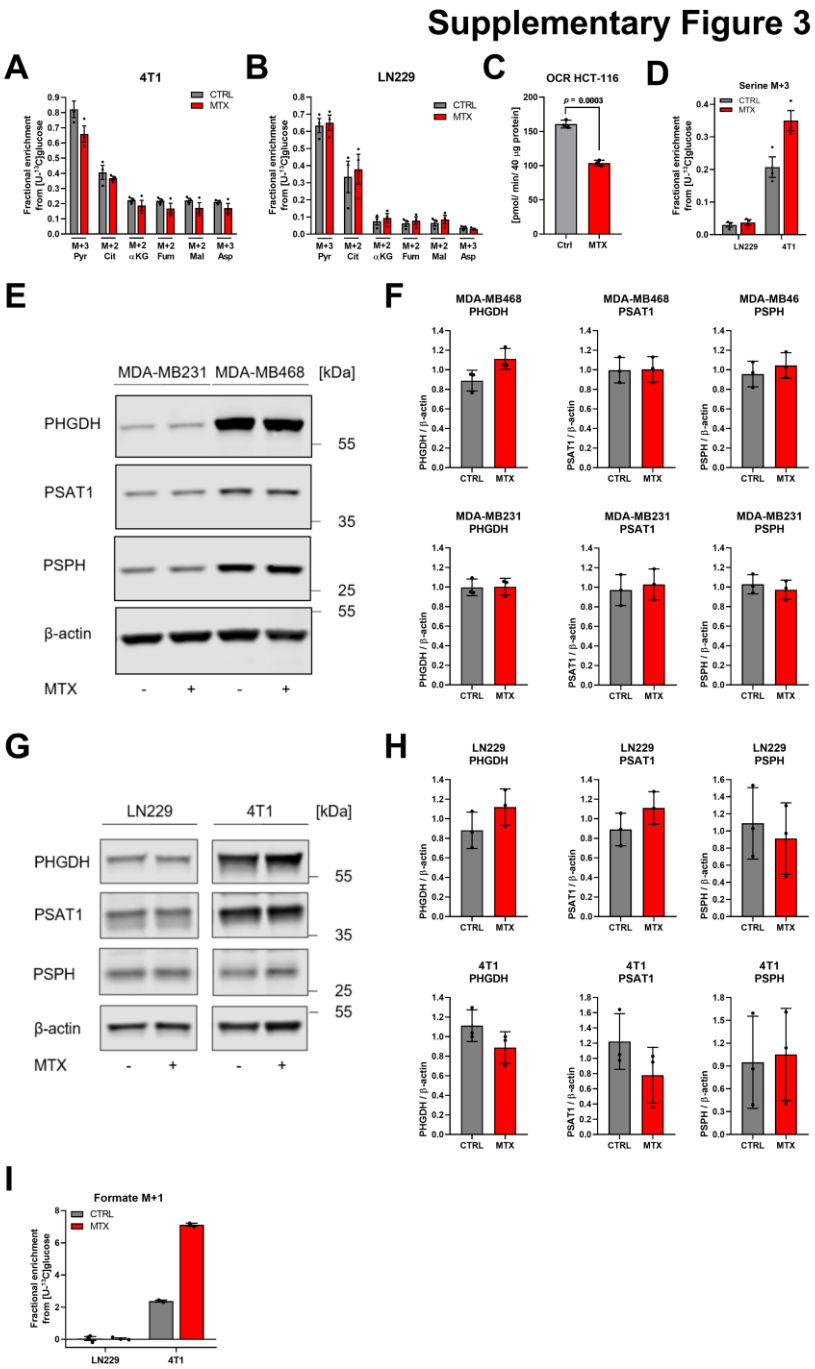


Supplementary Figure 1: (A) MDA-MB468 cells were treated for 48 h with 100 nM Sirolimus (SIR), 1 µM Simvastatin (SIM), 50 nM Rotenone (Rot), galactose (Gal) supplementation, 50 nM Methotrexate (MTX), 1 µM Pemetrexed (PEM), 0.5 mM hydroxyurea (HU), and 100 nM Clofarabine (CLO). Cells were analyzed for cell cycle distribution by flow cytometry of PI-stained cells; mean ± SD (n = 3 - 11). **(B)** Proliferation of LN229 cells in response to 50 nM MTX; mean ± SEM (n = 3). **(C)** Proliferation of 4T1 cells in response to 75 nM MTX; mean ± SEM (n = 4). **(D)** Migration of LN229 and 4T1 cells in response to 50 nM MTX and respective AUC; mean ± SEM (n = 5); unpaired t-test with Welch's correction. **(E), (F)** Migration and invasion of LN229 (E) and 4T1 (F) cells after 24h treatment with 50 nM (E), 75 nM (F) MTX was assessed using ECM-Collagen-coated or non-coated Boyden chambers. Each dot represents an independent experiment; mean ± SEM; Brown-Forsythe and Welch one-way ANOVA with Dunnett's multiple comparisons test.

Supplementary Figure 2

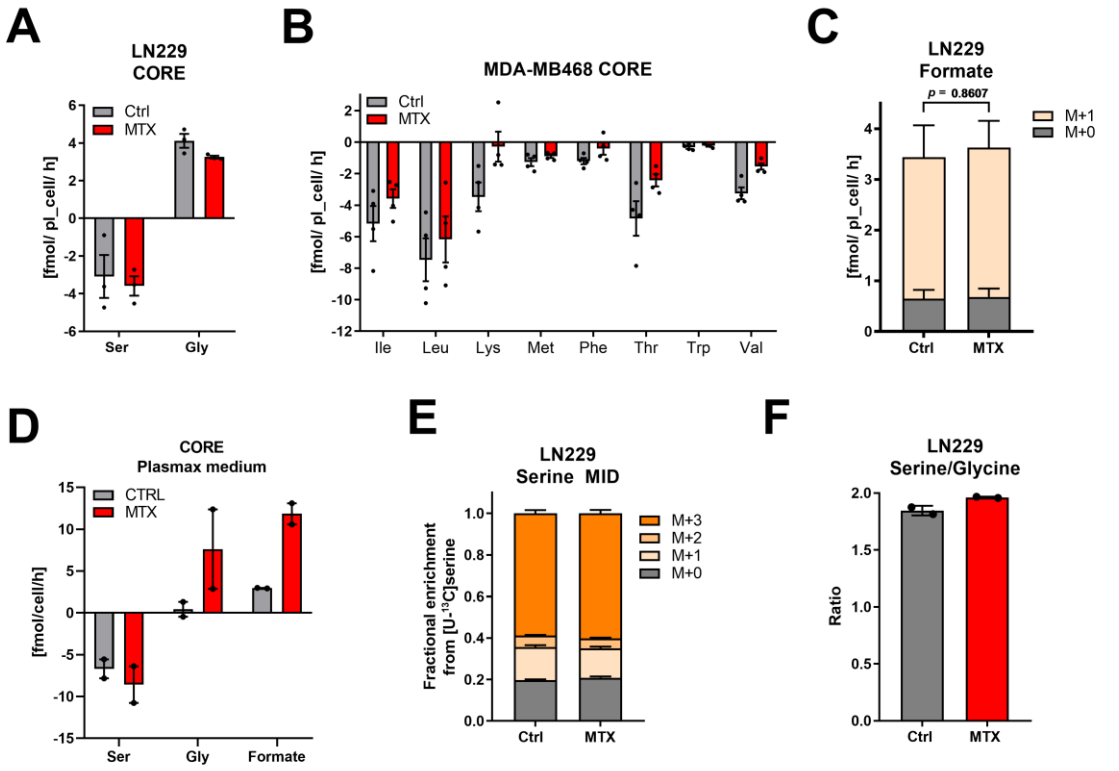


Supplementary Figure 2: (A) Proliferation of NucLight Rapid Red stained MDA-MB468 cells upon the indicated concentrations of MTX; mean ± SEM (n = 3). **(B)** Morphology of proliferating MTX-resistant MDA-MB468 cells. Bright-field images are representative of independent experiments. Scale bars correspond to 60 and 300 µm. **(C)** Growth of MDA-MB468 cells in response to 50 nM MTX and growth recovery upon removal of MTX after 38 h assessed as fold change of cell density; mean ± SEM (n = 3). **(D)** Recovery of cell cycle profile at the indicated time points after MTX removal as in (C). Histograms are representative of three independent experiments. **(E)** MID of intracellular ATP upon [U-^{13}C]serine tracer in response to 24 h 50 nM MTX in parental MDA-MB468 cells and MTX-resistant MDA-MB468 cells. Graph shows mean ± SEM of two independent experiments each measured in triplicate wells. **(F)** Heatmap of selected protein expressions from SILAC quantitative proteomics measurement with different MTX treatment durations. The log2 fold changes of each protein were standardized by row. Molecular function of proteins are indicated with colors.



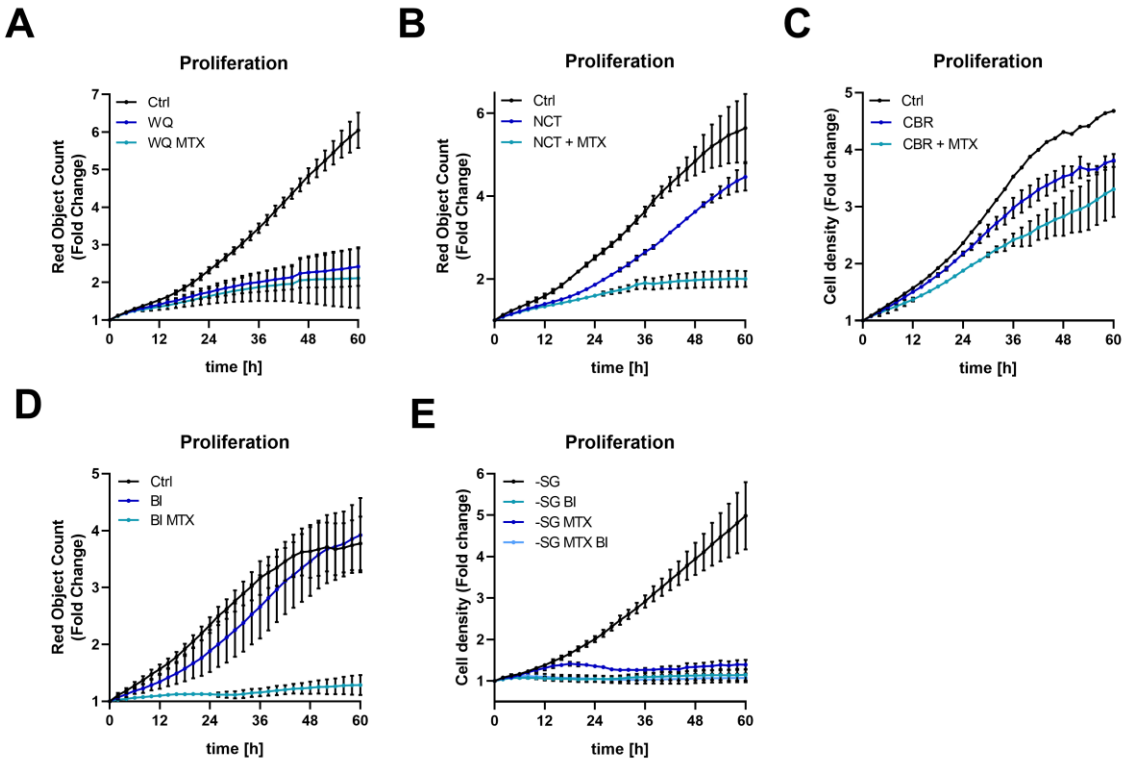
Supplementary Figure 3: (A), (B) Absolute CORE rates of glutamine and glutamate in the medium of 4T1 (A) and LN229 (B) cells in response to 24 h 50 nM MTX (A) and 75 nM MTX (B). Each dot represents an individual experiment composed of triplicate wells; mean ± SEM. **(C)** Basal cellular respiration in response to 24 h 50 nM MTX treatment was determined in HCT-116 cells as quantification of mitochondrial OCR. Each dot represents an individual experiment composed of six technical replicates; mean ± SEM; unpaired t-test with Welch's correction. **(D)** Enrichment of M+3 isotopologue of serine upon [U-^{13}C]glucose tracer in response to 24 h 50 nM MTX in LN229 and 24 h 75 nM MTX in 4T1 cells. Each dot represents an independent experiment in triplicate wells; mean ± SEM. **(E)** Expression of PHGDH, PSAT1, and PSPH in MDA-MB231 and MDA-MB468 cells upon 24 h 50 nM MTX treatment; β-actin serves as loading control. **(F)** Treatment as in (E). Quantification of PHGDH, PSAT1, and PSPH Western blot signal intensity relative to β-actin and normalized to global mean in MDA-MB231 and MDA-MB468 cells. Each dot represents an independent experiment; mean ± SD. **(G)** Expression of PHGDH, PSAT1, and PSPH in LN229 and 4T1 cells upon 24 h 50 nM MTX (LN229) and 75 nM MTX (4T1) treatment; β-actin serves as loading control. **(H)** Treatment as in (G). Quantification of PHGDH, PSAT1, and PSPH Western blot signal intensity relative to β-actin and normalized to global mean in LN229 and 4T1 cells. Each dot represents an independent experiment; mean ± SD. **(I)** M+1 isotopologue of extracellular formate in LN229 and 4T1 cells using [U-^{13}C]glucose tracer upon 24 h treatment with 50 nM (LN229) and 75 nM (4T1) MTX. Each dot indicates an independent experiment measured in triplicate wells; mean ± SEM.

Supplementary Figure 4



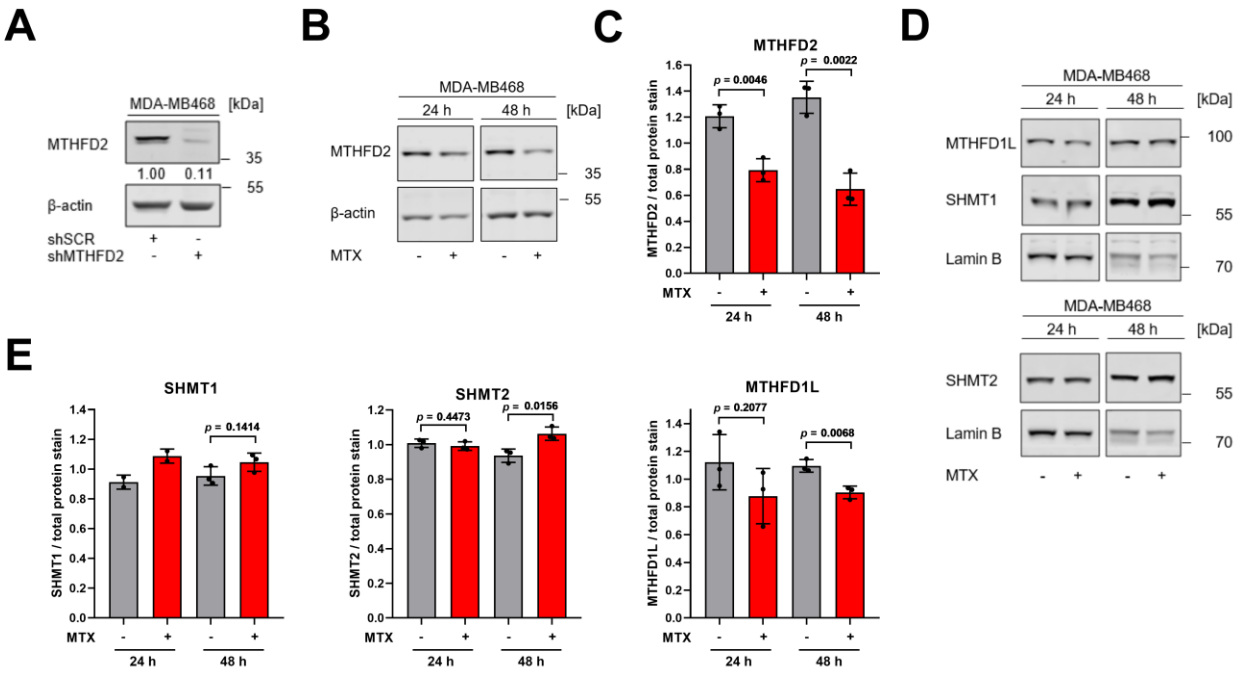
Supplementary Figure 4: (A) Absolute CORE rates of serine and glycine of LN229 cells in response to 24 h 50 nM MTX. Each dot represents the mean of an individual experiment each measured in triplicate wells; mean ± SEM. **(B)** Absolute CORE rates of essential amino acids as indicated in response to 24 h 50 nM MTX. Each dot represents an independent experiment in triplicate wells; mean ± SEM. **(C)** MID of extracellular formate upon [U-^{13}C]serine tracer in LN229 cells in response to 24 h 50 nM MTX. Graph shows mean ± SEM of three independent experiments each measured in triplicate wells; unpaired t-test with Welch's correction. **(D)** Absolute CORE rates of serine, glycine, and formate upon [U-^{13}C]serine tracer in MDA-MB468 cells in response to 24 h 50 nM MTX in Plasmax medium. Each dot represents an independent experiment in triplicate wells; mean ± SEM. **(E)** MID of intracellular serine upon [U-^{13}C]serine tracer in LN229 cells in response to 24 h 50 nM MTX; mean ± SEM of two independent experiments each measured in triplicate wells. **(F)** Ratio of absolute intracellular serine and glycine in response to 24 h 50 nM MTX in LN229 cells. Each dot represents an independent experiment in triplicate wells; mean ± SEM.

Supplementary Figure 5



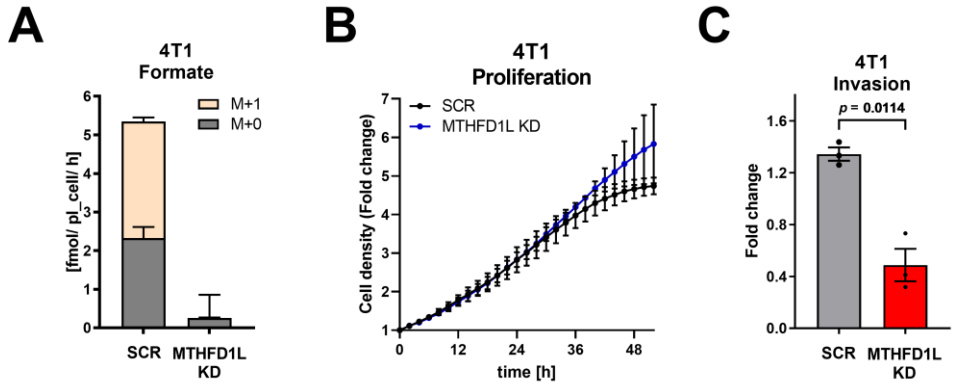
Supplementary Figure 5: (A) Proliferation of NucLight Rapid Red stained MDA-MB468 cells upon treatment with 10 µM WQ-2101 and 50 nM MTX as indicated; mean ± SEM (n = 6 - 7). **(B)** Proliferation of NucLight Rapid Red stained MDA-MB468 cells upon treatment with 10 µM NCT-502 and 50 nM MTX as indicated; mean ± SEM (n = 4). **(C)** Proliferation of MDA-MB468 cells measured as fold cell density upon treatment with 30 µM CBR-5884 and 50 nM MTX as indicated; mean ± SEM (n = 1 - 2). **(D)** Proliferation of NucLight Rapid Red stained MDA-MB468 cells upon treatment with 15 µM BI-4916 and 50 nM MTX as indicated; mean ± SEM (n = 2 - 3). **(E)** Proliferation of serine- and glycine-starved MDA-MB468 cells measured as fold cell density upon treatment with 15 µM BI-4916 and 50 nM MTX as indicated; mean ± SEM (n = 3).

Supplementary Figure 6



Supplementary Figure 6: (A) Expression of MTHFD2 in MDA-MB468 cells upon MTHFD2 KD using shRNA; β-actin serves as loading control. Quantification of MTHFD2 Western blot signal intensity relative to total protein stain and normalized to vehicle transfected control. **(B)** Expression of MTHFD2 in MDA-MB468 cells 24 h and 48 h 50 nM MTX; β-actin serves as loading control. **(C)** Treatment as in (B). Quantification of MTHFD2 Western blot signal intensity relative to total protein stain and normalized to GM in MDA-MB468 cells. Each dot represents an independent experiment; mean ± SD; unpaired t-test with Welch's correction. **(D)** Expression of MTHFD1L, SHMT1, and SHMT2 in MDA-MB468 cells 24 h and 48 h 50 nM MTX; Lamin B serves as loading control. **(E)** Treatment as in (D). Quantification of MTHFD1L, SHMT1, and SHMT2 Western blot signal intensity relative to total protein stain and normalized to GM in MDA-MB468 cells. Each dot represents an independent experiment; mean ± SD; unpaired t-test with Welch's correction.

Supplement Figure 7



Supplementary Figure 7: (A) MID of extracellular formate upon [U-^{13}C]serine tracer in 4T1 cells in response to *MTHFD1L* knockdown; mean ± SEM of one experiments measured in technical triplicates. **(B)** Proliferation of 4T1 cells measured as fold cell density upon *MTHFD1L* knockdown; mean ± SEM (n = 2). **(C)** Invasion of 4T1 cells in response to *MTHFD1L* knockdown was assessed using ECM-Collagen-coated Boyden chambers. Each dot represents an independent experiment; mean ± SEM; unpaired t-test with Welch's correction.