Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis

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An inborn error of metabolism associated with mutations in the human methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*) gene has been identified. The proband presented with SCID, megaloblastic anemia, and neurologic abnormalities, but the causal metabolic impairment is unknown. SCID has been associated with impaired purine nucleotide metabolism, whereas megaloblastic anemia has been associated with impaired de novo thymidylate (dTMP) biosynthesis. *MTHFD1* functions to condense formate with tetrahydrofolate and serves as the primary entry point of single carbons into folate-dependent one-carbon metabolism in the cytosol. In this study, we examined the impact of *MTHFD1* loss of function on folate-dependent purine, dTMP, and methionine biosynthesis in fibroblasts from the proband with *MTHFD1* deficiency. The flux of formate incorporation into methionine and dTMP was decreased by 90% and 50%, respectively, whereas formate flux through de novo purine biosynthesis was unaffected. Patient fibroblasts exhibited enriched *MTHFD1* in the nucleus, elevated uracil in DNA, lower rates of de novo dTMP synthesis, and increased salvage pathway dTMP biosynthesis relative to control fibroblasts. These results provide evidence that impaired nuclear de novo dTMP biosynthesis can lead to both megaloblastic anemia and SCID in *MTHFD1* deficiency.

folate | *MTHFD1* | SCID | megaloblastic anemia | thymidylate

The mechanisms underlying pathologies resulting from impaired folate- and vitamin B₁₂-mediated one-carbon (1C) metabolism are not completely resolved. This knowledge gap is, in part, because the associated metabolic pathways, namely de novo purine and de novo thymidylate (dTMP) biosynthesis, and homocysteine remethylation to methionine are tightly interconnected. Isotope tracer studies show that the 1C units carried by tetrahydrofolate (THF) in the cytosol are derived directly from the hydroxymethyl group of serine through serine hydroxymethyltransferase 1 (SHMT1) and SHMT2 α activity or formate through methyleneTHF dehydrogenase 1 (*MTHFD1*) (1). Formate originates from glycine and serine catabolism in mitochondria (2, 3). Recently, we showed that dTMP synthesis is unique among the nucleotides, in that it occurs at sites of DNA synthesis (4–6). During S phase, the folate enzymes that constitute the de novo dTMP pathway, namely SHMT1, SHMT2 α , dihydrofolate reductase, and dTMP synthase (TYMS), undergo small ubiquitin-like modifier (SUMO)-dependent translocation to the nucleus (Fig. 1). There, they physically interact with the DNA replication machinery and the folate-dependent enzyme *MTHFD1* for deoxythymidine triphosphate (dTTP) synthesis (Fig. 1) (6). In mice, inhibition of nuclear localization of the pathway results in depressed dTMP biosynthesis and elevated uracil incorporation into DNA (4).

Recently, a patient who presented with elevated plasma homocysteine, slightly decreased plasma methionine, SCID, megaloblastic anemia, and neurological impairment was shown to have inherited mutations in both *MTHFD1* alleles (7, 8). *MTHFD1* is a tri-functional enzyme responsible for generating and interconverting

1C-substituted THF cofactors from formate (Fig. 1). The N-terminal domain encodes the active site for 5,10-methenylTHF cyclohydrolase (C) and dehydrogenase (D) activities, whereas the C-terminal domain contains 10-formylTHF synthetase activity; the active sites of the C and D activities are overlapping (3). The synthetase activity is required to catalyze the ATP-dependent condensation of formate and THF to 10-formylTHF, the cofactor required for de novo purine biosynthesis (2, 9). The C activity converts 10-formylTHF to 5,10-methenylTHF, which is converted to 5,10-methyleneTHF by the D activity; 5,10-methyleneTHF can be either (i) used by TYMS to convert deoxyuridine monophosphate to dTMP or (ii) irreversibly reduced by 5,10-methyleneTHF reductase to 5-methylTHF, the cofactor required for homocysteine remethylation (Fig. 1). The proband exhibited a mutation (c.727+1 G > A) in the paternal *MTHFD1* allele, which perturbs a splice acceptor site in intron 7, creating an early stop codon in the C/D domain. It is not known whether this truncated protein is stably expressed and/or retains any C/D activity. The mutation on the maternal allele (c.517C > T) C517T results in an R173C amino acid change in the NADP binding site (10) that may affect D activity (7, 11). In this study, we examined the effects of these mutations on purine, dTMP, and homocysteine remethylation to understand the role of *MTHFD1* in the clinical presentation. The data indicate that *MTHFD1* mutations in the proband do not affect purine biosynthesis but do impact both homocysteine remethylation and dTMP biosynthesis. Compensatory elevations in the dTMP salvage pathway and enrichment of *MTHFD1* in the nucleus in the patient fibroblasts are insufficient to prevent elevated uracil accumulation in DNA, implicating impaired de novo dTMP synthesis in the SCID phenotype.

Results

***MTHFD1* Protein Expression and Nuclear Localization Are Altered in *MTHFD1*-Deficient Patient Fibroblasts.** *MTHFD1* protein levels in patient fibroblasts were 50% lower than levels in matched control

Significance

These studies have identified that human genetic mutations, which impair the function of the folate-dependent enzyme methylene tetrahydrofolate dehydrogenase I (*MTHFD1*), depress rates of de novo thymidylate synthesis, elevate uracil levels in human DNA, and increase genome instability. These findings provide insights into the role of *MTHFD1* and thymidylate biosynthesis in the etiology of SCID and megaloblastic anemia.

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Fig. 1. De novo and salvage pathway synthesis of dTMP in the nucleus. 1C metabolism is required for the synthesis of purines, dTMP, and methionine. Formate is a major source of 1C units, which are generated in the mitochondria. Mitochondrial-derived formate can enter the cytoplasm and function as a 1C unit for folate metabolism through the activity of MTHFD1. At S phase, the enzymes of the dTMP synthesis pathway undergo SUMO-dependent translocation to the nucleus. The 1C is labeled in bold. *Inset* shows the thymidylate synthesis cycle, which involves the enzymes MTHFD1, SHMT1, SHMT2 α , TYMS, and DHFR as well as the salvage pathway. AdoHcy, S-adenosylhomocysteine; AICAR Tfase, aminoimidazolecarboxamide ribonucleotide transformylase; AdoMet, S-adenosylmethionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dUMP, deoxyuridine monophosphate; GAR Tfase, glycylamide ribonucleotide transformylase; MTHFD1 (C), MTHFD1 C activity; MTHFD1 (D), MTHFD1 D activity; MTHFD1 (S), MTHFD1 synthase activity; MTR, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SUMO, small ubiquitin-like modifier.

fibroblasts using an antibody directed toward amino acids 1–120 of MTHFD1 (Fig. 2A). The MTHFD1 protein from the control and patient fibroblasts exhibited the same molecular mass, indicating that at least one of the mutant *MTHFD1* alleles is producing a full-length protein. Smaller molecular weight bands were not detected, indicating that the *MTHFD1* c.727+1G > A gene product is not expressed or does not accumulate in cells. The proband's maternal allele produces an R173C mutation in the MTHFD1 C/D domain, and other studies have shown that R173 is critical for MTHFD1 D activity, because human recombinant R173A and R173K mutant MTHFD1 C/D domain proteins exhibited increased K_m values for NADP⁺ (11). Relative to the WT recombinant MTHFD1 C/D domain, recombinant R173C MTHFD C/D mutant protein exhibited 2.5-fold increased K_m for NADP⁺ ($P < 0.01$), suggesting that the enzyme produced from this allele has compromised D activity.

MTHFD1 Preferentially Localizes to the Nucleus in MTHFD1-Deficient Patient Fibroblasts. MTHFD1 was enriched in the nuclei of the patient fibroblasts compared with control fibroblasts (Fig. 2B). The mean nuclear-to-cytosolic ratio in the patient WG3607 cells was 1.87 ± 0.56 , whereas the mean nuclear-to-cytosolic ratios in the control MCH058 and MCH064 fibroblasts were 0.89 ± 0.16

and 0.73 ± 0.22 , respectively (Fig. 2 *B–D*). The patient cells exhibited a 2.1-fold increase in the MTHFD1 nuclear-to-cytosolic ratio ($P = 5.09737\text{E-}18$) compared with MCH058 cells and a 2.5-fold increase in the MTHFD1 nuclear-to-cytosolic ratio ($P = 3.20927\text{E-}24$) compared with MCH064 cells. There was no difference in the MTHFD1 nuclear-to-cytosolic MTHFD1 ratio between the control fibroblast cell lines ($P = 0.13$).

The de novo dTMP synthesis pathway translocates to the nucleus in S phase, and impaired de novo dTMP synthesis has been shown to result in S-phase arrest in numerous cell types (12–17). The increase in the MTHFD1 nuclear-to-cytosolic ratio in the patient fibroblasts is only partially explained by the four-fold increase in S-phase cells relative to control cells (Fig. 2 *E*

Fig. 2. MTHFD1 protein level is decreased in patient fibroblasts but preferentially enriched in the nucleus. (A) MTHFD1 protein levels in whole-cell lysates from control (MCH058 and MCH064) and patient (WG3607) fibroblasts. (B) MTHFD1 nuclear localization in control (MCH064) and patient (WG3607) fibroblasts (green) and DNA stain Draq5 (blue) using confocal microscopy. (C and D) Effect of S-phase arrest [1 mM hydroxyurea (HU)] and impaired MTHFD1 nuclear export [20 nM Leptomycin B (LpmB)] on MTHFD1 nuclear localization in control (MCH058) and patient (MCH067) fibroblasts. The ratio of the cytosolic to nuclear MTHFD1 signal intensity (number of cells per condition was $n > 30$) is shown as mean and SD. (E) FACS analysis of control (MCH058) and patient (WG3607) fibroblasts after HU treatment. (F) Histogram showing distribution of nuclear-to-cytosolic ratios in control (MCH064 and MCH058) and patient (WG3607) fibroblasts. (G and H) Effect of MTHFD1 R173C mutation on MTHFD1 subcellular localization. MTHFD1-GFP and MTHFD1-R173C-GFP fusion proteins were expressed in HeLa cells with or without HU and imaged using confocal microscopy. The cytosolic-to-nuclear MTHFD1-GFP and MTHFD1-R173C-GFP mutant signal intensity ratios are shown as means and SDs (number of cells per condition was $n > 30$). The statistical significance is represented as follows: $P > 0.05$ was nonsignificant (NS) and $***0.001 > P$. async, Asynchronous cells; Nuc/Cyt, nuclear to cytosolic; NT, no treatment.

and *F*). Analysis of single asynchronous cells indicates that the highest nuclear-to-cytosolic ratio for the control MCH058 and MCH064 fibroblasts was less than the lowest nuclear-to-cytosolic ratio for the patient cells (Fig. 2*F*), with the exception of a single control cell. Furthermore, arresting cells at S phase with hydroxyurea treatment increased the MTHFD1 nuclear-to-cytosolic ratios in control cells but not patient fibroblasts (Fig. 2*D–F*). Similarly, inhibiting MTHFD1 nuclear export with Lep-tomycin B treatment increased the MTHFD1 nuclear-to-cytosolic ratios in control but not patient fibroblasts. These results indicate that patient fibroblasts, but not control fibroblasts, accumulate MTHFD1 in the nucleus independent of cell cycle and MTHFD1 nuclear export (Fig. 2*E* and *F*). To determine if nuclear MTHFD1 localization in the patient cells was caused by the p.R173C mutation from the maternal allele, MTHFD1-R173C-GFP and MTHFD1-GFP fusion proteins were expressed in HeLa cells (Fig. 2*G*). The nuclear-to-cytosolic ratio of the MTHFD1-R173C fusion protein was 30% greater than the MTHFD1-GFP fusion protein in asynchronous cells and comparable after S-phase arrest. This observation indicates that the p.R173C mutation slightly favors MTHFD1 nuclear localization but alone, cannot account for the 2.5-fold increased nuclear enrichment of endogenous MTHFD1-R173C protein observed in the patient fibroblasts. The observations that MTHFD1 is enriched in the nucleus because of increased enrichment in S phase as well as with the p.R173C mutation prompted us to assess the contribution of MTHFD1 to nuclear dTMP synthesis, purine biosynthesis, and homocysteine remethylation in the patient fibroblasts.

Enhanced MTHFD1 Nuclear Localization Supports dTMP Synthesis at the Expense of Homocysteine Remethylation in Patient Fibroblasts. The flux of L-[2,3,3-³H]-serine through the de novo dTMP synthesis and homocysteine remethylation pathways, as indicated by the D1/D1 + D2 ratio, was 50% lower in thymidine and nearly 90% lower in methionine in patient fibroblasts compared with control lines (Table 1). These findings show that reduced MTHFD1 expression has a greater impact on the flux of 1C units into the homocysteine remethylation pathway than de novo dTMP biosynthesis. This observation indicates that MTHFD1 enrichment in the nucleus in support of dTMP synthesis occurs

at the expense of providing 1C units to homocysteine remethylation in patient fibroblasts. The patient fibroblasts compensated by increasing the direct use of serine through SHMT as a 1C source for dTMP synthesis compared with the control fibroblasts, which primarily rely on formate as a 1C source (Fig. 1 and Table 1).

De Novo Purine Biosynthesis Is Not Impaired in Patient Fibroblasts. The ratio of the incorporation of [¹⁴C]-formate (an indicator of de novo purine synthesis) to [³H]-hypoxanthine (an indicator of salvage purine biosynthesis) into nuclear DNA did not vary among cell lines (Table 1). The [¹⁴C]-formate-to-³H]-hypoxanthine ratio in purines dA and dG in DNA was not different among cell lines (Table 1), indicating that purine synthesis is not compromised in patient fibroblasts.

Patient Fibroblasts Up-Regulate the Salvage Pathway to Meet Cellular dTMP Requirements. In many cell types, the salvage pathway is quantitatively more important to overall dTMP synthesis than the de novo synthesis pathway (5). The ratio of [¹⁴C]-deoxyuridine (an indicator of de novo dTMP synthesis) to [³H]-thymidine (an indicator of salvage pathway dTMP synthesis) was sevenfold lower in patient fibroblasts compared with control fibroblasts ($P < 0.001$ compared with both lines) (Table 1). The efficiency of de novo dTMP synthesis was also investigated in fibroblasts from patients with adenosine deaminase deficiency (WG0549 and WG0290), which is known to be associated with SCID. The ratio of [¹⁴C]-deoxyuridine to [³H]-thymidine in WG0549 and WG0290 cells did not differ between MCH058 and MCH064 control fibroblasts (Fig. 3*A*), indicating that the SCID phenotype resulting from adenosine deaminase deficiency is not related to dTMP synthesis.

The sevenfold decrease in the ratio of de novo/salvage thymidylate synthesis in the MTHFD1-deficient cells was driven primarily by a fourfold increase in the contribution of the salvage pathway to DNA synthesis in patient fibroblasts when cells were cultured in 2 mM glycine ($P = 0.02$ and $P = 0.03$) (Fig. 3*B*). This increase in salvage pathway dTMP synthesis in patient fibroblasts was associated with a twofold increase in the level of thymidine kinase (TK1) protein in patient fibroblasts compared with control

Table 1. 1C metabolism in patient and control fibroblasts

Cell line and type	dU suppression (¹⁴ C-deoxyuridine/ ³ H-thymidine in nuclear DNA)	Uracil in DNA (pg uracil/μg DNA)	Metabolic assays					
			3-[³ H]-serine isotope tracer		Formate suppression [¹⁴ C]-formate/ [³ H]-hypoxanthine			
			Thymidine (D1/D1 + D2)	Methionine (D1/D1 + D2)	Nuclear DNA	dG	dA	
MCH058 control	0.054 ± 0.002 (6.92)	0.50 ± 0.01 (0.87)	0.818 ± 0.009	0.872 ± 0.043	1.72 ± 0.08	2.99 ± 1.00	0.89 ± 0.06	
MCH064 control	0.058 ± 0.005 (7.43)	0.49 ± 0.004 (0.85)	0.821 ± 0.013	0.866 ± 0.005	1.74 ± 0.13	3.09 ± 1.53	0.78 ± 0.24	
WG3607 patient	0.0078 ± 0.0005	0.57 ± 0.01	0.398 ± 0.063	0.083 ± 0.070	1.76 ± 0.47	3.93 ± 0.63	1.11 ± 0.32	
<i>P</i> value MCH058 vs. MCH064	NS	NS	NS	NS	NS	NS	NS	
<i>P</i> value MCH058 vs. WG3607	$P < 0.001$	$P = 0.009$	$P = 0.002$	$P = 0.008$	NS	NS	NS	
<i>P</i> value MCH064 vs. WG3607	$P < 0.001$	$P < 0.001$	$P = 0.003$	$P = 0.007$	NS	NS	NS	

The dU suppression and formate suppression assays quantify the capacity of de novo nucleotide synthesis relative to the salvage pathway for dTMP and purine biosynthesis, respectively. Cells were cultured in DMEM containing 200 μM methionine and 20 nM leucovorin and supplemented with either 500 nM [³H]thymidine and 10 μM [¹⁴C]deoxyuridine (for the dU suppression assay) or 200 nM [³H]hypoxanthine and 20 μM [¹⁴C]formate (for the formate suppression assay). All data are means of $n = 3$ biological replicates per cell line, and the data are shown as means ± SDs. Numbers in parentheses represent the ratios of control to patient fibroblasts. For 3-[³H]-serine isotope tracer studies, cells were cultured in DMEM supplemented with 10 μM methionine, 20 nM leucovorin, and 250 μM L-[2,3,3-³H]-serine as described in *Materials and Methods*. Isotopic enrichment of L-[2,3,3-³H]-serine into thymidine in DNA and methionine in protein is shown as the ratio of carbons containing one deuterium atom divided by the total number of carbons containing one and two deuterium atoms (D1/D1 + D2). For isotope tracer studies, samples were analyzed as $n = 2$ biological replicates per cell line, and data are shown as means ± SDs. For all assays, *P* values result from Student's *t* tests with Bonferroni corrections for multiple comparisons. NS, not significant ($P > 0.1$).

Fig. 3. dTMP synthesis and DNA damage in patient and matched control fibroblasts. (A) dU suppression assay in control (MCH058 and MCH064) and adenosine deaminase-deficient fibroblasts (WG0549 and WG0290). Results are shown as the ratio of decays per minute (DPM) from [^{14}C]-deoxyuridine to DPM from [^3H]-thymidine in DNA, with $n = 3$ biological replicates per cell line. Data are shown as means $\pm$ SDs and were analyzed using Student's t test with Bonferroni correction for multiple comparisons. There were no significant differences. (B) [^3H]-thymidine incorporation (DPM) in DNA as a function of exogenous glycine concentration. Patient and control fibroblasts were cultured for four doublings in DMEM containing either 2 or 5 mM glycine. Data are shown as means $\pm$ SDs of three biological replicates and analyzed by Student's t test with Bonferroni correction for multiple comparisons. (C) TK1 levels as a function of exogenous glycine in patient and matched control fibroblast cell lysates. Bands were quantified using the ratio of TK1-to-GAPDH expression, and densitometry was performed using ImageJ software. (D and E) MTHFD1-deficient patient fibroblasts (WG3607) exhibit elevated γH2AX -positive foci per nuclei compared with (D) control (MCH058) fibroblasts and (E) control (MCH058 and MCH064) and adenosine deaminase-deficient fibroblasts (WG0549 and WG0290). DNA damage was quantified as γH2AX -positive area percentage for each cell imaged above a set threshold and presented as a mean of all cells imaged per cell line $\pm$ SEM. Number of cells imaged: MCH058, $n = 914$; MCH064, $n = 758$; WG0290, $n = 653$; WG0549, $n = 633$; WG3607, $n = 1,049$. The statistical significance was assessed by t test with Bonferroni correction for multiple comparisons and is represented as follows: $P > 0.05$ was nonsignificant (NS), $0.05 > P > 0.01$, and $***0.001 > P$. DIC, differential image contrast. (F) MTHFD1-deficient patient fibroblasts display more DNA damage when folate-depleted ($P = 0.02$). MTHFD1-deficient patient fibroblasts were cultured for five doublings in defined media supplemented ($n = 286$) or not ($n = 400$) with folic acid.

fibroblasts (Fig. 3C). The increased reliance on SHMT-derived 5,10-methyleneTHF for de novo dTMP synthesis in patient fibroblasts (Table 1) suggested that they may be more sensitive to exogenous glycine concentrations, because the SHMT reaction is reversible, and its directionality in cells is influenced by exogenous glycine (1). Fig. 3B shows that the contribution of the salvage pathway to dTMP synthesis was increased by exogenous

glycine in patient but not control fibroblasts ($P = 0.03$), but the difference was not statistically significant after correction for multiple comparisons.

Patient Fibroblasts Exhibit Increased DNA Damage Relative to Control Fibroblasts. Phosphorylated variant histone H2A (γH2AX) levels, markers of double-strand breaks in DNA, were markedly increased in WG3607 fibroblasts relative to both control lines (MCH058 and MCH064) as well as the adenosine deaminase-deficient fibroblast lines (WG0290 and WG0549) (Fig. 3D and E). γH2AX staining increased in WG3607 fibroblasts when cultured in folate-deficient medium (Fig. 3F), indicating that the MTHFD1-deficient WG3607 fibroblasts are sensitized to folate deficiency-induced DNA damage.

Patient Fibroblasts Exhibit Elevated Uracil Content in Nuclear DNA. Uracil levels in nuclear DNA from patient fibroblasts were increased by 15% relative to controls ($P < 0.01$) (Table 1), indicating insufficient dTMP synthesis to support DNA replication. The increase in DNA uracil indicates that the increases in MTHFD1 nuclear localization and up-regulation of salvage pathway synthesis were not sufficient to meet dTMP demands in the proband.

Discussion

This study provides the first evidence, to our knowledge, that MTHFD1 nuclear localization is critical for de novo dTMP synthesis, and it provides a mechanism for the role of MTHFD1 in megaloblastic anemia, which most commonly results from impaired dTMP biosynthesis caused by folate deficiency and/or vitamin B $_{12}$ deficiency. Patient fibroblasts exhibited impairments in two of three primary anabolic pathways associated with 1C metabolism (namely de novo dTMP biosynthesis and homocysteine remethylation to methionine), whereas de novo purine biosynthesis was unaffected.

MTHFD1 activity is known to be essential for purine biosynthesis through its 10-formylTHF synthetase activity, which incorporates formate in the purine ring through the cofactor 10-formylTHF. Murine ES cells lacking *Mthfd1* expression are auxotrophic for purines (18). In this study, patient fibroblasts, which do not exhibit mutations in the 10-formylTHF synthetase domain (Fig. 2A), did not exhibit impairments in folate-dependent de novo purine biosynthesis relative to purine salvage pathway biosynthesis (Table 1). These findings are consistent with previous studies of the *Mthfd1*^{tg/+} mouse model, which also exhibits a 50% reduction in 10-formylTHF synthetase activity without decreased de novo purine biosynthesis (19). Although SCID can result from loss of adenosine deaminase activity and the resulting imbalance in purine metabolite pools (20), the findings from the MTHFD1-deficient proband do not support a role for impaired de novo purine biosynthesis in the patient's SCID phenotype.

Isotope tracer studies showed that loss of MTHFD1 C/D activity, resulting from both decreased MTHFD1 protein expression and decreased affinity for NADP $^{+}$, in the patient cells had the greatest impact on the flux of formate into the homocysteine remethylation pathway, which was reduced 10-fold (Table 1). Consistent with these findings, previous studies of the *Mthfd1*^{tg/+} mouse model showed that a 50% reduction in MTHFD1 protein led to reduced hepatic S-adenosylmethionine levels (21), lower methylation potential (19), elevated plasma homocysteine (22), and lower levels of methionine (23), indicating impaired function of the homocysteine remethylation cycle. However, the proband exhibited relatively mild elevations in plasma homocysteine concurrent with lower serum methionine and elevated homocysteine (8). Inborn errors of metabolism that severely impair homocysteine remethylation are not associated with SCID.

The enrichment of formate into de novo dTMP was impaired in patient fibroblasts by 50% (Table 1), with a 15% increase in uracil content in DNA, likely resulting from uracil misincorporation during DNA synthesis as occurs in folate deficiency (24, 25). Formate incorporation into dTMP has been shown in isolated nuclei (12), and this study shows the importance of MTHFD1 and formate in nuclear dTMP and DNA synthesis. Interestingly, patient fibroblasts compensated for the depressed MTHFD1 activity by (i) increasing the flux through SHMT to provide serine-derived 5,10-methyleneTHF for dTMP synthesis, (ii) up-regulating the expression of TK1 to support salvage pathway dTMP biosynthesis, and (iii) enriching MTHFD1 protein in the nucleus to support de novo dTMP biosynthesis (Figs. 2 and 3). This latter compensatory response further compromised the impact of MTHFD1 mutations on homocysteine remethylation but likely spared further increases in uracil accumulation in DNA. The accumulation of MTHFD1-deficient cells in the S phase of the cell cycle (Fig. 2E) is likely caused by impaired dTMP synthesis in the proband, because impaired dTMP synthesis causes S-phase arrest (13, 16, 17, 26). The increase in nuclear accumulation of the MTHFD1-R173C-GFP enzyme compared with the MTHFD1-GFP fusion protein is only observed in asynchronous HeLa cells (Fig. 2G) and not HeLa cells arrested in S phase, when dTMP synthesis occurs (12).

The results from this study indicate that impairment in nuclear de novo dTMP synthesis is the most likely cause of both the megaloblastic anemia and SCID phenotypes in the proband. SCID is a heterogeneous group of more than 20 monogenic disorders typically resulting from severe defects in T-cell differentiation and/or lack of survival of lymphocyte precursors because of elevated apoptosis (15). Impaired dTMP synthesis can result in elevated rates of apoptosis, and the sensitivity to apoptosis resulting from impaired dTMP synthesis and/or DNA damage differs among cell types (13, 14). Fibroblasts lacking adenosine deaminase activity, which causes impaired DNA synthesis and reduced B- and T-cell division, leading to SCID, did not exhibit impaired dTMP synthesis or increased DNA damage as did fibroblasts lacking MTHFD1 (Fig. 3). The mechanisms used to regulate dTMP synthesis in combined B- and T-cell immunodeficiencies require additional investigations, including whether compensatory changes of MTHFD1 nuclear localization and up-regulation of TK1 activity occur in all cell types. Uracil levels in DNA differ among mouse tissues (27), indicating that there may be cell-specific differences in the regulation of dTMP biosynthesis. Excessive uracil accumulation in nuclear DNA can decrease rates of cell proliferation and increase rates of apoptosis; cell-specific differences in the capacity to regulate uracil accumulation in DNA could account for the SCID phenotype. Finally, the up-regulation of TK1 and increased reliance on SHMT for de novo dTMP biosynthesis in the MTHFD1-deficient proband may indicate some benefit from thymidine supplementation and perhaps, limited glycine to better support dTMP biosynthesis.

Materials and Methods

Cell Culture. Patient (WG3607 MTHFD-deficient and WG0549 and WG0290 adenosine deaminase-deficient; W and G are the initials of the two individuals who started the cell bank and are used for coding cells) and two Montreal Children's Hospital (MCH) control fibroblasts cell lines (MCH058 and MCH064) were maintained in MEM, α -modification (α -MEM; HyClone) supplemented with 10% (vol/vol) FBS. For tracer and uracil in DNA measurements, the medium consisted of DMEM (HyClone) that lacked glycine, serine, methionine, pyridoxine, folate, and all nucleosides/nucleotides but was supplemented with 10% (vol/vol) dialyzed FBS.

Immunoblotting. Cells were plated at 30% confluence in 100-mm dishes, allowed to grow to 90% confluence, harvested, and pelleted. Cellular proteins were extracted in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 5 mM DTT, and Protease Inhibitor Mixture (Sigma) diluted 1:100. Total protein was quantified (28). Proteins (30 μ g per lane) were

resolved on 4–15% (vol/vol) gradient SDS/PAGE gels (Bio-Rad) and transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blocked overnight at 4 °C in 10% (wt/vol) nonfat dry milk in PBS with 1% Nonidet P-40 (US Biologicals). MTHFD1 was detected using 2 μ g/mL primary antibody generated against the first 120 aa of the MTHFD1 protein (Santa Cruz Biotechnology) and a 1:10,000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Pierce). TK1 was detected using 1 μ g/mL rabbit anti-TK1 antibody generated against the N-terminal region of human TK1 (Abcam) and a 1:10,000 dilution of HRP-conjugated donkey anti-rabbit secondary antibody (Pierce). β -Actin was detected using a 1:20,000 dilution of HRP-conjugated mouse anti- β -actin antibody (Abcam); GAPDH was detected using a 1:4,000,000 mouse anti-GAPDH (Novus Biologicals) followed by a 1:10,000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Pierce). The membranes were then visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to autoradiography. Densitometry was performed with Image J software.

Cloning, Purification, and Kinetic Characterization of His-MTHFD1C/D and His-R173C-MTHFD1C/D. The C/D domain was amplified from both the MTHFD1 and R173C-MTHFD1 cDNAs using a forward primer (5'-ATATATGGATCCATGGC-GCCAGCAGAAATC-3') and a reverse primer (5'-ATATATGCGGCCGCTAA-ATCATCCACTTTCCTG-3'; restriction sites underlined). PCR was performed using GoTaq (Promega) according to the manufacturers' instructions, and the C/D domains of both MTHFD1 and R173C-MTHFD1 were cloned into the BamHI and NotI sites of the pet28a(+) vector. His-MTHFD1C/D and His-R173CMTHFD1C/D recombinant proteins were expressed in transfected BL21(DE3) cells (Invitrogen) grown to midlate log phase in Luria-Bertani broth containing 50 μ g/mL kanamycin. Expression was induced overnight with isopropyl-beta-D-thiogalactopyranoside (750 μ M). Proteins were purified according to the protocol designed for His-MTHFS (29). D activity was quantified using an enzyme-coupled assay with SHMT generating 5,10-methyleneTHF from serine and THF. D activity was measured by converting NADP⁺ to NADPH and monitoring absorbance at 340 nm. All reactions were carried out at room temperature in 50 mM potassium phosphate buffer with 14.3 mM β -mercaptoethanol, 20 mM L-serine, 0.33 μ M SHMT, 150 μ M THF, 20 nM MTHFD1 or R173C-MTHFD1, and 0–200 μ M NADP⁺. K_m and k_{cat} values were determined from Lineweaver–Burke plots, and values are represented as the ratio of the average and SD of three biological replicates. Statistical significance was determined by Student's *t* test.

Uracil Content in Nuclear DNA. Cells were plated and grown for four doublings in DMEM supplemented with 200 μ M methionine and 20 nM Leucovorin. Nuclear DNA was extracted and uracil was quantified as described previously (4).

[2,3,3-²H₃]-Serine Isotope Tracer Studies. The flux of 1C units into the de novo dTMP synthesis pathway and the homocysteine remethylation pathway was quantified using [2,3,3-²H₃]-serine as described previously (1). The hydroxymethyl group of serine can be directly converted to 5,10-methyleneTHF containing 2 deuterium atoms by SHMT in support of dTMP synthesis and homocysteine remethylation to methionine. Alternatively, serine can be converted to formate in mitochondria and used to generate 5,10-methyleneTHF containing one deuterium atom in the 1C through the activity of MTHFD1. The ratio of carbons containing one deuterium atom divided by the total number of carbons containing one and two deuterium atoms (D1/D1 + D2) in thymidine isolated from DNA and methionine in cellular protein serves as a measure of the ability of formate to serve as a 1C source through MTHFD1. Cells were plated and grown for four doublings in DMEM supplemented with 10 μ M methionine, 20 nM leucovorin, and 250 μ M L-[2,3,3-²H₃]-serine. Cells were pelleted, washed with 1 $\times$ PBS, and stored as frozen pellets. Isotopic enrichment of methionine in proteins and thymidine in DNA was determined as described previously (1).

dU Suppression Assay. The contribution of the de novo and salvage pathways to dTMP synthesis was performed by exposing fibroblasts to [¹⁴C]-deoxyuridine and [³H]-thymidine as described elsewhere (1). The ratio of [¹⁴C]/³H DPM in nuclear DNA is a measure of relative capacity of de novo synthesis and salvage pathway dTMP synthesis. [¹⁴C]-deoxyuridine is incorporated into DNA by TYMS in a folate-dependent manner, whereas [³H]-thymidine is incorporated into DNA through the salvage pathway after its phosphorylation by TK1. Cells were plated in triplicate in six-well plates and allowed to grow for two doublings in DMEM supplemented with 200 μ M methionine, 20 nM Leucovorin, 500 nM [³H]-thymidine (Perkin-Elmer), and 10 μ M [¹⁴C]-deoxyuridine (Moravsek). In a separate experiment, cells were plated

in the medium described above that was also supplemented with 2 or 5 mM glycine.

Formate Suppression Assay. The formate suppression assay quantifies the contribution of the de novo and salvage pathways to purine synthesis by exposing fibroblasts to [^{14}C]-formate and [^3H]-hypoxanthine and was performed as described previously (21). [^{14}C]-formate is incorporated into purine nucleotides through the folate-dependent de novo synthesis pathway, whereas [^3H]-hypoxanthine is incorporated into DNA through the purine salvage pathway. Cells were plated in triplicate on 100-mm plates and allowed to grow for two doublings in DMEM supplemented with 200 μM methionine, 20 nM Leucovorin, 200 nM [^3H]-hypoxanthine (Moravek), and 20 μM [^{14}C]-formate (Moravek). Data are shown as the averages and SDs of the ratio of [^{14}C]-DPM/[^3H]-DPM per fraction for eluted nucleosides, with $n = 3$ biological replicates per cell line.

Microscopy. Cells were plated in duplicate in six-well plates containing microscopy cover glass #1 (Fisher Scientific) on the bottom of each well and allowed to grow for 36–48 h in α -MEM supplemented with 10% (vol/vol) FBS. Cell fixation and immunostaining were performed as described (30) with minor modifications. Briefly, cells were washed two times with PBS, fixed with 4% (vol/vol) formaldehyde in PBS for 10 min, washed two times with PBS, permeabilized with 0.2% Triton in PBS for 10 min, and washed an additional four times; 5 μM DRAQ5 (Thermo Scientific) was used for nuclear staining following the manufacturer's protocol. Anti-MTHFD1 mouse mAb C-3 (Santa Cruz) was diluted 80 $\times$ in PBS, added to fixed cells, and incubated at 4 $^{\circ}\text{C}$ overnight. After four washes with PBS (at least 15 min for each wash), cells were incubated for 1 h at room temperature with 1:400 dilution of goat anti-mouse IgG coupled to Alexa Fluor 488 (Molecular Probes). Cells were washed with PBS four times and mounted on microscopy slides with Fluoromount G (SouthernBiotech). Cells were visualized with a Leica confocal microscope at the Cornell Microscope and Imaging Facility. Nuclear and cytosolic signal intensities were quantified using Leica Lite software, and the ratio of MTHFD1 signal in the nucleus and cytosol was calculated for at least 30 individual cells per cell type and graphed as the mean and SD. The statistical significance of the differences between the

means was estimated using a bilateral Student's t test for unpaired data with Bonferroni correction for multiple comparisons. The MTHFD1-GFP construct is described elsewhere (12). The MTHFD1-R173C-GFP mutant was generated with primers 5'-TGCTGTGGTGGTGGGTGCAGTAAATAGTTGGG-3' and 5'-CCCAACTATTTTACTGCACCAACCACACAGCA-3' and MTHFD1-GFP as the template using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions.

γH2AX Staining and Microscopy. Cells were plated in triplicates in six-well plates with cover glass #1 (Fisher Scientific) on the bottom of each well and allowed to grow for 36–48 h in α -MEM supplemented with 10% (vol/vol) FBS or DMEM with or without folate addition where indicated. Cells were treated with 20 nM Leptomycin B or 1 mM hydroxyurea to inhibit nuclear export or induce S-phase arrest, respectively. Cell fixation and immunostaining were performed as described (15) with minor modifications. Briefly, cells were washed two times with PBS, fixed with 4% (vol/vol) formaldehyde in PBS for 10 min, washed two times with PBS, permeabilized with 0.2% Triton in PBS for 10 min, and washed an additional four times; 5 μM DRAQ5 (Thermo Scientific) was used for nuclear staining following the manufacturer's protocol. Anti- γH2AX mouse mAb (05–636; Millipore) was diluted 1,000 $\times$ in PBS, added to fixed cells, and incubated at 4 $^{\circ}\text{C}$ overnight. After four washes with PBS, cells were incubated for 1 h at room temperature with a 1:400 dilution of goat anti-mouse IgG coupled to Alexa Fluor 488 (Molecular Probes). Cells were washed with PBS four times and mounted on microscopy slides with Fluoromount G (SouthernBiotech). Cells were visualized with a Leica confocal microscope. The increase in the area of the γH2AX -positive signal as an indicator of genome instability was quantified using Metamorph imaging software package as follows: γH2AX -positive area (defined as γH2AX signal above a set threshold) was expressed as a percentage of the total area of the nucleus in the images as labeled by draq5 stain. The percentage of the γH2AX -positive area was calculated for each nucleus individually and then presented as a mean $\pm$ SEM for all cells imaged ($n =$ between 650 and 1,040 cells per condition).

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