Published in final edited form as:

Mol Aspects Med. 2017 February ; 53: 48–56. doi:10.1016/j.mam.2016.11.005.

Targeting Nuclear Thymidylate Biosynthesis

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Abstract

Thymidylate (dTMP) biosynthesis plays an essential and exclusive function in DNA synthesis and proper cell division, and therefore has been an attractive therapeutic target. Folate analogues, known as antifolates, and nucleotide analogs that inhibit the enzymatic action of the *de novo* thymidylate biosynthesis pathway and are commonly used in cancer treatment. In this review, we examine the mechanisms by which the antifolate 5-fluorouracil, as well as other dTMP synthesis inhibitors, function in cancer treatment in light of emerging evidence that dTMP synthesis occurs in the nucleus. Nuclear localization of the *de novo* dTMP synthesis pathway requires modification of the pathway enzymes by the small ubiquitin-like modifier (SUMO) protein. SUMOylation is required for nuclear localization of the *de novo* dTMP biosynthesis pathway, and disruption in the SUMO pathway inhibits cell proliferation in several cancer models. We summarize evidence that the nuclear localization of the dTMP biosynthesis pathway is a critical factor in the efficacy of antifolate-based therapies that target dTMP synthesis.

Keywords

Nuclear thymidylate synthesis; antifolate; folate-mediated one-carbon metabolism; sumoylation; thymidylate synthase

1. Overview of folate-mediated one-carbon metabolism (FOCM)

Folate functions as a family of enzyme cofactors that chemically activate and transfer one-carbon units for fundamental cellular processes including DNA replication and repair, mitochondrial protein synthesis and amino acid interconversions and catabolism¹. The single carbons carried by tetrahydrofolate (THF) are generated from the catabolism of the one-carbon donors that include serine, glycine, sarcosine, dimethylglycine and histidine. Folate-activated single carbons are required for *de novo* purine synthesis, *de novo* thymidylate (dTMP) biosynthesis, for the remethylation of homocysteine to methionine and for the synthesis of ^{fmet}tRNA^{Met}¹. Collectively, these pathways are known as folate-

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mediated one-carbon metabolism (FOCM), which is a network of interconnected folate-dependent metabolic pathways that are compartmentalized in the mitochondria, cytosol and the nucleus.

In mitochondria, the inner membrane folate transporter, SLC25A32, is essential for mitochondrial folate accretion², and the mitochondrial and cytoplasmic folate pools don't freely exchange³. In mitochondria, one-carbon donors are catabolized to generate formaldehyde in the form of 5,10-methylene-tetrahydrofolate, which is subsequently oxidized to 10-formyl-tetrahydrofolate. Formate is then liberated from 10-formyl-tetrahydrofolate in an ATP-generating reaction, and translocates to the cytosol and nucleus to support FOCM in those compartments⁴. Mitochondria also contain an anabolic pathway to generate dTMP for mitochondrial DNA synthesis⁵, and mitochondrial FOCM is also required for ^{fm}etRNAmet synthesis, which is involved in the initiation of mitochondrial protein synthesis.

In the cytosol, serine and mitochondrial-derived formate are the two primary sources of folate-activated one-carbons for folate-dependent biosynthetic reactions. Formate accounts for 70-90% of total one-carbon units for cytoplasmic FOCM in MCF-7 cells^{6,7}, whereas serine catabolism in the cytoplasm accounts for 10-30% of total one-carbon units. Serine is a direct source of single carbons in the cytoplasm through the activity of serine hydroxymethyltransferase 1 (SHMT1) which catalyzes the transfer of the one-carbon unit from serine to tetrahydrofolate to form glycine and 5,10-methylene-tetrahydrofolate (Figure 1).

Mitochondrially-derived formate is also a source of 5,10-methylene-tetrahydrofolate. It is condensed with tetrahydrofolate to form 10-formyl-tetrahydrofolate (Figure 1) in an ATP-dependent reaction catalyzed by the synthase (S) activity of the trifunctional enzyme methylenetetrahydrofolate dehydrogenase (MTHFD1). 10-formyl-tetrahydrofolate can serve as a cofactor that supplies the 2 and 8 carbon of the purine ring for *de novo* purine synthesis. Alternatively, MTHFD1 can further process 10-formyl-tetrahydrofolate to 5,10-methenyl-tetrahydrofolate through its cyclohydrolase (C) activity, and then to 5,10-methylene-tetrahydrofolate through the NADPH-requiring dehydrogenase (D) activity of MTHFD1. 5,10-methylene-tetrahydrofolate exists at a branch-point in the FOCM network. It is the substrate for 5,10-methylene-tetrahydrofolate reductase (MTHFR). The NADPH-dependent synthesis of 5-methyl-tetrahydrofolate by MTHFR is essentially irreversible *in vivo*, hence 5-methyl-tetrahydrofolate is committed to serving as a cofactor for homocysteine remethylation to methionine, catalyzed by the vitamin B12-dependent enzyme methionine synthase (MTR). Methionine is a precursor of *S*-adenosylmethionine (SAM), which is a required cofactor for over 100 cellular methylation reactions including DNA, histone, protein, and phospholipid methylation and for neurotransmitter synthesis¹. 5,10-methylene-tetrahydrofolate is also the source of one-carbons for *de novo* dTMP biosynthesis (Figure 1).

1.1 Overview of dTMP biosynthesis pathways

The *de novo* dTMP synthesis pathway catalyzes conversion of deoxyuridylate (dUMP) to dTMP, and involves four enzymes. SHMT1 and MTHFD1 each independently generate 5,10-methylene-tetrahydrofolate from tetrahydrofolate using serine and formate as one-

carbon sources, respectively, as described above. Thymidylate synthase (TYMS) utilizes 5,10-methylene-tetrahydrofolate as a cofactor in the conversion of dUMP to dTMP (Figure 1), oxidizing tetrahydrofolate to dihydrofolate. Dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate (Figure 1).

In addition to folate-dependent *de novo* synthesis, thymidylate synthesis can occur through a salvage pathway. Salvage synthesis occurs through the action of thymidine kinase (TK), which catalyzes the ATP-dependent phosphorylation of the nucleoside thymidine to the nucleotide dTMP. TK1 is found in the cytoplasm and nucleus whereas TK2 is found within the mitochondria⁸. Both TK1 expression and nuclear localization increase during S-phase of the cell cycle and in response to DNA damage⁹, but TK1 activity is low in quiescent cells. TK2 is not cell cycle regulated, rather it is constitutively expressed and contributes to the mitochondrial dTMP pool to support mitochondrial DNA synthesis, which is required even in post-mitotic tissues¹⁰. In animal cells, salvage pathway synthesis generally does not provide sufficient levels of dTMP to meet DNA synthesis requirements.

2. Causes and consequences of impaired *de novo* thymidylate biosynthesis

Impaired *de novo* dTMP synthesis creates imbalances in dNTP pools, which are mutagenic¹¹, stall replication forks, and induce cell cycle arrest and apoptosis¹². Impaired *de novo* dTMP synthesis also increases rates of uracil misincorporation into DNA^{13,14}. DNA polymerases can incorporate either dTTP or dUTP during DNA replication when there is an “A” base on the template strand, and will increase dUTP incorporation into DNA when dTTP becomes limiting. Both rates of cell division and of uracil accumulation into DNA may be affected by the dUTP/dTTP ratio¹⁵.

The main consequence of uracil in DNA is DNA single- and double-strand breaks generated by DNA repair enzymes. Mammalian cells possess redundant pathways to both limit and repair uracil misincorporation. Deoxyuridine triphosphatase (dUTPase) catabolizes dUTP making it unavailable for DNA synthesis¹⁵. Once dUTP is incorporated into DNA, one of several uracil DNA glycosylases initiate base excision repair. Uracil DNA glycosylase (UNG2 is the nuclear isozyme and UNG1 is the mitochondrial isozyme) is considered to be the primary glycosylase that targets uracil in DNA. However, other glycosylases contribute to uracil excision including Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1 (SMUG1). In base excision repair, DNA glycosylases cleaves uracil from the sugar phosphate backbone, leaving an abasic (AP) site. This site is then cleaved by AP endonuclease (APE1) creating a single-strand break. Single-strand breaks can either be processed by short- or long-patch repair, removing a single or several nucleotides, respectively. DNA polymerase β fills these sites^{16,17}. Hence, excision of uracil directly leads to the formation single- and double-strand breaks that can be lethal¹⁸. Excessive repair of double-strand breaks can lead to activation of p53-mediated apoptosis¹⁹.

The impact of altered expression of the enzymes that constitute the *de novo* dTMP pathway on the functioning of *de novo* dTMP biosynthesis and DNA stability has been studied in genetic knockout mouse models, and in studies of gene variants and inborn errors of metabolism (IEMs).

2.1 Thymidylate synthase

Mice homozygous for a single amino acid change at position 106 in TYMS, generated through *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, display an early embryonic lethal phenotype, demonstrating that TYMS is essential. Interestingly, mice heterozygous for the mutation are viable but compensate for the mutation by increasing TYMS protein synthesis such that heterozygous mutants express more TYMS in the liver than wild-type littermates²⁰.

TYMS expression is highly regulated at the transcriptional and translational levels. TYMS transcription is regulated by activating factors that bind to 28-bp tandem repeats in the promoter. The 28-bp tandem repeats in the 5'UTR most commonly exist as either two repeats (2R) or three repeats (3R), though some populations have been reported to have four to nine repeats^{21,22}. These repeat sequences contain enhancer box (E-box) sequences, which bind numerous upstream stimulating factors to increase TYMS transcription²¹. TYMS expression is higher in individuals with the 3R allele compared to the 2R allele, and this affects both response to chemotherapeutic agents and risk for several types of cancer²¹. For instance, those carrying the 2R allele may have increased risk for colorectal cancer and head and neck squamous cell cancer, though risk may be different among different populations²¹. Recent investigations have assessed the relationship between TYMS polymorphisms and cancer risk on treatment response, but findings are not consistent²³⁻²⁷.

TYMS mRNA levels increase as much as 20-fold in S-phase for DNA synthesis²⁸, driven by the E2F transcription factor²⁹. E2F also drives expression of other proteins required for cell cycle progression, DNA damage response, and stress response³⁰. E2F is regulated by the retinoblastoma (Rb) tumor suppressor protein. In quiescent cells, hypophosphorylated Rb binds E2F and represses transcription. However, in proliferating cells Rb becomes hyperphosphorylated which causes dissociation of Rb from E2F, allowing for E2F-activated transcription. E2F hyperphosphorylation also contributes to stimulating the G1 to S transition in some cell types³⁰⁻³². The TYMS protein binds to its own mRNA, which serves to suppress its own translation. Interestingly, TYMS protein can only bind its mRNA when its active site is unoccupied by folate or nucleotide substrates. This regulatory response can modulate response to 5-FU and possibly other antifolate compounds by modulating rates of TYMS translation³³.

2.2 Dihydrofolate reductase

There are no reports characterizing a DHFR-deficient mouse, although given its role in regenerating tetrahydrofolate cofactors from dihydrofolate, it is assumed to be essential for life. Patients with IEMs in DHFR usually present with megaloblastic anemia as a result of inadequate RBC precursor synthesis due to insufficient dTMP synthesis to support DNA replication, resulting in impaired cell division^{34,35}. These patients also exhibit neurological and psychomotor impairments due to low folate levels within the cerebrospinal fluid. Both hematological and neurological symptoms improve clinical symptoms in most cases treated with high doses of reduced folate such as folinic acid^{34,35}.

Transcription of DHFR, like TYMS, in S-phase is driven by the E2F transcription factor which (for DHFR) works in concert with the Sp1 transcription factor³². DHFR protein also binds its own mRNA when its active site is unoccupied and thereby autoregulates its translation. One of the first successful and still widely used antifolates, methotrexate (MTX), targets DHFR³⁶. Binding of MTX to DHFR inhibits DHFR activity, and also disrupts the interaction between DHFR and its own mRNA, relieving the repressed DHFR translation and leading to increased DHFR protein levels^{32,37,38}. Many cells develop resistance to MTX due to de-repression of DHFR translation by preventing DHFR from binding to its own mRNA³⁶. A 19-bp deletion polymorphism in intron 1 of DHFR affects both DHFR transcription and translation, and has been linked to increased risk for several folate-associated pathologies, but not all studies agree and/or risk varies by population (reviewed extensively in Abali et al³²).

2.3 Serine hydroxymethyltransferase

The *Shmt1* knockout mouse is viable and fertile, and its primary phenotype is decreased rates of *de novo* dTMP synthesis and increased incorporation of uracil in nuclear DNA^{39,40}. The *Shmt1* mouse is the only FOCM enzyme knockout mouse model to develop folate-responsive NTDs⁴¹. *Shmt1* heterozygosity on the *Apc*^{min/+} background increased tumor number and tumor load compared to *Shmt1*^{+/+} mice on the *Apc*^{min/+} background. A common polymorphism in the *SHMT1* gene, C1420T (rs 1979277), exacerbated the effect of the MTHFR polymorphism that increased the risk of cardiovascular disease in the Nurses' Health Study⁴² and also contributed to reduced risk of ALL in individuals who are homozygous for the TYMS 3R allele⁴³.

SHMT1 is translated through both a cap-dependent mechanism and through an internal-ribosome entry sequence (IRES) (cap-independent) mechanism⁴⁴⁻⁴⁶. IRES-mediated translation of SHMT1 is mediated by the mRNA binding protein eIF2 and is responsive to several factors including heavy-chain ferritin binding and UV-induced DNA damage^{44,46}. Interestingly, although the SHMT1 IRES is part of the 5'UTR of the mRNA, cooperation between ITAFs in both the 5'UTR and the 3'UTR work in concert to facilitate SHMT1 translation⁴⁵.

2.4 Methylenetetrahydrofolate dehydrogenase

Mthfd1^{gt/gt} embryos exhibit an early embryonic lethal phenotype; *Mthfd1*^{gt/+} embryos are viable and do not develop NTDs⁴⁷. Recently, several IEMs in MTHFD1 in humans have been identified using exome-capture sequencing, and these mutations result in clinical phenotypes including severe combined immunodeficiency (SCID)^{48,49}. One proband inherited two mutations resulting in decreased MTHFD1 protein levels and severely impaired cyclohydrolase/dehydrogenase (C/D) enzyme activity. Cultured fibroblasts from the proband exhibited elevated homocysteine, impaired *de novo* dTMP synthesis and DNA damage, while *de novo* purine biosynthesis was unaffected⁵⁰. The common MTHFD1 G1958A (R653Q) polymorphism (rs2236225) results in decreased 10-formyltetrahydrofolate synthetase activity⁵¹. The rs2236225 polymorphism is associated with increased risk for neural tube defects and fetal loss as well as breast and gastric cancers⁵²⁻⁵⁵.

2.5 Mitochondrial thymidylate synthesis

Mitochondria also synthesize dTMP from both salvage (via TK2) and *de novo* (via SHMT2, DHFRL1, and TYMS) pathways. IEMs resulting from either compromised mitochondrial DNA (mtDNA) synthesis or nucleotide precursor synthesis lead to mitochondrial depletion syndromes (MDS). IEMs in genes including *TK2*, *TYMP*, *RRM2B*, *POLG*, and *DGUOK* are some examples of genes associated with MDS, and this topic has been recently reviewed elsewhere ⁵⁶. Chinese Hamster Ovary (CHO) cells lacking SHMT2 exhibit increased uracil in mtDNA and impaired mitochondrial *de novo* dTMP synthesis ⁵.

3. *De novo* thymidylate biosynthesis is a target for cancer therapy

Shortly after the discovery of folate as an essential factor for mammalian DNA synthesis, folate antagonists were developed as anticancer agents to impair DNA synthesis in transformed cells. Pharmaceuticals that target enzymes in the folate-dependent dTMP biosynthesis pathway were developed including aminopterin, methotrexate, and nucleoside analogs ⁵⁷. 5-fluorouracil (5-FU) is the most widely used of the therapeutic agents that target TYMS and is used in the treatment of breast, colorectal, epithelial, esophageal and pancreatic cancers ⁵⁸. The antifolates Pemetrexed and Raltitrexed also inhibits TYMS and are used to treat lung and colorectal cancer ⁵⁷.

3.1 TYMS inhibition

The canonical mechanism of 5-FU-mediated cell death is TYMS inhibition. Thymidine phosphorylase (TP) converts 5-FU into 5-fluorodeoxyuridine (5-FdUrd), and TK1 phosphorylates 5-FdUrd, generating 5-fluorodeoxyuracil monophosphate (FdUMP) (Figure 2). The 5-FU metabolite, FdUMP, acts as a dUMP substrate analogue for TYMS. FdUMP, TYMS, and 5,10-methylene-tetrahydrofolate form a stable ternary complex ^{59,60} resulting in covalent bond formation between FdUMP and TYMS. The inactivation of TYMS activity causes dUMP accumulation and depleted dTTP pools, resulting in “thymineless cell death” ⁶¹. Impaired dTMP biosynthesis results in accelerated rates of genomic uracil incorporation ^{39,40} and DNA repair ^{16,17}. The resulting futile cycles of uracil misincorporation and excision result in an accumulation of DNA strand breaks, as described earlier.

3.2 5-FU incorporation into DNA

In addition to TYMS inhibition, FdUTP can be incorporated into DNA. Thymidylate kinase (TMPK) converts FdUMP to FdUDP, and nucleoside diphosphate kinase (NDPK) phosphorylates FdUDP to FdUTP (Figure 2). There is evidence to suggest that FdUTP incorporation contributes significantly to 5-FU-mediated toxicity. In mammalian cells, 5-FU treatment leads to 5-FdU incorporation into DNA at levels higher than uracil incorporation in DNA. However in yeast exposed to 5FU, uracil levels in DNA exceed genomic 5-FdUTP levels ⁶². Inhibition of UNG expression sensitizes the ovarian cancer cell line OVCAR-5 to 5-FdU, but not to Raltitrexed. This suggests that 5-FdU in DNA has a significant toxic effect ⁶³ compared to dU incorporation alone, as would be expected from Raltitrexed treatment.

Current evidence suggests that cytotoxicity of 5-FdUMP present in the genome is a consequence of DNA repair and the resulting lesions. *In vitro* assays with DNA polymerase δ indicate that synthetic oligonucleotides containing 5-FdU do not decrease rates of DNA synthesis or stall DNA replication itself⁶³. 5-FdUTP that is incorporated into DNA is recognized and excised by base excision repair machinery by the same mechanisms that remove genomic uracil⁶⁴. In the absence of UNG, 5-FdUMP in DNA is removed by another DNA glycosylase, thymine DNA glycosylase (TDG). TDG-mediated excision results in persistent strand breaks and delayed S-phase progression. Unlike other uracil DNA glycosylases, TDG is post-translationally modified by the Small Ubiquitin-like Modifier (SUMO), and SUMOylation is required for TDG dissociation from AP sites and subsequent APE1 loading⁶⁵. This requirement for SUMO modification is hypothesized to delay onset of base excision repair, leading to increased half-life of these intermediate sites which can lead to replication fork stalling and collapse. Additionally, MEFs derived from *Tdg*^{-/-} mice are resistant to 5-FU⁶⁶. Hence repair of repair of 5FdU in DNA by TDG plays a meaningful role in 5-FU mediated toxicity.

3.3 5-FU incorporation into RNA

5-FU incorporation into RNA may or may not make meaningful contributions to its cytotoxicity. 5-FU is converted to 5-fluorouracil triphosphate (FUTP), through the collective actions of orotate phosphoribosyltransferase (ORPT), uridine monophosphate kinase (UMPK), and uridine diphosphate kinase (UDPK) (Figure 2). Alternatively, 5-fluorouridine (5-FUrd) can be administered and converted to FUMP via uridine kinase (UK), and subsequently converted to FUTP via UMPK and UDPK. FUTP can be incorporated into newly synthesized RNA. Following exposure to 5-FU, its incorporation into RNA is reported to be 3,000 – 15,000 times higher than its incorporation into DNA; furthermore, 5-FU cytotoxicity is partially rescued by ribonucleosides, but not deoxyribonucleosides in HeLa cells and SW480 colon cancer cells⁶⁷. HeLa cells exposed to 5-FUrd, but not 5-FdUrd, exhibit stress granule formation. Stress granules are cytosolic aggregations of RNA and protein formed under stress, which house stalled translation complexes⁶⁸. Stress granules are hypothesized to be a defense to prevent improper translation, and can undergo re-initiation of translation or degradation. This effect is also seen with other RNA incorporating drugs⁶⁸.

The cytotoxicity of 5-FU in RNA appears to be caused by impaired RNA processing, as opposed to direct translational inhibition as 5-FU substitution in mRNA does not affect the rate of *in vitro* translation⁶⁹. 5-FU incorporation into rRNA *in vitro* prevents its degradation by exoribonuclease Rrp6, suggesting turnover of rRNA is impaired in yeast cells⁷⁰. 5-FU incorporation into mRNA also alters splicing *in vitro*⁷¹, as 5-FU treated HeLa cells exhibited impaired splicing of pre-mRNA. Injection of 5-FU synthesized mRNA did not exhibit impaired splicing⁷², rather incorporation of 5-FU into snRNA impairs the function of the splicing machinery. However, investigations of 5-FU tumor resistance have not revealed a role for 5-FU incorporation into RNA as having a primary effect in 5-FU efficacy as a chemotherapeutic agent.

4. Mechanisms of 5-FU resistance

4.1 5-FU catabolism

Cancer cells can become resistant to 5-FU by increasing rates of 5-FU catabolism or by increasing rates of *de novo* dTMP biosynthesis. Dihydropyrimidine dehydrogenase (DPD) in the liver catabolizes 5-FU into dihydrofluorouracil (DHFU), metabolizing 80% of the 5-FU⁷³ (Figure 2). Mutations in the *DPYD* gene, which encodes DPD, result in impaired liver DPD activity, a condition known as DPD deficiency. DPD deficiency is linked to increased cytotoxicity in 5-FU treated patients⁷⁴. Conversely, the efficient degradation of 5-FU by DPD limits the usefulness of the drug. The oral 5-FU prodrug Capecitabine circumvents this degradation.

4.2 Salvage enzyme and TYMS expression

TYMS levels can increase in response to 5-FU treatment⁵⁸, and increased TYMS gene expression is associated with poor response to 5-FU treatment⁷⁵⁻⁷⁸. Elevated levels of DPD, TYMS, and thymidine phosphorylase (TP) are correlated with increased 5-FU chemoresistance and decreased survival Bai, et al.⁷⁹. However, Li et al found there was no correlation observed between mRNA levels of TP, DPD, or TYMS and breast carcinoma tumor size, node status, or histological grade, although elevated TYMS mRNA levels were found to be correlated with shorter survival. The mRNA expression of these genes had high variation, which may have obscured significant correlations⁸⁰. Increased TYMS and TP levels were correlated with lymph node recurrence in advanced gastric cancer⁸¹, and consistent with observations that injected TP overexpressing cells in BALB/c mice are sensitized to 5-FU⁸². As extensively reviewed in Bronckaers *et al*, TP promotes tumor growth and metastasis, but is also necessary to activate 5-FU prodrugs⁷³.

4.3 Folate status modifies 5-FU sensitivity

In humans the addition of leucovorin to 5-FU treatment has been shown to be more effective than 5-FU alone⁸³. Leucovorin, which is a natural form of folate, 5-formyl-tetrahydrofolate, can be converted to 5,10-methylene-tetrahydrofolate in the cell, supporting the formation of the TYMS-5-FdUMP-5,10-methylene-tetrahydrofolate ternary complex^{59,60}. The addition of leucovorin has been shown to sensitize cancer cells to 5-FU⁸⁴. Leucovorin supplementation has been shown to increase ternary complex formation in MCF-7 breast cancer cells and NCI H630 colon cancer cells⁸⁵. These findings indicate that elevated cellular folate levels increase 5-FU efficacy. The FOLFOX regimen, which is formulated to include oxaliplatin, (a platinum-based agent which forms intra- and inter-strand crosslinks in DNA), leucovorin and 5-FU, has been shown to further increase 5-FU efficacy in the treatment of metastatic colorectal cancer⁸⁶.

5. Emergence of nuclear OCM as the source of thymidylate synthesis in mammals

Until recently, it was assumed that dTMP synthesis occurred in the cytoplasm, with dTMP translocating to the mitochondria or nucleus for DNA replication. The enzymes that constitute the *de novo* dTMP synthesis pathway were originally localized to the cytosol in

both mammalian cells and yeast. However, in the 1980s, there were reports demonstrating that TYMS could also be localized to the nucleus. Thereafter, co-sedimentation and co-fractionation experiments in mammalian cells indicated that some, but not all, of the enzymes involved in deoxynucleotide biosynthesis were found to co-localize in a multiprotein complex with the DNA replication machinery, and this complex was referred to as the “replitase”⁸⁷⁻⁸⁹. Interestingly, the nuclear localization of TYMS was not observed in yeast, rather *S. cerevisiae* TYMS was found to localize to the nuclear periphery⁹⁰. Hence, it was not clear whether the entire *de novo* biosynthesis pathway was present in the nucleus, or if dTMP biosynthesis occurred in the nucleus.

Radiolabeling experiments using isolated nuclei from S-phase blocked cells demonstrated that nuclei are capable of *de novo* thymidylate biosynthesis using either serine (via SHMT) or formate (via MTHFD1) as one-carbon donors^{7,91}. This raises the possibility that dTMP synthesis occurs exclusively in the nucleus only during S phase. Formation of a multienzyme complex and the associated protein-protein interactions are essential for the functioning of the *de novo* dTMP synthesis pathway, as sonicated nuclei have greatly reduced *de novo* dTMP biosynthesis activity⁹². Furthermore, the *de novo* dTMP biosynthesis complex was found to localize to sites of DNA synthesis and associate with nuclear lamin proteins and other components of the DNA replication machinery including PCNA⁹². The co-localization of the *de novo* dTMP synthesis pathway to cellular compartments associated with DNA synthesis is not unique to the nucleus, as the *de novo* dTMP synthesis pathway also has been found to function in purified mitochondria, involving the enzymes TYMS, DHFR2, and SHMT2⁵.

The mammalian enzymes that constitute the *de novo* dTMP biosynthesis pathway including DHFR, SHMT1, TYMS, and MTHFD1 undergo SUMOylation at the G1/S boundary and in response to UV-induced DNA damage leading to SUMO-dependent nuclear import^{46,93,94}. The multienzyme complex responsible for nuclear *de novo* dTMP biosynthesis is essential to prevent high levels of uracil incorporation into DNA in mice⁴⁰. Previous studies have demonstrated that SHMT1 expression can determine rates of *de novo* thymidylate biosynthesis in cultured cells⁹⁵ and expression of catalytically-inactive SHMT1 mutants also stimulate *de novo* dTMP biosynthesis⁹². Yet, formate, through the activity of MTHFD1, is the primary source folate-activated one-carbons for *de novo* dTMP synthesis⁶. These experimental findings were explained by the observation that SHMT1 acts as a scaffold protein that is essential for the co-localization of MTHFD1, TYMS and DHFR, and that SHMT1 nuclear localization is necessary for *de novo* dTMP complex formation^{44,50,92}. SHMT1 binds tightly to lamin A and lamin B proteins, and serves to tether TYMS, DHFR and MTHFD1 to the nuclear lamina^{50,92}. Interestingly, overexpression of the SHMT1 cDNA in mice results in high levels of SHMT1 protein in liver, but impaired nuclear localization of SHMT1, leading to depressed rates of *de novo* dTMP biosynthesis in isolated nuclei, and up to 10-fold increased levels of uracil in mouse liver nuclear DNA⁴⁰. These studies demonstrate the importance of nuclear localization of the *de novo* dTMP synthesis complex in preventing uracil accumulation in DNA and maintaining genome stability.

Formation of a nuclear *de novo* dTMP synthesis complex appears to be unique to mammalian cells, and the evolution of nuclear dTMP synthesis may provide several

advantages in limiting genome instability. In response to DNA damage, yeast increase dNTP concentrations 6-8 fold ⁹⁶ and *E. coli* increase dNTP concentration 1.8-3.7 fold ⁹⁷, whereas mammalian cells do not show increased dNTP concentrations following DNA damage ^{11,98}. In both *S. cerevisiae* and *E. coli*, the increase in dNTP levels results in higher rates of mutagenesis ^{96,97}. Thus, dTMP synthesis localized to sites of DNA synthesis may lower mutation rates. Nuclear dTMP synthesis at sites of DNA synthesis allows for selective concentration of this deoxyribonucleotide at its only site of utilization, thereby limiting unnecessary synthesis and its incorporation into RNA in the cytosol. Provision of dTTP directly at sites of DNA synthesis may also permit the regulated and selective incorporation of dTTP or dUTP into DNA, although there is no evidence currently that uracil incorporation into the genome is regulated or selectively incorporated.

6.0 Role of SUMOylation in cancer

SUMOylation of proteins is known to affect protein-protein interactions, cellular localization, and substrate stability ⁹⁹. SUMOylation is necessary for proper functioning of many proteins involved in critical cellular processes such as DNA replication and repair ¹⁰⁰⁻¹⁰², transcriptional regulation ¹⁰³⁻¹⁰⁵, maintenance of chromosomal structure ¹⁰⁶, nuclear morphology, and nuclear import. For example, the E3 SUMO ligase PIASy stimulates SUMOylation of both p53 and Rb, inducing premature senescence ¹⁰⁷, indicating SUMOylation is used as a regulator of gene transcription by the cell. Disruption of SUMOylation machinery results in DNA replication and repair defects, suggesting SUMOylation of these components is critical. Loss of the SUMO-conjugating protein ligase Ubc9 leads to chromosome condensation and segregation defects in mouse embryos, and Ubc9-deficient blastocysts have defects in nuclear and subnuclear architecture. Loss of Ubc9 leads to a collapse in the RanGDP-GTP gradient, which is critical for nuclear import and export ¹⁰⁸. Cancer models show upregulation of the various SUMO components. Ubc9 is upregulated in HPV-positive cervical lesions ¹⁰⁹, and in malignant and peritumoral squamous cell carcinoma tissues ¹¹⁰. As reviewed in Mattoscio *et. al.*, upregulation of several SUMO proteases, which remove SUMO modifications (SEN1, SENP3, SENP5), has been shown to be critical for some cancer cell proliferation ¹¹¹⁻¹¹⁴, whereas SENP2 is downregulated in hepatocellular carcinoma and bladder cancer ^{115,116}.

The SUMO machinery has been suggested to be an attractive target for the development of anticancer therapies, as disruption of SUMO components has been shown to affect cancer cell proliferation. Reduced Ubc9 expression suppresses growth of KRAS mutant colorectal cancer cells ¹¹⁷, and Spectinomycin B1, an inhibitor of Ubc9, inhibits proliferation of MCF-7 breast cancer cells ¹¹⁸. Reduced expression of the SUMO-activating enzyme subunit 2 (SAE2) and Ubc9 impair cell proliferation in cancer cells as does reduced expression of SAE2 alone in U2OS osteosarcoma cells. HCT116 colorectal carcinoma cells with reduced SAE2 expression exhibit elevated rates of apoptosis and senescence, with xenografts exhibiting lower rates of tumor growth in mice ¹¹⁹. Disruption of deSUMOylating enzymes also impairs cancer cell proliferation. Reduced expression of SENP1 leads to growth arrest and apoptosis in multiple myeloma cells ¹²⁰ and inhibits cell proliferation in prostate cancer cells ¹²¹. Reduced SENP5 expression inhibits cell proliferation and causes defects in nuclear morphology in HeLa cells ¹²².

7. Reevaluating mechanisms of 5-FU toxicity and its function in the nucleus

Advances in the fundamental understanding of the nuclear compartmentation of *de novo* dTMP biosynthesis prompts a reevaluation of the mechanisms of 5-FU cytotoxicity. There is evidence that nuclear compartmentation of the *de novo* dTMP synthesis pathway modifies 5-FU efficacy. A higher ratio of nuclear:cytoplasmic TYMS was shown to be significantly correlated with decreased survival in colorectal cancer¹²³. Additionally, increased nuclear TYMS expression is associated with poor response to 5-FU in colorectal carcinoma¹²⁴. Other antifolates may function to impair nuclear dTMP synthesis, as MTX has been shown to almost completely block nuclear translocation of DHFR in acute myeloid leukemia cells¹²⁵. Thus, nuclear localization of the *de novo* dTMP biosynthesis pathway may be a critical predictor of 5-FU mediated toxicity. Interestingly, a common SHMT1 variant, *SHMT1* C1420T (rs 1979277), results in an amino acid substitution (Leu474Phe) located at the interface where Ubc9 and SHMT1 interact when SHMT1 undergoes SUMOylation⁹³. This SHMT1 polymorphism impairs the post-translational SUMO modification of SHMT1 and its nuclear localization⁹⁴, and hence would be expected to decrease total dTMP synthesis capacity. SHMT1 C1420T was associated with better response and longer progression-free survival in patients with metastatic colorectal cancer treated with bevacizumab+ FOLFIRI (5-FU, leucovorin, irinotecan)¹²⁶. Barcelos *et. al.* also found that SHMT1 C1420T was associated longer progression-free survival with FOLFIRI treatment in metastatic colorectal cancer¹²⁷. The discovery of SUMO-dependent nuclear *de novo* dTMP synthesis in mammalian cells raises the possibility of novel strategies to target *de novo* dTMP biosynthesis, with a focus on SHMT1 SUMOylation as it is essential for formation of the metabolic complex responsible for nuclear dTMP synthesis.

8. Concluding remarks and future studies

To improve efficacy of antifolate based therapies, subcellular localization and the factors which modify localization of target proteins must be taken into account. Considering that nuclear localization of the *de novo* dTMP biosynthesis complex is critical for proper dTMP synthesis, further studies are needed to determine if pharmaceuticals that target enzymes in dTMP synthesis affect nuclear localization of TYMS (as shown with DHFR and MTX), and if nuclear localization of the pathway enzymes influences drug efficacy and cancer survival. At a more fundamental level, further studies are needed to determine if antifolate binding to their respective enzymes induce conformational changes that could affect SUMOylation and nuclear import. Because of SHMT1's critical role in nuclear localization of the *de novo* dTMP biosynthesis complex, impairing SHMT1 nuclear import or lamin-binding capacity may improve antifolate based therapies. Considering that SUMOylation plays a critical role in nuclear localization of the *de novo* dTMP biosynthesis complex, and in DNA replication and repair in general, targeted SUMO-based inhibitors could be used to augment antifolate treatments.

References

1. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. *Vitam Horm.* 2008; 79:1–44. [PubMed: 18804690]

2. Titus SA, Moran RG. Retrovirally mediated complementation of the glyB phenotype. Cloning of a human gene encoding the carrier for entry of folates into mitochondria. *J Biol Chem.* 2000; 275:36811–7. [PubMed: 10978331]
3. Lawrence SA, et al. Mammalian mitochondrial and cytosolic folylpolyglutamate synthetase maintain the subcellular compartmentalization of folates. *J Biol Chem.* 2014; 289:29386–96. [PubMed: 25164808]
4. Tibbetts AS, Appling DR. Compartmentalization of Mammalian folate-mediated one-carbon metabolism. *Annu Rev Nutr.* 2010; 30:57–81. [PubMed: 20645850]
5. Anderson DD, Quintero CM, Stover PJ. Identification of a de novo thymidylate biosynthesis pathway in mammalian mitochondria. *Proc Natl Acad Sci U S A.* 2011; 108:15163–8. [PubMed: 21876188]
6. Herbig K, et al. Cytoplasmic serine hydroxymethyltransferase mediates competition between folate-dependent deoxyribonucleotide and S-adenosylmethionine biosyntheses. *J Biol Chem.* 2002; 277:38381–9. [PubMed: 12161434]
7. Field MS, et al. Nuclear enrichment of folate cofactors and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) protect de novo thymidylate biosynthesis during folate deficiency. *J Biol Chem.* 2014; 289:29642–50. [PubMed: 25213861]
8. Pontarin G, Gallinaro L, Ferraro P, Reichard P, Bianchi V. Origins of mitochondrial thymidine triphosphate: dynamic relations to cytosolic pools. *Proc Natl Acad Sci U S A.* 2003; 100:12159–64. [PubMed: 14519855]
9. Chen YL, Eriksson S, Chang ZF. Regulation and functional contribution of thymidine kinase 1 in repair of DNA damage. *J Biol Chem.* 2010; 285:27327–35. [PubMed: 20554529]
10. Dorado B, Area E, Akman HO, Hirano M. Onset and organ specificity of Tk2 deficiency depends on Tk1 down-regulation and transcriptional compensation. *Hum Mol Genet.* 2011; 20:155–64. [PubMed: 20940150]
11. Niida H, Shimada M, Murakami H, Nakanishi M. Mechanisms of dNTP supply that play an essential role in maintaining genome integrity in eukaryotic cells. *Cancer Sci.* 2010; 101:2505–9. [PubMed: 20874841]
12. Wataya Y, et al. Molecular mechanism of cell death induced dNTP pool imbalance. *Nucleic Acids Symp Ser.* 1993:109–10. [PubMed: 8280290]
13. Blount BC, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A.* 1997; 94:3290–5. [PubMed: 9096386]
14. Goulian M, Bleile B, Tseng BY. Methotrexate-induced misincorporation of uracil into DNA. *Proc Natl Acad Sci U S A.* 1980; 77:1956–60. [PubMed: 6929529]
15. Vertessy BG, Toth J. Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. *Acc Chem Res.* 2009; 42:97–106. [PubMed: 18837522]
16. Schormann N, Ricciardi R, Chattopadhyay D. Uracil-DNA glycosylases-structural and functional perspectives on an essential family of DNA repair enzymes. *Protein Sci.* 2014; 23:1667–85. [PubMed: 25252105]
17. Schrader CE, Guikema JE, Wu X, Stavnezer J. The roles of APE1, APE2, DNA polymerase beta and mismatch repair in creating S region DNA breaks during antibody class switch. *Philos Trans R Soc Lond B Biol Sci.* 2009; 364:645–52. [PubMed: 19010771]
18. Fenech M. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. *Mutat Res.* 2012; 733:21–33. [PubMed: 22093367]
19. Sakaguchi K, et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev.* 1998; 12:2831–41. [PubMed: 9744860]
20. Ching YH, et al. High resolution mapping and positional cloning of ENU-induced mutations in the Rw region of mouse chromosome 5. *BMC Genet.* 2010; 11:106. [PubMed: 21118569]
21. Lima A, Azevedo R, Sousa H, Seabra V, Medeiros R. Current approaches for TYMS polymorphisms and their importance in molecular epidemiology and pharmacogenetics. *Pharmacogenomics.* 2013; 14:1337–51. [PubMed: 23930679]

22. Mandola MV, et al. A novel single nucleotide polymorphism within the 5' tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. *Cancer Res.* 2003; 63:2898–904. [PubMed: 12782596]
23. Bolufer P, et al. Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. *Leuk Res.* 2006; 30:1471–91. [PubMed: 17023046]
24. Figueiredo JC, Levine AJ, Crott JW, Baurley J, Haile RW. Folate-genetics and colorectal neoplasia: what we know and need to know next. *Mol Nutr Food Res.* 2013; 57:607–27. [PubMed: 23401104]
25. Gusella M, Padriani R. G>C SNP of thymidylate synthase with respect to colorectal cancer. *Pharmacogenomics.* 2007; 8:985–96. [PubMed: 17716232]
26. Wang J, Wang B, Bi J, Di J. The association between two polymorphisms in the TYMS gene and breast cancer risk: a meta-analysis. *Breast Cancer Res Treat.* 2011; 128:203–9. [PubMed: 21188629]
27. Weng Y, Zhang J, Tang X, Xie X, Chen G. Thymidylate synthase polymorphisms and hematological cancer risk: a meta-analysis. *Leuk Lymphoma.* 2012; 53:1345–51. [PubMed: 22166040]
28. Navalgund LG, Rossana C, Muench AJ, Johnson LF. Cell cycle regulation of thymidylate synthetase gene expression in cultured mouse fibroblasts. *J Biol Chem.* 1980; 255:7386–90. [PubMed: 7391086]
29. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol Cell Biol.* 1995; 15:4215–24. [PubMed: 7623816]
30. Cecchini MJ, et al. A retinoblastoma allele that is mutated at its common E2F interaction site inhibits cell proliferation in gene-targeted mice. *Mol Cell Biol.* 2014; 34:2029–45. [PubMed: 24662053]
31. Ikeda MA, Jakoi L, Nevins JR. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. *Proc Natl Acad Sci U S A.* 1996; 93:3215–20. [PubMed: 8622916]
32. Abali EE, Skacel NE, Celikkaya H, Hsieh YC. Regulation of human dihydrofolate reductase activity and expression. *Vitam Horm.* 2008; 79:267–92. [PubMed: 18804698]
33. Berger SH, Berger FG, Lebioda L. Effects of ligand binding and conformational switching on intracellular stability of human thymidylate synthase. *Biochim Biophys Acta.* 2004; 1696:15–22. [PubMed: 14726200]
34. Banka S, et al. Identification and characterization of an inborn error of metabolism caused by dihydrofolate reductase deficiency. *Am J Hum Genet.* 2011; 88:216–25. [PubMed: 21310276]
35. Cario H, et al. Dihydrofolate reductase deficiency due to a homozygous DHFR mutation causes megaloblastic anemia and cerebral folate deficiency leading to severe neurologic disease. *Am J Hum Genet.* 2011; 88:226–31. [PubMed: 21310277]
36. Bertino JR, et al. Increased level of dihydrofolic reductase in leucocytes of patients treated with amethopterin. *Nature.* 1962; 193:140–2. [PubMed: 13868435]
37. Appleman JR, Prendergast N, Delcamp TJ, Freisheim JH, Blakley RL. Kinetics of the formation and isomerization of methotrexate complexes of recombinant human dihydrofolate reductase. *J Biol Chem.* 1988; 263:10304–13. [PubMed: 3292526]
38. Bystroff C, Kraut J. Crystal structure of unliganded Escherichia coli dihydrofolate reductase. Ligand-induced conformational changes and cooperativity in binding. *Biochemistry.* 1991; 30:2227–39. [PubMed: 1998681]
39. Macfarlane AJ, Perry CA, McEntee MF, Lin DM, Stover PJ. Shmt1 heterozygosity impairs folate-dependent thymidylate synthesis capacity and modifies risk of Apc(min)-mediated intestinal cancer risk. *Cancer Res.* 2011; 71:2098–107. [PubMed: 21406397]
40. MacFarlane AJ, et al. Nuclear localization of de novo thymidylate biosynthesis pathway is required to prevent uracil accumulation in DNA. *J Biol Chem.* 2011; 286:44015–22. [PubMed: 22057276]
41. Beaudin AE, et al. Shmt1 and de novo thymidylate biosynthesis underlie folate-responsive neural tube defects in mice. *Am J Clin Nutr.* 2011; 93:789–98. [PubMed: 21346092]

42. Wernimont SM, et al. Polymorphisms in serine hydroxymethyltransferase 1 and methylenetetrahydrofolate reductase interact to increase cardiovascular disease risk in humans. *J Nutr.* 2011; 141:255–60. [PubMed: 21178087]
43. Skibola CF, et al. Polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and risk of adult acute lymphocytic leukemia. *Blood.* 2002; 99:3786–91. [PubMed: 11986237]
44. Woeller CF, Fox JT, Perry C, Stover PJ. A ferritin-responsive internal ribosome entry site regulates folate metabolism. *J Biol Chem.* 2007; 282:29927–35. [PubMed: 17702748]
45. Fox JT, Stover PJ. Mechanism of the internal ribosome entry site-mediated translation of serine hydroxymethyltransferase 1. *J Biol Chem.* 2009; 284:31085–96. [PubMed: 19734143]
46. Fox JT, Shin WK, Caudill MA, Stover PJ. A UV-responsive internal ribosome entry site enhances serine hydroxymethyltransferase 1 expression for DNA damage repair. *J Biol Chem.* 2009; 284:31097–108. [PubMed: 19734144]
47. Beaudin AE, Perry CA, Stabler SP, Allen RH, Stover PJ. Maternal Mthfd1 disruption impairs fetal growth but does not cause neural tube defects in mice. *Am J Clin Nutr.* 2012; 95:882–91. [PubMed: 22378735]
48. Watkins D, et al. Novel inborn error of folate metabolism: identification by exome capture and sequencing of mutations in the MTHFD1 gene in a single proband. *J Med Genet.* 2011; 48:590–2. [PubMed: 21813566]
49. Burda P, et al. Characterization and review of MTHFD1 deficiency: four new patients, cellular delineation and response to folic and folinic acid treatment. *J Inherit Metab Dis.* 2015; 38:863–72. [PubMed: 25633902]
50. Field MS, Kamynina E, Watkins D, Rosenblatt DS, Stover PJ. Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis. *Proc Natl Acad Sci U S A.* 2015; 112:400–5. [PubMed: 25548164]
51. Christensen KE, et al. The MTHFD1 p.Arg653Gln variant alters enzyme function and increases risk for congenital heart defects. *Hum Mutat.* 2009; 30:212–20. [PubMed: 18767138]
52. De Marco P, et al. Evaluation of a methylenetetrahydrofolate-dehydrogenase 1958G>A polymorphism for neural tube defect risk. *J Hum Genet.* 2006; 51:98–103. [PubMed: 16315005]
53. Parle-McDermott A, et al. Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. *Eur J Hum Genet.* 2006; 14:768–72. [PubMed: 16552426]
54. Stevens VL, et al. Association of polymorphisms in one-carbon metabolism genes and postmenopausal breast cancer incidence. *Cancer Epidemiol Biomarkers Prev.* 2007; 16:1140–7. [PubMed: 17548676]
55. Brody LC, et al. A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group. *Am J Hum Genet.* 2002; 71:1207–15. [PubMed: 12384833]
56. El-Hattab AW, Scaglia F. Mitochondrial DNA depletion syndromes: review and updates of genetic basis, manifestations, and therapeutic options. *Neurotherapeutics.* 2013; 10:186–98. [PubMed: 23385875]
57. Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. *Drug Resist Updat.* 2012; 15:183–210. [PubMed: 22921318]
58. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer.* 2003; 3:330–8. [PubMed: 12724731]
59. Santi DV, McHenry CS, Sommer H. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry.* 1974; 13:471–81. [PubMed: 4203910]
60. Sommer H, Santi DV. Purification and amino acid analysis of an active site peptide from thymidylate synthetase containing covalently bound 5-fluoro-2'-deoxyuridylate and methylenetetrahydrofolate. *Biochem Biophys Res Commun.* 1974; 57:689–95. [PubMed: 4275130]
61. Ahmad SI, Kirk SH, Eisenstark A. Thymine metabolism and thymineless death in prokaryotes and eukaryotes. *Annu Rev Microbiol.* 1998; 52:591–625. [PubMed: 9891809]

62. Seiple L, Jaruga P, Dizdaroglu M, Stivers JT. Linking uracil base excision repair and 5-fluorouracil toxicity in yeast. *Nucleic Acids Res.* 2006; 34:140–51. [PubMed: 16407331]
63. Huehls AM, et al. Genomically Incorporated 5-Fluorouracil that Escapes UNG-Initiated Base Excision Repair Blocks DNA Replication and Activates Homologous Recombination. *Mol Pharmacol.* 2016; 89:53–62. [PubMed: 26494862]
64. Wyatt MD, Wilson DM 3rd. Participation of DNA repair in the response to 5-fluorouracil. *Cell Mol Life Sci.* 2009; 66:788–99. [PubMed: 18979208]
65. Steinacher R, Schar P. Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol.* 2005; 15:616–23. [PubMed: 15823533]
66. Kunz C, et al. Base excision by thymine DNA glycosylase mediates DNA-directed cytotoxicity of 5-fluorouracil. *PLoS Biol.* 2009; 7:e91. [PubMed: 19402749]
67. Pettersen HS, et al. UNG-initiated base excision repair is the major repair route for 5-fluorouracil in DNA, but 5-fluorouracil cytotoxicity depends mainly on RNA incorporation. *Nucleic Acids Res.* 2011; 39:8430–44. [PubMed: 21745813]
68. Kaehler C, Isensee J, Hucho T, Lehrach H, Krobitsch S. 5-Fluorouracil affects assembly of stress granules based on RNA incorporation. *Nucleic Acids Res.* 2014; 42:6436–47. [PubMed: 24728989]
69. Takimoto CH, et al. Effects of 5-fluorouracil substitution on the RNA conformation and in vitro translation of thymidylate synthase messenger RNA. *J Biol Chem.* 1993; 268:21438–42. [PubMed: 8407987]
70. Silverstein RA, Gonzalez de Valdivia E, Visa N. The incorporation of 5-fluorouracil into RNA affects the ribonucleolytic activity of the exosome subunit Rrp6. *Mol Cancer Res.* 2011; 9:332–40. [PubMed: 21289297]
71. Doong SL, Dolnick BJ. 5-Fluorouracil substitution alters pre-mRNA splicing in vitro. *J Biol Chem.* 1988; 263:4467–73. [PubMed: 3346255]
72. Zhao X, Yu YT. Incorporation of 5-fluorouracil into U2 snRNA blocks pseudouridylation and pre-mRNA splicing in vivo. *Nucleic Acids Res.* 2007; 35:550–8. [PubMed: 17169984]
73. Bronckaers A, Gago F, Balzarini J, Liekens S. The dual role of thymidine phosphorylase in cancer development and chemotherapy. *Med Res Rev.* 2009; 29:903–53. [PubMed: 19434693]
74. Chazal M, et al. Link between dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells and liver. *Clin Cancer Res.* 1996; 2:507–10. [PubMed: 9816197]
75. Bathe OF, et al. Increased thymidylate synthase gene expression in liver metastases from colorectal carcinoma: implications for chemotherapeutic options and survival. *Cancer J Sci Am.* 1999; 5:34–40. [PubMed: 10188059]
76. Aschele C, et al. Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. *J Clin Oncol.* 1999; 17:1760–70. [PubMed: 10561213]
77. Cascinu S, et al. Thymidylate synthase protein expression in advanced colon cancer: correlation with the site of metastasis and the clinical response to leucovorin-modulated bolus 5-fluorouracil. *Clin Cancer Res.* 1999; 5:1996–9. [PubMed: 10473077]
78. Johnston PG, et al. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res.* 1995; 55:1407–12. [PubMed: 7882343]
79. Bai W, Wu Y, Zhang P, Xi Y. Correlations between expression levels of thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase, and efficacy of 5-fluorouracil-based chemotherapy for advanced colorectal cancer. *Int J Clin Exp Pathol.* 2015; 8:12333–45. [PubMed: 26722420]
80. Li H, et al. The prognostic significance of thymidine phosphorylase, thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expressions in breast carcinomas. *Histol Histopathol.* 2004; 19:129–36. [PubMed: 14702180]
81. Suda Y, et al. Expression of thymidylate synthase and thymidine phosphorylase in recurrence and survival rates of advanced gastric cancer. *Gastric Cancer.* 1999; 2:165–172. [PubMed: 11957091]

82. Evrard A, et al. Enhancement of 5-fluorouracil cytotoxicity by human thymidine phosphorylase expression in cancer cells: in vitro and in vivo study. *Int J Cancer*. 1999; 80:465–70. [PubMed: 9935191]
83. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. Advanced Colorectal Cancer Meta-Analysis Project. *J Clin Oncol*. 1992; 10:896–903. [PubMed: 1534121]
84. Nakamura A, et al. Enhancement of 5-fluorouracil-induced cytotoxicity by leucovorin in 5-fluorouracil-resistant gastric cancer cells with upregulated expression of thymidylate synthase. *Gastric Cancer*. 2014; 17:188–95. [PubMed: 23494117]
85. Drake JC, Voeller DM, Allegra CJ, Johnston PG. The effect of dose and interval between 5-fluorouracil and leucovorin on the formation of thymidylate synthase ternary complex in human cancer cells. *Br J Cancer*. 1995; 71:1145–50. [PubMed: 7779703]
86. Giacchetti S, et al. Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. *J Clin Oncol*. 2000; 18:136–47. [PubMed: 10623704]
87. Murthy S, Reddy GP-V. Replisome: Complete machinery for DNA synthesis. *Journal of Cellular Physiology*. 2006; 209:711–717. [PubMed: 17001698]
88. Prem veer Reddy G, Pardee AB. Multienzyme complex for metabolic channeling in mammalian DNA replication. *Proc Natl Acad Sci U S A*. 1980; 77:3312–16. [PubMed: 6251456]
89. Wickremasinghe RG, Yaxley JC, Hoffbrand AV. Gel filtration of a complex of DNA polymerase and DNA precursor-synthesizing enzymes from a human lymphoblastoid cell line. *Biochim Biophys Acta*. 1983; 740:243–8. [PubMed: 6307381]
90. Poon PP, Storms RK. Thymidylate synthase is localized to the nuclear periphery in the yeast *Saccharomyces cerevisiae*. *J Biol Chem*. 1994; 269:8341–7. [PubMed: 8132557]
91. Anderson DD, Stover PJ. SHMT1 and SHMT2 are functionally redundant in nuclear de novo thymidylate biosynthesis. *PLoS One*. 2009; 4:e5839. [PubMed: 19513116]
92. Anderson DD, Woeller CF, Chiang EP, Shane B, Stover PJ. Serine hydroxymethyltransferase anchors de novo thymidylate synthesis pathway to nuclear lamina for DNA synthesis. *J Biol Chem*. 2012; 287:7051–62. [PubMed: 22235121]
93. Woeller CF, Anderson DD, Szebenyi DM, Stover PJ. Evidence for small ubiquitin-like modifier-dependent nuclear import of the thymidylate biosynthesis pathway. *J Biol Chem*. 2007; 282:17623–31. [PubMed: 17446168]
94. Anderson DD, Eom JY, Stover PJ. Competition between sumoylation and ubiquitination of serine hydroxymethyltransferase 1 determines its nuclear localization and its accumulation in the nucleus. *J Biol Chem*. 2012; 287:4790–9. [PubMed: 22194612]
95. Oppenheim EW, Adelman C, Liu X, Stover PJ. Heavy chain ferritin enhances serine hydroxymethyltransferase expression and de novo thymidine biosynthesis. *J Biol Chem*. 2001; 276:19855–61. [PubMed: 11278996]
96. Chabes A, et al. Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell*. 2003; 112:391–401. [PubMed: 12581528]
97. Gon S, Napolitano R, Rocha W, Coulon S, Fuchs RP. Increase in dNTP pool size during the DNA damage response plays a key role in spontaneous and induced-mutagenesis in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 2011; 108:19311–6. [PubMed: 22084087]
98. Hakansson P, Hofer A, Thelander L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem*. 2006; 281:7834–41. [PubMed: 16436374]
99. Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol*. 2007; 8:947–56. [PubMed: 18000527]
100. Lee M-T, Bachant J. SUMO MODIFICATION OF DNA TOPOISOMERASE II: TRYING TO GET A CENSe OF IT ALL. *DNA repair*. 2009; 8:557–568. [PubMed: 19230795]
101. Mohan RD, Rao A, Gagliardi J, Tini M. SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment. *Mol Cell Biol*. 2007; 27:229–43. [PubMed: 17060459]

102. Gali H, et al. Role of SUMO modification of human PCNA at stalled replication fork. *Nucleic Acids Res.* 2012; 40:6049–59. [PubMed: 22457066]
103. Verger A, Perdomo J, Crossley M. Modification with SUMO. A role in transcriptional regulation. *EMBO Rep.* 2003; 4:137–42. [PubMed: 12612601]
104. Lyst MJ, Stancheva I. A role for SUMO modification in transcriptional repression and activation. *Biochemical Society transactions.* 2007; 35:1389–1392. [PubMed: 18031228]
105. Rosonina E, Duncan SM, Manley JL. SUMO functions in constitutive transcription and during activation of inducible genes in yeast. *Genes Dev.* 2010; 24:1242–52. [PubMed: 20504900]
106. Srikumar T, et al. Global analysis of SUMO chain function reveals multiple roles in chromatin regulation. *J Cell Biol.* 2013; 201:145–63. [PubMed: 23547032]
107. Bischof O, et al. The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis. *Mol Cell.* 2006; 22:783–94. [PubMed: 16793547]
108. Nacerddine K, et al. The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. *Dev Cell.* 2005; 9:769–79. [PubMed: 16326389]
109. Mattoscio D, Casadio C, Fumagalli M, Sideri M, Chiocca S. The SUMO conjugating enzyme UBC9 as a biomarker for cervical HPV infections. *Ecancermedicallscience.* 2015; 9:534. [PubMed: 26015803]
110. Ronen O, et al. Expression of a novel marker, Ubc9, in squamous cell carcinoma of the head and neck. *Head Neck.* 2009; 31:845–55. [PubMed: 19309722]
111. Mattoscio D, Chiocca S. SUMO pathway components as possible cancer biomarkers. *Future Oncol.* 2015; 11:1599–610. [PubMed: 26043214]
112. Nagaria P, et al. SMUG1 but not UNG DNA glycosylase contributes to the cellular response to recovery from 5-fluorouracil induced replication stress. *Mutat Res.* 2013; 743-744:26–32. [PubMed: 23253900]
113. Sun Z, et al. Overexpression of SENP3 in oral squamous cell carcinoma and its association with differentiation. *Oncol Rep.* 2013; 29:1701–6. [PubMed: 23467634]
114. Ding X, et al. Overexpression of SENP5 in oral squamous cell carcinoma and its association with differentiation. *Oncol Rep.* 2008; 20:1041–5. [PubMed: 18949399]
115. Tan M, et al. SENP2 regulates MMP13 expression in a bladder cancer cell line through SUMOylation of TBL1/TBLR1. *Sci Rep.* 2015; 5:13996. [PubMed: 26369384]
116. Tan MY, et al. SUMO-specific protease 2 suppresses cell migration and invasion through inhibiting the expression of MMP13 in bladder cancer cells. *Cell Physiol Biochem.* 2013; 32:542–8. [PubMed: 24008762]
117. Yu B, et al. Oncogenesis driven by the Ras/Raf pathway requires the SUMO E2 ligase Ubc9. *Proc Natl Acad Sci U S A.* 2015; 112:E1724–33. [PubMed: 25805818]
118. Hirohama M, et al. Spectomycin B1 as a novel SUMOylation inhibitor that directly binds to SUMO E2. *ACS Chem Biol.* 2013; 8:2635–42. [PubMed: 24143955]
119. He X, et al. Characterization of the loss of SUMO pathway function on cancer cells and tumor proliferation. *PLoS One.* 2015; 10:e0123882. [PubMed: 25860128]
120. Xu J, et al. SENP1 inhibition induces apoptosis and growth arrest of multiple myeloma cells through modulation of NF-kappaB signaling. *Biochem Biophys Res Commun.* 2015; 460:409–15. [PubMed: 25791478]
121. Wang C, et al. Tumor-suppressive microRNA-145 induces growth arrest by targeting SENP1 in human prostate cancer cells. *Cancer Science.* 2015; 106:375–382. [PubMed: 25645686]
122. Di Bacco A, et al. The SUMO-Specific Protease SENP5 Is Required for Cell Division. *Molecular and Cellular Biology.* 2006; 26:4489–4498. [PubMed: 16738315]
123. Gustavson MD, Molinaro AM, Tedeschi G, Camp RL, Rimm DL. AQUA analysis of thymidylate synthase reveals localization to be a key prognostic biomarker in 2 large cohorts of colorectal carcinoma. *Archives of Pathology & Laboratory Medicine.* 2008; 132:1746. [PubMed: 18976010]
124. Wong NA, et al. Nuclear thymidylate synthase expression, p53 expression and 5FU response in colorectal carcinoma. *Br J Cancer.* 2001; 85:1937–43. [PubMed: 11747337]

125. Yuan TT, et al. Nuclear translocation of dihydrofolate reductase is not a pre-requisite for DNA damage induced apoptosis. *Apoptosis*. 2009; 14:699–710. [PubMed: 19360472]
126. Budai B, et al. Impact of SHMT1 polymorphism on the clinical outcome of patients with metastatic colorectal cancer treated with first-line FOLFIRI+bevacizumab. *Pharmacogenet Genomics*. 2012; 22:69–72. [PubMed: 22044939]
127. Barcelos A, et al. Polymorphisms correlated with the clinical outcome of locally advanced or metastatic colorectal cancer patients treated with ALIRI vs. FOLFIRI. *Pteridines*. 2013; 24:69–79.

Figure 1. Overview of folate-mediated one-carbon metabolism

Folate carries one-carbon units required for de novo thymidylate synthesis, de novo purine synthesis, and methylation reactions. THF, tetrahydrofolate; DHF, dihydrofolate; dUMP, deoxyuracil monophosphate; dTMP, deoxythymidine monophosphate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MTHFD1, methylenetetrahydrofolate dehydrogenase; (S), synthase; (C), cyclohydrolase; (D), dehydrogenase; SHMT1, serine hydroxymethyltransferase; TYMS, thymidylate synthase; DHFR, dihydrofolate reductase; MTHFR, methylenetetrahydrofolate reductase.

Figure 2. Known and potential mechanisms of 5-FU mediated cell death

5-FU can be incorporated into DNA, RNA, or bind to TYMS, increasing uracil in DNA. It is unknown whether FdUMP binding impairs TYMS nuclear localization. It is also unknown how TYMS inhibition and impaired formation of the complex of *de novo* dTMP synthesis enzymes each contribute to uracil incorporation in DNA. Noncoding RNA refers to snRNA and rRNA incorporation, as described in section 3.3. 5-FU, 5-fluorouracil; 5-FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuracil monophosphate; FdUDP, 5-fluorodeoxyuracil diphosphate; FdUTP, 5-fluorodeoxyuracil triphosphate; FUMP, 5-fluorouracil monophosphate; FUDP, 5-fluorouracil diphosphate; FUTP, 5-fluorouracil triphosphate; OPRT, orotate phosphoribosyltransferase; UMPK, uridine monophosphate kinase; UDPK, uridine diphosphate kinase; TK1, thymidine kinase 1; TMPK, thymidylate kinase; NDPK, Nucleoside diphosphate kinase; TYMS, thymidylate synthase.