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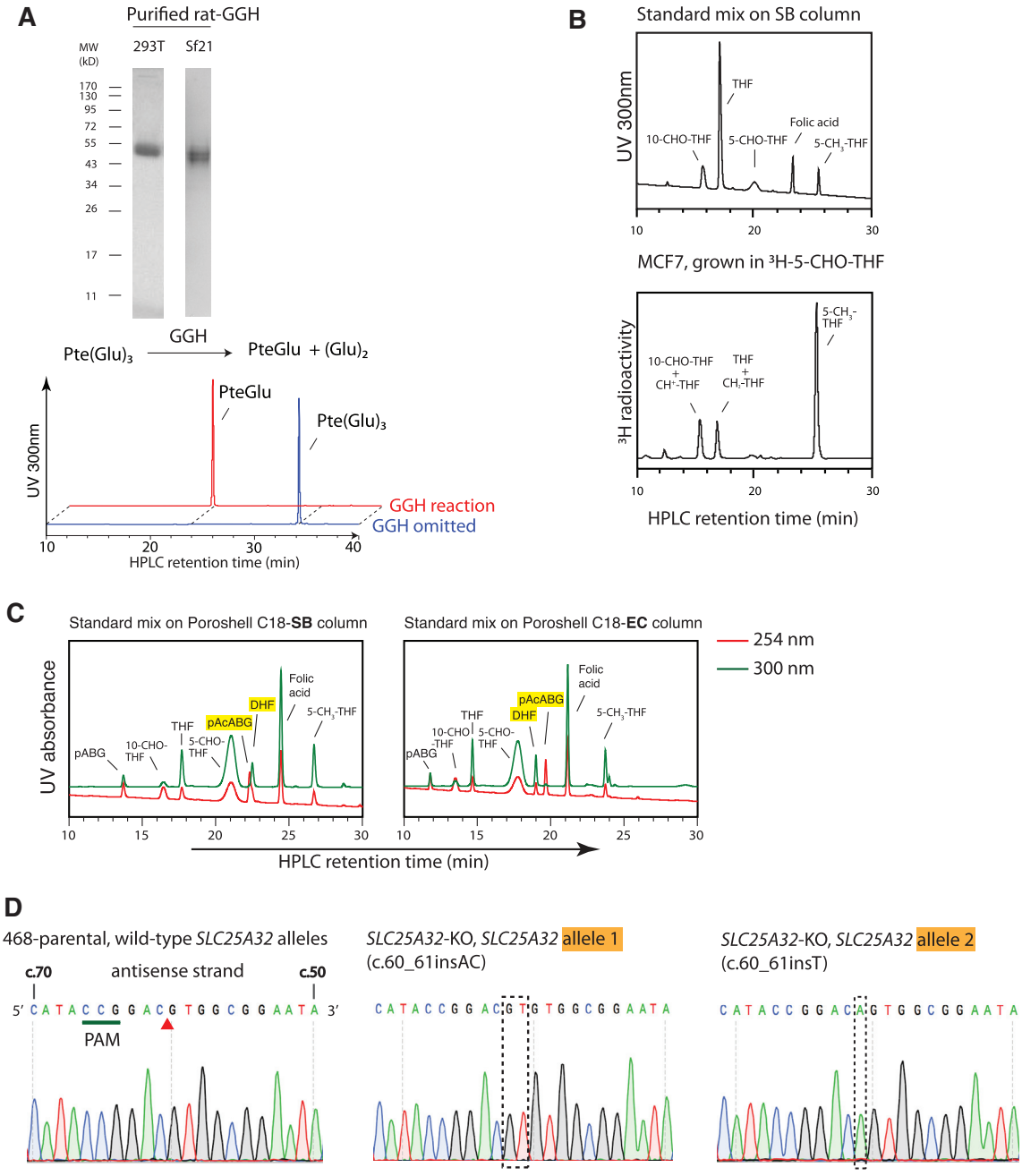


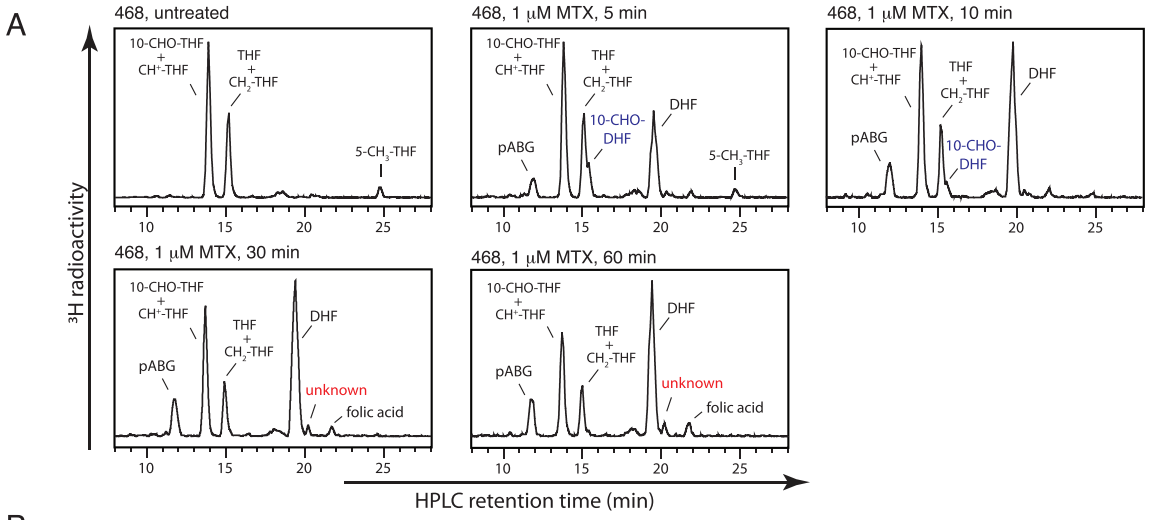
Figure S1. Development of an HPLC Assay for Intracellular Folates and Sanger Sequencing for *SLC25A32*-Knockout Verification, Related to Figure 1

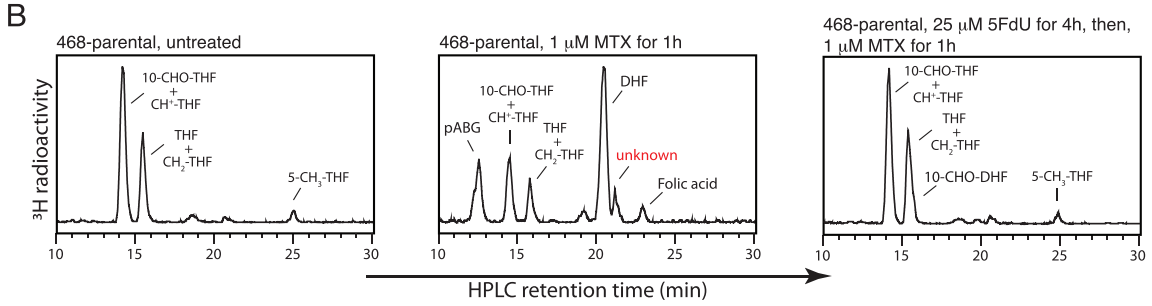
(A) Top, Coomassie Blue staining for rat GGH purified from 293T and Sf21 (insect) culture media. GGH is glycosylated, and migrates on the gel as one "fuzzy" band or multiple bands. Bottom, purified rat GGH is active.

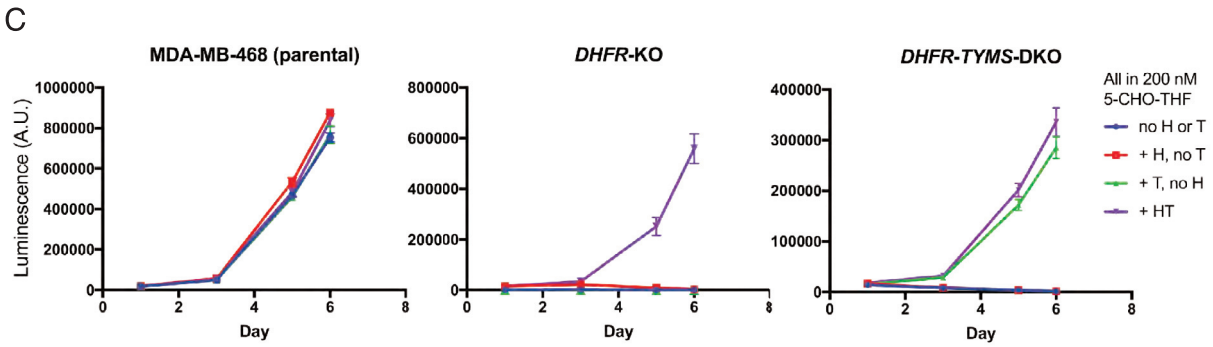
(B) HPLC identification of ^{3}H-labeled intracellular folates from MCF7 cells by comparison with authentic standards.

(C) The order of elution of pAcABG and DHF standards varies, depending on the type of column.

(D) Genotyping of the MDA-MB-468 *SLC25A32*-knockout clone. Individual alleles were cloned into PCR4-TOPO vector and sequenced.







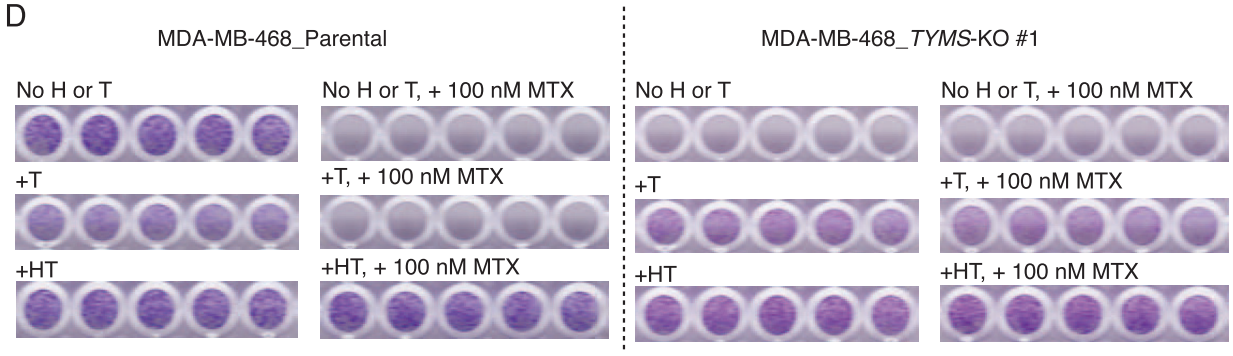


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Figure S2. *TYMS* Inhibition or Knockout Suppressed the Biochemical and Growth Phenotypes of MDA-MB-468 Cells upon Methotrexate Treatment, or *DHFR* Knockout, Related to Figure 2.

(A) HPLC analysis of folates from MDA-MB-468 exposed to 1 μM methotrexate on a short timescale. 10-CHO-DHF eluted as a shoulder of the THF/5,10-CH^+-THF peak.

(B) HPLC analysis of folates from MDA-MB-468 cells, untreated (left), treated with 1 μM methotrexate for 1 h (middle), or pretreated with 25 μM 5-fluoro-2′-deoxyuridine for 4h before being treated with 1 μM methotrexate for 1 h (right).

(C) Growth curves of MDA-MB-468 parental, *DHFR*-knockout and *DHFR-TYMS*-double knockout cells. The cells were placed in DMEM + 10% dialyzed FBS + 200 nM 5-CHO-THF + the indicated supplementation. "H" denotes 100 μM hypoxanthine, and "T" denotes 16 μM thymidine (T).

(D) Crystal violet staining of MDA-MB-468 parental, *DHFR*-knockout and *DHFR-TYMS*-double knockout cells. The cells were seeded at 1000 cells per well on day 0 and placed in DMEM + 10% dialyzed FBS + the indicated supplementation. "H" denotes 100 μM hypoxanthine, and "T" denotes 16 μM thymidine (T). "MTX" denotes 100 nM MTX. The staining was performed on day 5.

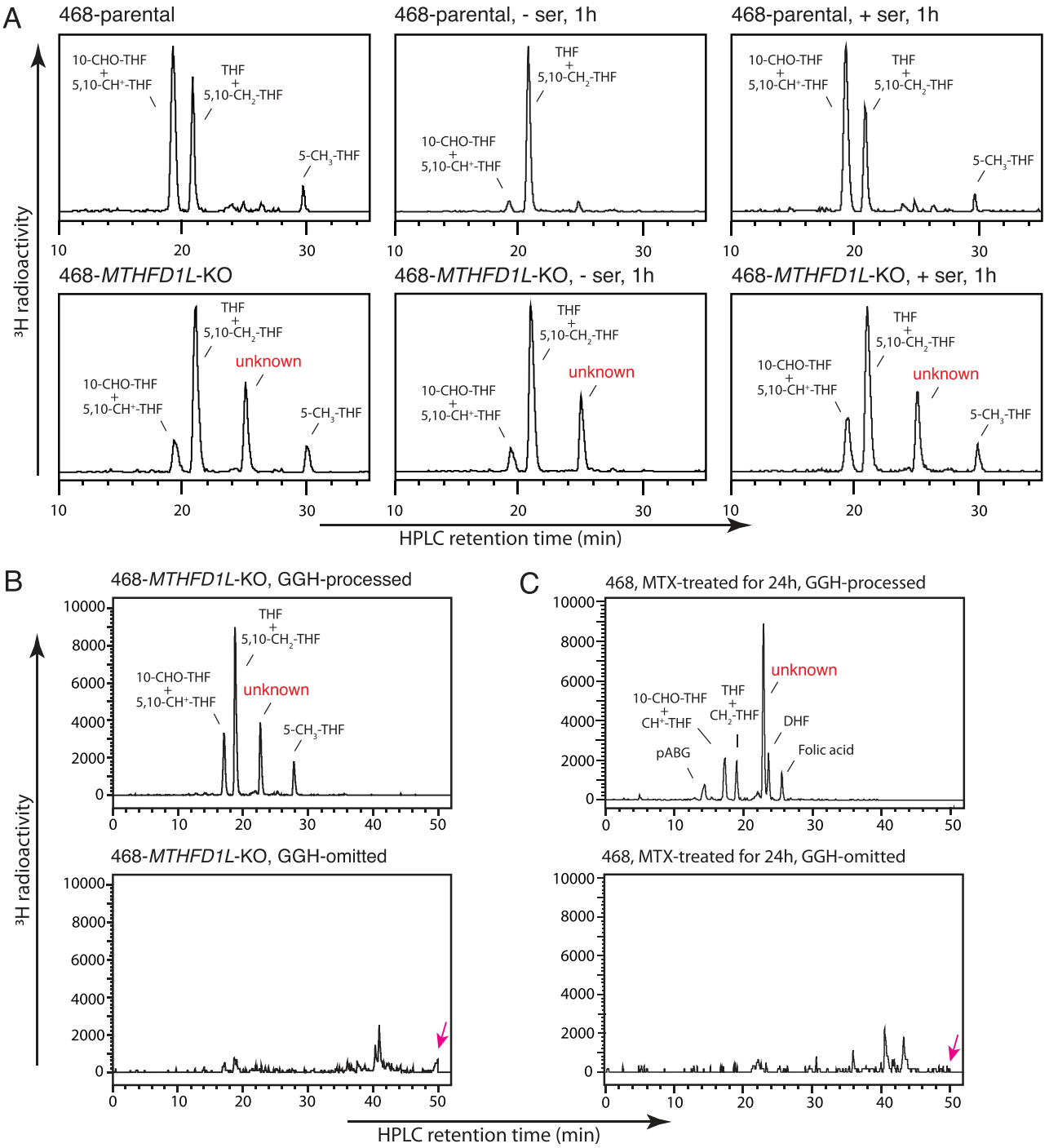

Figure S3. The Unusual Folate Metabolite Does Not Interconvert with Other Folates and Contains a Polyglutamate Tail, Related to Figure 3

(A) HPLC analysis of folates from MDA-MB-468 parental and *MTHFD1L*-knockout cells, untreated, upon acute serine deprivation for 1 h, or upon + serine medium change for 1 h.

(B) HPLC analysis of folates from MDA-MB-468 *MTHFD1L*-knockout cells, unprocessed (bottom) or processed (top) with GGH. At 50 min (red arrow), radiation detection was switched off.

(C) HPLC analysis of folates from MDA-MB-468 cells treated with 1 μM methotrexate for 24 h, unprocessed (bottom) or processed (top) with GGH. At 50 min (red arrow), radiation detection was switched off.

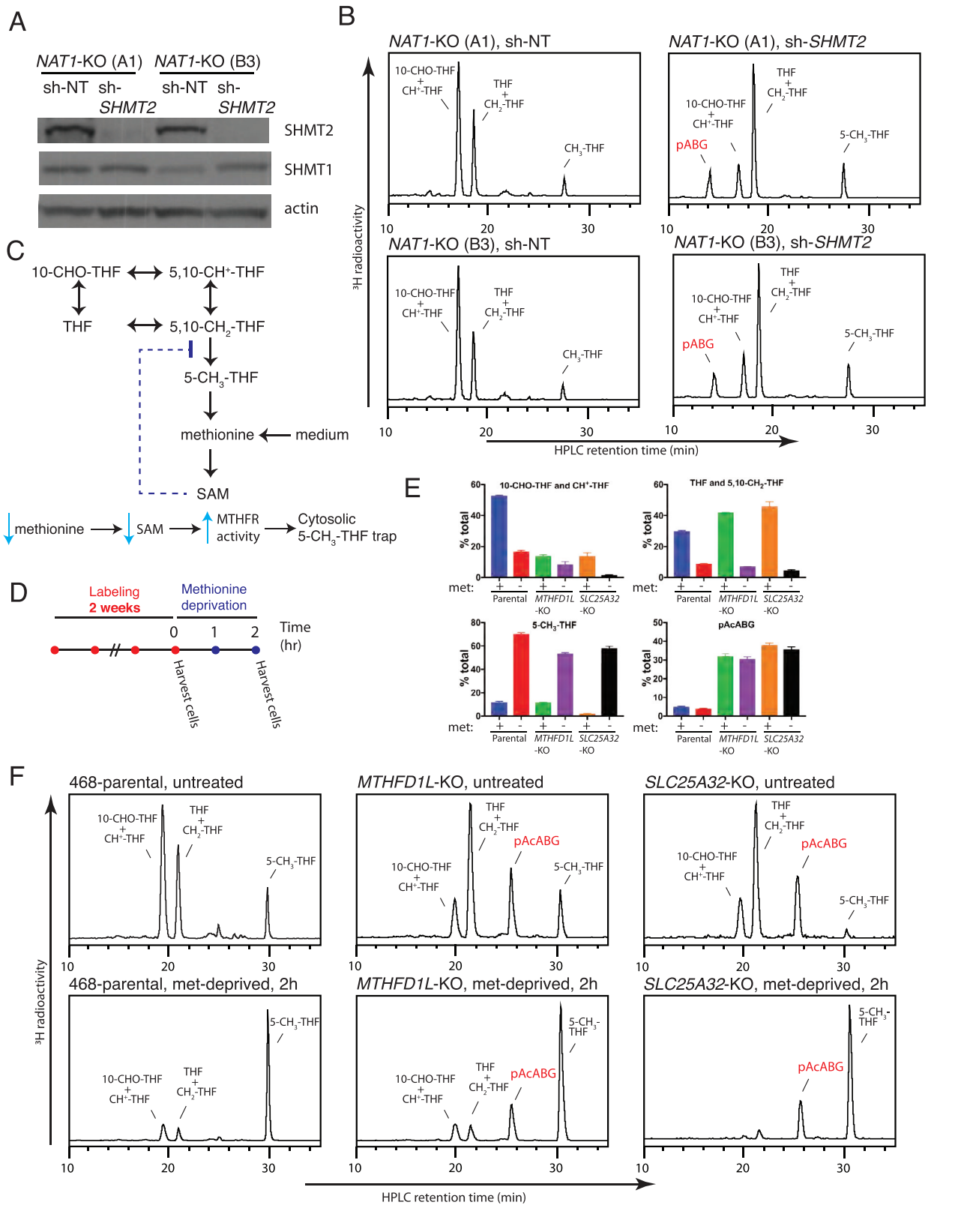

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Figure S4. NAT1 Is Responsible for pABG Acetylation, and Folate Degradation Seen in the Mitochondrial-1C-Pathway-Knockout Cells Occurs in the Cytosol, Related to Figure 4

(A) Western blot analysis of MDA-MB-468 *NAT1*-knockout cells stably expressing non-targeting or *SHMT2*-targeting shRNAs. A1 and B3 refer to two different clones generated using two different CRISPR guides.

(B) HPLC analysis of folates from MDA-MB-468 *NAT1*-knockout cells stably expressing non- targeting or *SHMT2*-targeting shRNAs.

(C) Schematic of the "methyl-folate trap" concept.

(D) Schematic of the methionine deprivation experimental design.

(E and F) HPLC analysis and quantification of folates from MDA-MB-468 parental, *MTHFD1L*-, and *SLC25A32*-knockout cells treated as in (D).

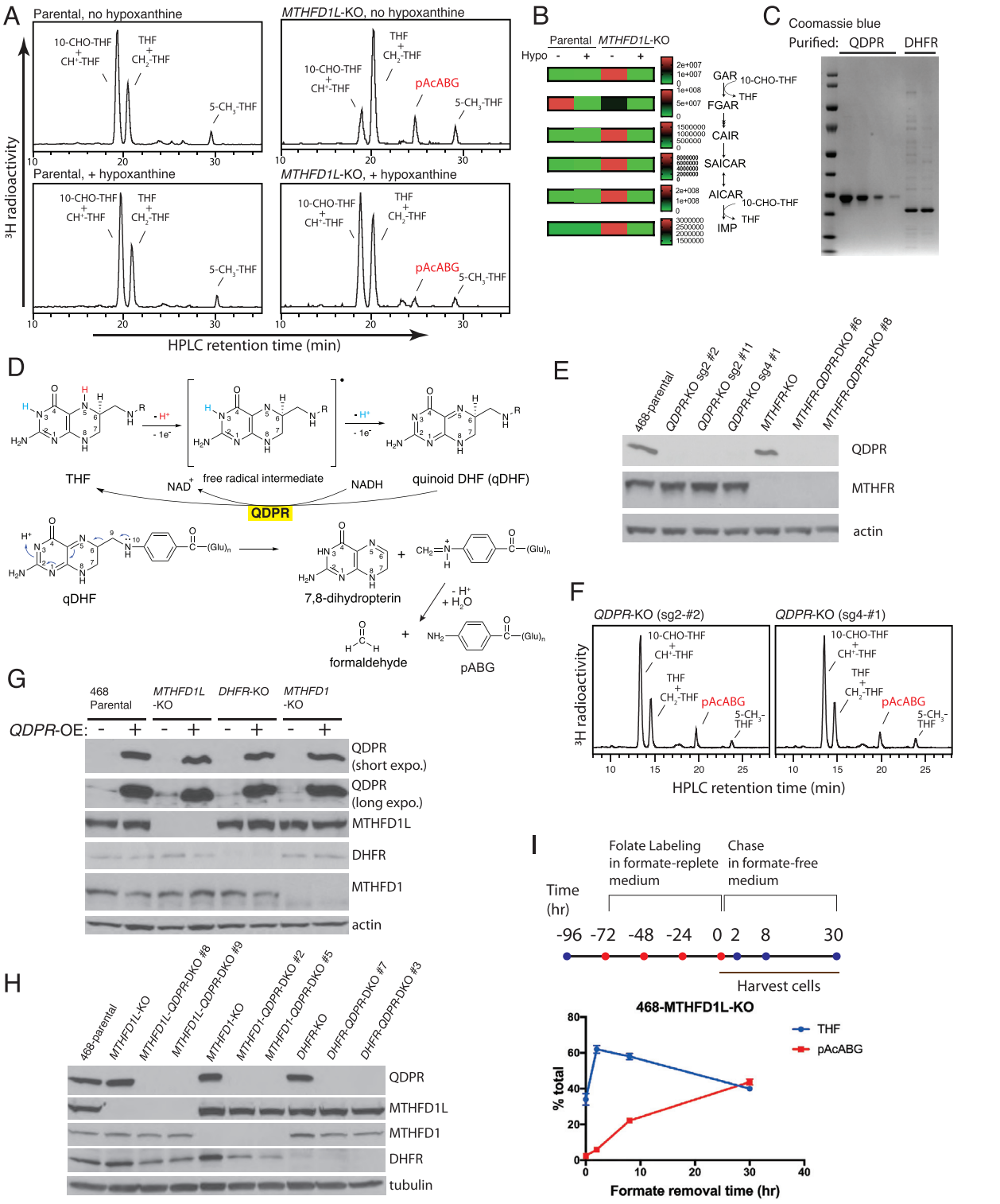

Figure S5. QDPR Repairs Oxidative Damage to Unsubstituted THF in Cells, Related to Figure 5

(A) HPLC analysis of folates from MDA-MB-468 parental, *MTHFD1L*-, and *SLC25A32*-knockout cells, cultured in DMEM/10% dialyzed FBS, with or without 100 μM hypoxanthine supplementation.

(B) Heat-map for the LC-MS analysis of purine-related metabolites from MDA-MB-468 parental and *MTHFD1L*-knockout cells, cultured in DMEM/10% dialyzed FBS, with or without 100 μM hypoxanthine supplementation. The scale bars indicate peak area intensity.

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(C) SDS-PAGE and Coomassie blue analysis of human QDPR and human DHFR expressed and purified from *E. coli*.
(D) A plausible chemical mechanism by which QDPR repairs oxidative damage to THF. The putative substrate for QDPR, quinoid DHF (or 6(H),7,8-DHF) is a distinct tautomer from the substrate for DHFR, 3(H),7,8-DHF (the latter not shown).
(E, G, and H) western blot analysis of the indicated cells.
(F) HPLC analysis of folates from MDA-MB-468 *QDPR*-knockout cells generated using two different CRISPR guides (sg2 and sg4).
(I) Top, schematic of the modified pulse-chase experiment to examine the rate of THF decomposition in MDA-MB-468 *MTHFD1L*-knockout cells. Bottom, time course of THF and pAcABG dynamics in *MTHFD1L*-knockout cells treated as in the top schematic.

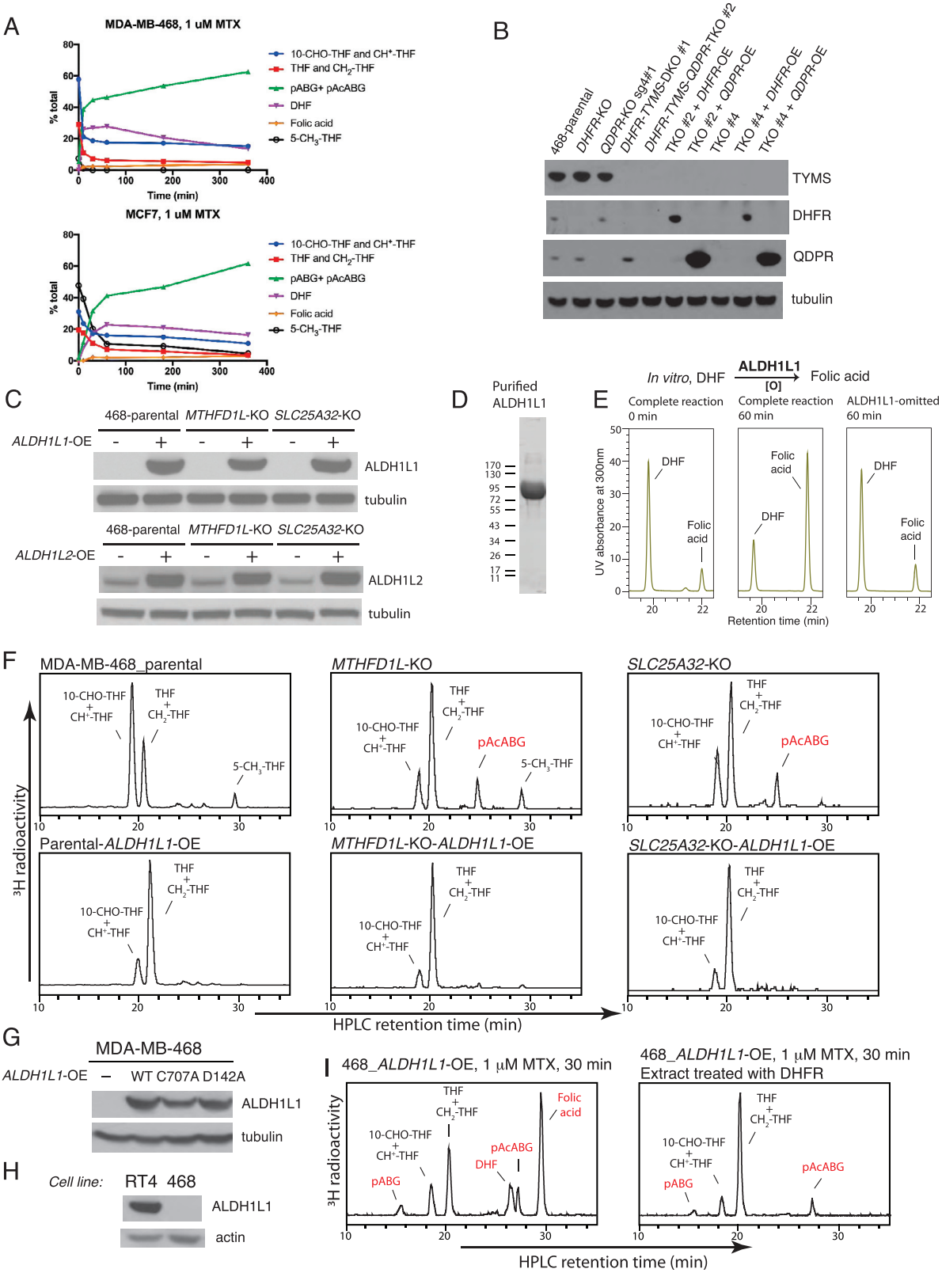

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Figure S6. *QDPR* Genetically Interacts with *DHFR*, and *ALDH1L1* Expression Alters the Folate Response to Methotrexate, Related to Figure 6

(A) Longer time courses of folate interconversion and decomposition in MDA-MB-468 and MCF7 cells upon 1 μM methotrexate treatment.

(B and C) Western blot analysis for the indicated cells.

(D) SDS-PAGE and Coomassie blue analysis of human ALDH1L1 that were expressed and purified from Sf21 insect cells.

(E) HPLC-UV analysis of the *in vitro* reaction of ALDH1L1 with DHF, related to Figure 6N.

(F) HPLC analysis of folates from MDA-MB-468 parental, *MTHFD1L*-, or *SLC25A32*-knockout cells, with or without ectopic *ALDH1L1* overexpression.

(G and H) Western blot analysis for the indicated cells.

(I) HPLC analysis of folates from MDA-MB-468 *ALDH1L1*-overexpressing cells upon methotrexate treatment. Treating the extract with DHFR converted folic acid and DHF into THF, thus confirming the peak eluting at ~30 min to be folic acid.

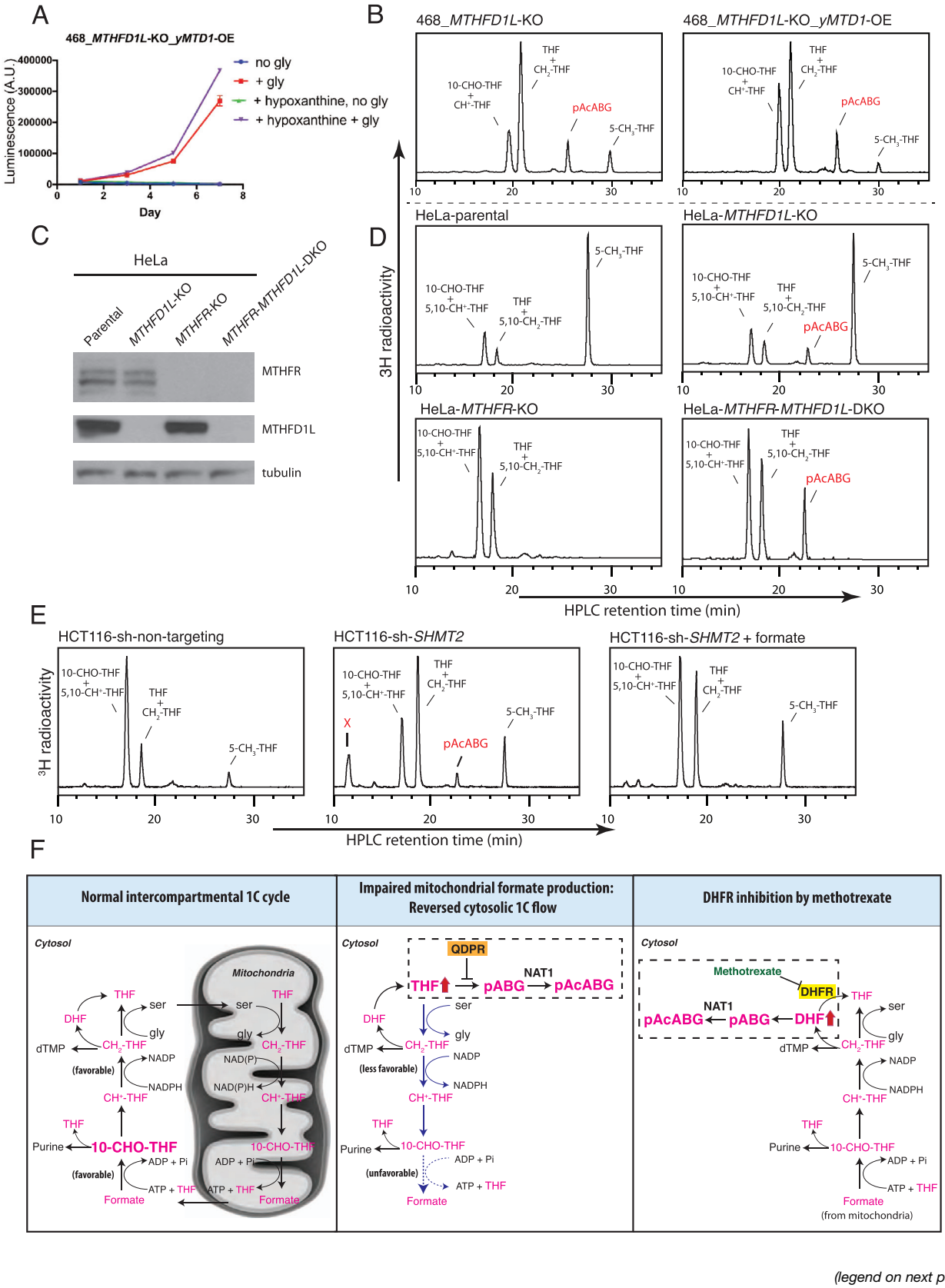

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Figure S7. Phenotypic Variation across Cell Lines and the Overall Model, Related to Figures 1–7

(A) Growth curves of MDA-MB-468 *MTHFD1L*-knockout cells with ectopic expression of yeast *MTD1*, with or without supplementation with 400 μM glycine or 100 μM hypoxanthine.

(B) HPLC analysis of folates from MDA-MB-468 *MTHFD1L*-knockout cells, with or without ectopic expression of yeast *MTD1*.

(C) Western blots of a panel of isogenic HeLa cells, with or without *MTHFD1L* or *MTHFR* knockout.

(D) HPLC analysis of folates from the panel of isogenic HeLa cells in (C).

(E) HPLC analysis of folates from HCT116 cells expressing non-targeting or *SHMT2*-targeting shRNAs. An additional folate breakdown product (denoted by "X," which elutes before pABG on HPLC) was formed upon *SHMT2* knockdown, and was eliminated by 2 mM formate supplementation.

(F) Summary model.