

REVIEW

Mechanisms of genome instability from cellular folate stress

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Folate is the key cofactor in one-carbon metabolism, a universal metabolic pathway crucial for supporting the biosynthesis of nucleotides, several amino acids, and key redox regulators. Mammals are unable to synthesize folate de novo, and folate deficiency can result from several causes, including restricted dietary intake, genetic defects in folate absorption and its metabolism, and exposure to antimetabolite drugs. The link between depletion of folates and genetic instability has long been the subject of research and is implicated in the pathogenesis of human diseases associated with folate deficiency. In this review, we will discuss the different genotoxic mechanisms arising from folate deficiency and the impact on genome stability. Increasing our understanding of this topic is crucial for interpreting possible links between genetic instability downstream of folate stress and the healthcare impact of folate deficiency.

Introduction

In 1880, the renowned hematologist and histopathologist Dr. Paul Ehrlich used the term “megaloblasts” to describe bone marrow cells with abnormally large nuclei in patients with vitamin B12 or folate deficiency (Ehrlich, 1898; Ehrlich, 1910). The morphological characterization of megaloblasts was one of the earliest pieces of evidence that link perturbed one-carbon metabolism with genomic instability. We now understand these megaloblasts as red cell precursors with immature nuclei caused by incompletely replicated DNA.

The one-carbon cycle plays an essential role in the synthesis of substrates for DNA metabolism and homeostasis (Fox and Stover, 2008; Ducker and Rabinowitz, 2017). These substrates include the de novo synthesis of deoxythymidine monophosphate (dTMP) and purine precursors for DNA replication and repair and S-adenosylmethionine (SAM) for genome methylation reactions. To enable the cycling of one-carbon units, folates shuttle single carbons as methyl and formyl moieties to their intended substrates, while vitamin B12 is an enzyme cofactor that catalyzes the recycling of 5-methyl-tetrahydrofolate (5-methylTHF) to tetrahydrofolate (THF) (Fig. 1). Depletion of either folates or vitamin B12 can result in disruption of one-carbon metabolism. Multiple studies using a range of model systems, including cell lines, animal models, and human subjects, have demonstrated that disruption of one-carbon metabolism results in genotoxicity (Fenech, 2001; Duthie et al., 2002). Folate deficiency has, for example, been observed to lead to abnormal nuclear morphology and structures that includes micronuclei, nuclear buds, and nucleoplasmic bridges, which can be caused

by DNA damage (Everson et al., 1988; Macgregor et al., 1997; Beetstra et al., 2005; Leopardi et al., 2006; Lindberg et al., 2007; LeBlanc et al., 2018). At the chromosomal level, folate deficiency has also been associated with increased chromosome breaks and structural alterations (Menziez et al., 1966; Heath, 1966), aneuploidy (Wang et al., 2004; Beetstra et al., 2005), and increased sister chromatid exchanges, which are markers of homology-directed repair (Knuutila et al., 1978). Furthermore, studies using molecular techniques have directly measured increased DNA double-strand breaks (DSB) in folate-deficient cells (James and Yin, 1989; Duthie and Hawdon, 1998; Melnyk et al., 1999; Lamm et al., 2015).

The most extensively studied mechanism of genotoxicity from folate deficiency arises from disrupted dTMP synthesis that leads to increased uracil misincorporation into DNA during replication. Advances over the last decade in sensitive and high-throughput technologies in DNA damage detection and whole-genome sequencing have revealed novel insights into how folate deficiency impacts genome biology beyond uracil misincorporation. These insights include an understanding of how specific regions of the genome and chromosome are more vulnerable to DNA damage, a greater appreciation of the vulnerability of the mitochondrial genome, and an understanding of how DNA damage in different tissues drives degeneration and disease pathology. Despite national food programs in several countries to fortify core foods with folic acid (MRC Vitamin Study Research Group, 1991; Kancherla et al., 2022; US Preventative Services Task Force, 2023), folate deficiency remains a public health problem. Populations that show an increased risk of

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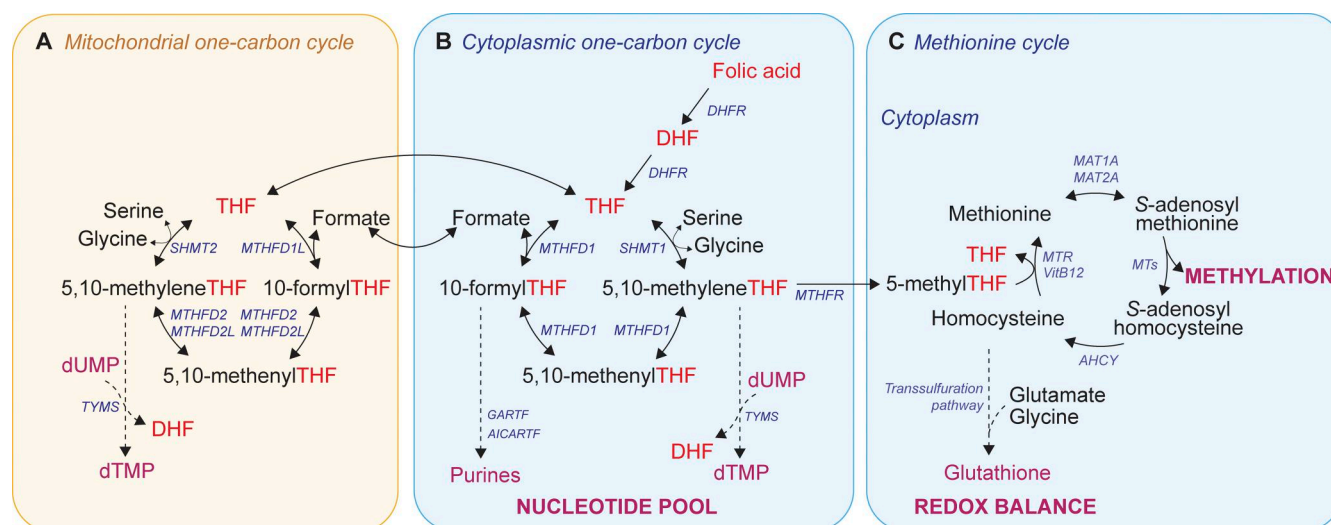


Figure 1. **Folate-dependent one-carbon cycle.** (A and B) Outline of the major steps within the one-carbon cycle that shuttles single-carbon units on different folate metabolites in the mitochondria (A) and the cytoplasm (B). (C) The folate-dependent one-carbon cycle is intrinsically linked to the methionine cycle through 5-methylTHF. The major products of the one-carbon cycle are nucleotides for DNA replication and maintenance, SAM as the methyl donor for methylation reactions, and homocysteine contributes to glutathione synthesis via the transsulfuration pathway. AHCY, adenosylhomocysteinase; AICARTF, aminoimidazolecarboxamide ribonucleotide transformylase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dUMP, deoxyuridine monophosphate; GARTF, glycinamide ribonucleotide transformylase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFD1L, methylenetetrahydrofolate dehydrogenase 1-like; MTHFR, methylene-tetrahydrofolate reductase; MTR, methionine synthase; MTs, methyltransferases; SHMT, serine hydroxymethyltransferase; TYMS, thymidylate synthase; dUMP, deoxyuracil monophosphate.

folate deficiency include pregnant people, people with alcohol dependence, those with malabsorption disorders, and those on antifolate therapy—which remains a common treatment modality in cancer chemotherapy and immunosuppression (Kovalev et al., 2022). Therefore, there remains a need to better understand the diverse mechanisms of genetic instability induced by folate deficiency and the impact on health. This review seeks to address the question of the cellular and biochemical mechanisms that link folate deficiency to DNA damage.

Mechanisms of genetic instability associated with folate deficiency

Nucleotide pool imbalance and genomic uracil misincorporation

Folate acts as a one-carbon donor in the de novo synthesis of the deoxynucleotide triphosphate substrates of DNA replication. In particular, the synthesis of the pyrimidine nucleotide thymidine proceeds through methylating deoxyuridine monophosphate (dUMP) to dTMP by thymidylate synthase, which utilizes 5,10-methylene-tetrahydrofolate (5,10-methyleneTHF) as the one-carbon donor (Fig. 1, A and B). Folate deficiency therefore limits nucleotide synthesis necessary to support DNA replication and repair. While nucleotide salvage pathways can provide a limited alternative source of purines, cytidine, and uridine, ultimately folate is still essential for the conversion of uridine to thymidine. Therefore, the most striking change in the nucleotide pool following folate deficiency is an increase in the dUMP/dTMP ratio (James et al., 2003; Tattersall and Harrap, 1973; Tattersall et al., 1973). Despite showing a preference for thymidine, uracil can be incorporated by DNA polymerase enzymes into DNA during replication (Bessman et al., 1958; Andersen

et al., 2005; El-Hajj et al., 1992), leading to accumulation of genomic uracil in folate-deficient cell lines, animal models, and human patients (Goulian et al., 1980; Duthie and Hawdon, 1998; Andersen et al., 2005; Duthie et al., 2000a; Duthie et al., 2000b; Blount et al., 1997; Luzatto et al., 1981; Wickramasinghe and Fida, 1994). As will be discussed later, mitochondrial DNA is also susceptible to uracil misincorporation. The blood and bone marrow of folate-normal individuals contain ~500,000 uracils per human diploid genome, but this increases by eight- to ninefold in folate-deficient individuals (Blount et al., 1997). To counter this pervasive incorporation of uracil into DNA, organisms have evolved DNA repair mechanisms to remove uracil through the activity of a family of uracil DNA glycosylases (UDGs) in coordination with base excision repair (BER) (Schormann et al., 2014; Krokan and Bjørås, 2013). Mammals possess multiple UDGs with distinct cellular locations and functions. The Uracil-DNA glycosylase (UNG) gene encodes two isoforms: mitochondrial UNG1 and nuclear UNG2. Three additional UDGs—Single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), Thymine DNA glycosylase (TDG), and Methyl-CpG binding domain protein 4 (MBD4)—are found exclusively in the nucleus (Visnes et al., 2008). The main role of UDGs is to directly excise uracil from the DNA backbone to form an abasic (AP) site. Among the nuclear UDGs, UNG2 is the primary enzyme responsible for uracil excision, particularly during DNA replication (Kavli et al., 2002). Following excision, cleavage of the AP site by the AP endonuclease APE1 creates a single-strand break, which can be filled either through short patch BER (generation and filling of a single nucleotide gap by DNA polymerase β) or long patch BER (where a 2–10 nucleotide gap is generated and filled) (Krokan and Bjørås, 2013).

How does the accumulation of genomic uracil lead to DNA damage? The genotoxic consequence of unrepaired genomic uracil has been studied using UDG-deficient *UNG*^{-/-} human cell lines that are unable to excise genomic uracil. When these UNG-deficient cells are treated with antifolate pemetrexed, genomic uracil accumulates and is correlated with increased markers of DNA damage, including γ H2AX, increased DNA strand breaks measured by the alkaline comet assay, cell cycle arrest, and apoptosis (Bulgar et al., 2012; Weeks et al., 2013; Weeks et al., 2014). In support of these findings using cell lines, folate-deficient *Ung*^{-/-} mice exhibited increased mitochondrial DNA deletions in the brain with selective loss of CA3 pyramidal neurons in the hippocampus (Kronenberg et al., 2011). By fluorescently labeling newly synthesized DNA in dividing cells, increased genomic uracil has been shown to delay DNA polymerase progression (Bulgar et al., 2012; Saxena et al., 2024). The stalling of DNA polymerase behind the DNA helicase at the replication fork reveals a long tract of single-stranded DNA (ssDNA) coated with replication protein A. This replication protein A-ssDNA accumulation recruits a primase enzyme Primase and polymerase (PRIMPOL) to reprime downstream of the DNA replication impediment, leaving an ssDNA gap in the newly synthesized DNA. If the PRIMPOL-mediated ssDNA gap is not repaired prior to the next cell cycle, the replication fork that encounters the persistent single-stranded gap can collapse to form a DNA DSB. In support of this, increased DSBs were observed in UNG-deficient cells when the resolution of the ssDNA gaps generated by PRIMPOL-mediated repriming was impaired through treatment with an Ataxia telangiectasia and Rad3-related protein (ATR) inhibitor. Conversely, the genetic knock-down of PRIMPOL in UNG-deficient cells was able to rescue the sensitivity to ATR inhibition, consistent with the PRIMPOL-dependent formation of DSBs (Saxena et al., 2024).

The outcomes of uracil repair can paradoxically lead to genotoxicity through several mechanisms. First, when thymidine becomes limiting, BER can enter futile cycles. In these cycles, uracil is excised but immediately reincorporated due to thymidine depletion in the nucleotide pool (Goulian et al., 1980). This repetitive process results in persistent DNA strand breaks, continuous activation of the DNA damage response, and ultimately apoptosis. Second, BER can generate DSBs when processing closely spaced uracils. When two uracils occur on opposite DNA strands, their excision creates opposing AP sites. APE1 then incises both sites to form single-strand breaks that, due to their proximity, spontaneously form a DSB. While this mechanism has been proposed to contribute to DNA breaks in folate-deficient cells (Blount et al., 1997), this mutagenic process is essential for immune function. During antibody class switch recombination, activated B lymphocytes express activation-induced cytidine deaminase, which converts cytosines to uracils at high density within immunoglobulin genes. The resulting DSBs generated via BER trigger intrachromosomal translocations that generate different antibody isotypes (Schrader et al., 2005; Stavnezer et al., 2008). A third genotoxic mechanism involves the chemical reactivity of BER intermediates. The deoxyribose sugar at AP sites can exist as an open-chain aldehyde (Wilde et al., 1989), which is highly reactive and

capable of forming both DNA interstrand cross-links (Price et al., 2014) and DNA-protein cross-links (Yudkina et al., 2023). Studies using polymerase β heterozygous mice, which have impaired BER completion and thus accumulate genotoxic intermediates, demonstrate this mechanism in vivo. When fed a folate-deficient diet, these mice exhibit elevated single-strand breaks and aldehydic DNA lesions (Cabelof et al., 2003; Cabelof et al., 2004).

Altogether, which mechanism of uracil genotoxicity underpins the DNA damage observed during folate deficiency? Under folate-deficient experimental conditions, the consensus indicates that the loss of uracil glycosylase results in elevated replication stress and chromosome breakages, thus indicating that it is the accumulation of genomic uracil that is more genotoxic than uracil excision and BER repair. In contrast, the mechanism of DNA damage arising from BER repair of uracil likely requires a very high concentration of genomic uracil localized within small genomic region. While this is physiologically possible through targeted enzymatic cytidine deamination at the immunoglobulin locus, folate deficiency may not trigger the same concentration of localized genomic uracil misincorporation.

Elevation of oxidative DNA damage

One key consequence of folate deficiency is the perturbation of cellular redox balance, leading to increased production of reactive oxygen species that can directly modify DNA bases (Markkanen, 2017; Poetsch, 2020). This oxidative stress represents another pathway through which folate deficiency promotes genomic instability. Evidence from both animal models and human studies demonstrates that folate deficiency elevates oxidative DNA damage. In animal models, folate-deficient mice subjected to cerebral ischemia show increased oxidative DNA damage signatures in brain tissue (Endres et al., 2005), while lymphocytes from folate-deficient rats exhibit elevated levels of 8-oxoguanine, a common oxidative DNA lesion (Duthie et al., 2010). Human studies corroborate these findings. Folate-deficient individuals display increased 8-oxoguanine in their urine and mitochondrial DNA from peripheral blood cells (Wang et al., 2012; Lv et al., 2019). In response to this oxidative challenge, cells upregulate DNA repair pathways during folate deficiency. Notably, 8-oxoguanine DNA glycosylase 1 (OGG1), which excises 8-oxoguanine from DNA, shows increased activity under folate-deficient conditions (Duthie et al., 2010).

Several mechanisms may contribute to oxidative stress during folate deficiency. First, folic acid and its derivatives possess direct antioxidant activity, acting as free radical scavengers (Joshi et al., 2001; Rezk et al., 2003; Gliszczynska-Swiglo, 2006; Gliszczynska-Swiglo, 2007). Second, folate deficiency leads to homocysteine accumulation, which independently promotes reactive oxygen species generation through multiple pathways: increased NADPH oxidase expression, decreased thioredoxin antioxidant protein (Tyagi et al., 2005), and mitochondrial dysfunction (Chen et al., 2017; Deep et al., 2024). Third, folate deficiency directly impairs mitochondrial integrity through disruption of mitochondrial one-carbon metabolism and mitochondrial DNA damage, independent of homocysteine effects

(see section on Mitochondrial DNA damage). Another proposed mechanism involves glutathione synthesis, though evidence for this pathway remains controversial. The one-carbon cycle generates metabolites theoretically important for synthesizing glutathione, a critical cellular antioxidant. These include glycine (generated from serine by serine hydroxymethyltransferase [SHMT] enzymes; Fig. 1 B) and cysteine (generated from homocysteine via transsulfuration; Fig. 1 C) (Ducker et al., 2016; Vitvitsky et al., 2006; Yoon et al., 2023). However, in vivo tracing experiments have challenged the physiological significance of this connection. When mice were traced with [¹³C]-serine, only a small fraction of glutathione in liver, pancreas, and kidney utilized glycine or cysteine generated from one-carbon metabolism (Yoon et al., 2023), suggesting that glutathione synthesis predominantly relies on dietary sources of these amino acids. Supporting this disconnect, folate deficiency fails to decrease glutathione levels across multiple models: cell lines (Maynard et al., 2024), rodents (Chang et al., 2007; Martínez-Vega et al., 2015), and porcine systems (Halsted et al., 2002). Furthermore, when SHMT is inhibited or genetically deleted in the liver, glycine uptake from the circulation can maintain glutathione synthesis (Ghrayeb et al., 2024; McBride et al., 2024).

Impacts of disrupted methylation on genome stability

Folate metabolism feeds the methionine cycle (Fig. 1 C), where methionine synthase transfers a methyl group from 5-methylTHF to homocysteine to synthesize methionine. Methionine is the critical precursor required to synthesize the cellular methyl donor SAM through the activity of methionine adenosyltransferase enzymes MAT1A/MAT2A. A major use of SAM is for methylation of cytosine in genomic DNA to 5-methylcytosine by methyltransferase enzymes. 5-methylcytosine acts as an important epigenetic mechanism used to downregulate gene expression (Mattei et al., 2022; Lister et al., 2009; Angeloni and Bogdanovic, 2021). While cellular methionine can also be obtained via uptake from exogenous dietary sources, this appears to be insufficient to support the cellular demand for SAM, as evidenced by decreased SAM levels in dietary and genetic models of folate deficiency (Balaghi et al., 1993; Kim et al., 1994; Miller et al., 1994; Kim et al., 1997; Chen et al., 2001; MacFarlane et al., 2009). Consequently, folate deficiency has been consistently observed to cause DNA hypomethylation in cell lines (Duthie et al., 2000b; Wasson et al., 2006), animal models (James et al., 2003; Linhart et al., 2009; Wainfan and Poirier, 1992; Pogribny et al., 1995; Padmanabhan et al., 2013; Bertozzi et al., 2021), and humans (Jacob et al., 1998; Rampersaud et al., 2000; Pufulete et al., 2005). Parental deficiency in folate, other B vitamins, and methionine also leads to hypomethylated DNA in the offspring of animal models (Cao et al., 2023; Sinclair et al., 2007; Kim et al., 2009; McKay et al., 2011).

There is a compelling body of work that links DNA hypomethylation with chromosomal instability. Genetic inactivation of the major DNA methyltransferase enzyme, DNMT1, results in increased aneuploidy, mitotic defects, and abnormal nuclear morphology (Chen et al., 1998; Eden et al., 2003; Karpf and Matsui, 2005; Chen et al., 2007; Sheaffer et al., 2016; Besselink

et al., 2023). Humans harboring homozygous inactivating mutations in DNMT3B, another DNA methyltransferase, are afflicted with ICF syndrome (named for immunodeficiency, centromere instability, and facial anomalies), which is characterized by hypomethylated CpG sites in pericentromeric regions that cause frequent breakage of chromosomes 1, 9, and 16 (Xu et al., 1999; Hansen et al., 1999). Loss of DNMT1, DNMT3a, and 3b also leads to hypomethylation at sub-telomeric regions that correlate with increased telomeric recombination and perturbed telomere length (Gonzalo et al., 2006). In support of the chromosomal instability phenotype from loss of DNMT activity, treatment of cells with the DNMT inhibitor 5-aza-2'-deoxycytidine results in elevated chromatin decondensation, aneuploidy, and chromosome breakages (Haaf, 1995; Costa et al., 2016). Loss of methylation can also lead to transcriptional reactivation of transposable repetitive elements in the genome, such as Long interspersed nuclear element-1 (LINE-1), that makes multiple copies of itself via reverse transcription and disrupt the genome through insertions, deletions, and rearrangements (Jönsson et al., 2019; Almeida et al., 1993; Daskalos et al., 2009). Furthermore, hypomethylated regions within the genome are correlated with increased formation of R-loops (Nadel et al., 2015), RNA-DNA hybrids formed during transcription that can promote DNA damage (García-Muse and Aguilera, 2019).

The well-established links between DNA hypomethylation and DNA damage raise an important question: does the disrupted DNA methylation caused by folate deficiency lead to genetic instability? With respect to telomere length, folate deficiency in the human WIL2-NS cell line caused global DNA hypomethylation, which was associated with abnormal elongation of telomeres (Bull et al., 2014). Interestingly, the effect size on telomere length from folate deficiency was similar to that induced by a DNA methyltransferase inhibitor, suggesting that DNA hypomethylation may play a causative role. Regarding LINE-1 transposon methylation, a study of 177 women found a correlation between low oral folate intake and decreased methylation of LINE-1 elements, although the participants' serum folate levels were not measured in this study (Agodi et al., 2015). A separate study found decreased global and LINE-1 DNA methylation in stillborn case subjects with neural tube defects (Wang et al., 2010b). Additionally, diet-induced folate deficiency in mice resulted in decreased levels of SAM with hypomethylation both globally across the genome and at LINE-1 elements (Lu et al., 2022). Furthermore, the authors of this study found that dietary folic acid supplementation was able to restore the methylation of LINE-1 elements. Beyond DNA hypomethylation, folate deficiency has been shown to cause increased Ten-eleven translocation 2 (TET2)-mediated generation of 5'-hydroxymethylcytosine in mouse embryonic stem cells (Wang et al., 2022a), an epigenetic modification that has been observed to coincide with an enrichment of DNA single-strand breaks (Wu et al., 2021). There is also evidence that methotrexate treatment of mouse embryonic stem cells induces DSBs that are enriched at hotspots of H3K4me1 histone methylation (Xie et al., 2017), raising the possibility that histone methylation could play a role in genomic instability during folate depletion. Overall, several lines of evidence suggest that folate stress-

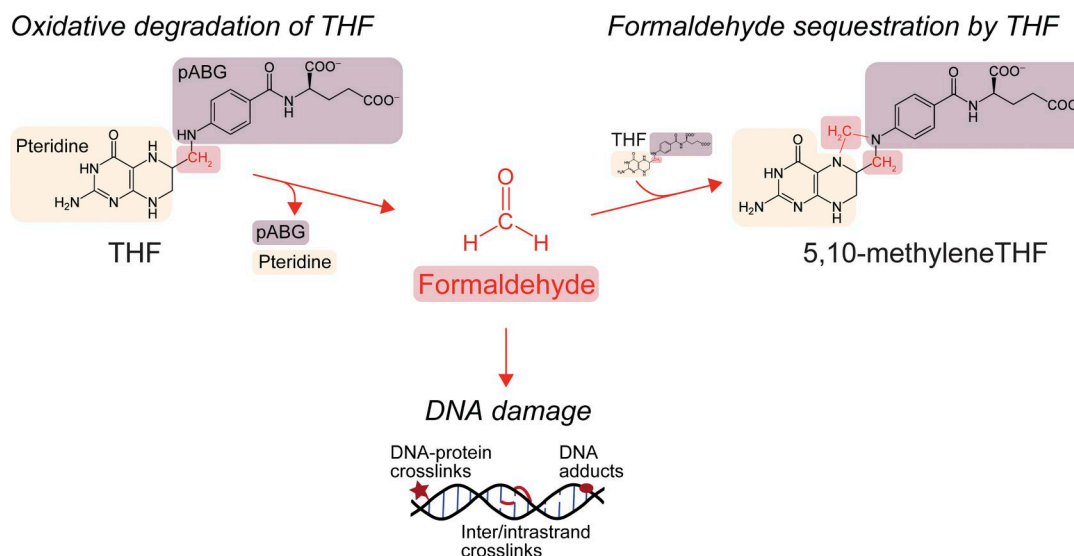


Figure 2. **Links between folate metabolism and formaldehyde.** The genotoxin formaldehyde and the folate-dependent one-carbon cycle are intrinsically linked. THF (as well as dihydrofolate and 5,10-methyleneTHF) are susceptible to oxidative degradation to release its constituents: pterine, para-aminobenzoylglutamate (pABG), and formaldehyde. Formaldehyde can directly damage DNA through the formation of a number of DNA lesions. THF can sequester formaldehyde to form 5,10-methyleneTHF.

induced DNA hypomethylation can impact telomere biology and LINE-1 transposon reactivation. Further studies are needed to better establish how much of the genome instability that arises from folate deficiency is attributable to hypomethylation.

Link between folate one-carbon metabolism and formaldehyde genotoxicity

Formaldehyde is commonly used as an industrial chemical but is also produced endogenously in mammals (Dingler et al., 2020; Wang et al., 2022b) at levels that can cause DNA damage (Pontel et al., 2015; Dingler et al., 2020; Swenberg et al., 2013). To counteract this genotoxic threat, cells rely on formaldehyde detoxification pathways and DNA repair mechanisms, which have been extensively reviewed elsewhere (Wang et al., 2022b; Valverde-Santiago and Pontel, 2025). The critical importance of these protective pathways is evident from genetic studies. Both humans and animal models with deficiencies in formaldehyde protection develop severe phenotypes, including early onset cancers and accelerated aging (Pontel et al., 2015; Mulderrig et al., 2021; Wang et al., 2023a; Oka et al., 2020). These observations raise a fundamental question: what metabolic processes determine endogenous formaldehyde generation? This question is particularly relevant to folate metabolism, as the chemistry of folate is intrinsically linked with formaldehyde production and processing.

The methylene bridge that links the pteridine ring and the para-aminobenzoylglutamate moiety in certain folate metabolites—specifically THF, 5,10-methyleneTHF, and DHF—is susceptible to oxidative decomposition, releasing formaldehyde as a byproduct (Fig. 2) (García-Calderón et al., 2018; Chippel and Scrimgeour, 1970; Burgos-Barragan et al., 2017). Excess THF supplementation to cell lines has been shown to increase formaldehyde levels and induce genotoxicity (Burgos-Barragan et al., 2017; García-Calderón et al., 2018). However,

whether increased dietary folate supplementation causes elevated endogenous formaldehyde in vivo has not been determined. In vivo, additional mechanisms may operate to protect intracellular THF from oxidative decomposition. The intracellular level of THF may be kept low by rapid conversion to the decomposition-resistant 10-formylTHF (Zheng et al., 2018). Furthermore, the Quinoid dihydropteridine reductase (QDPR) enzyme has been shown to repair partially oxidized THF to prevent irreversible decomposition (Zheng et al., 2018). Beyond its role as a toxic byproduct, formaldehyde may also function as a metabolic regulator. Recent evidence suggests formaldehyde alters MAT1A activity and expression (Pham et al., 2023), potentially creating a feedback mechanism between folate decomposition and one-carbon metabolism. However, the physiological significance of this regulatory connection requires further investigation.

If unstable folate derivatives are a source of formaldehyde, reducing their abundance should logically decrease endogenous formaldehyde levels. While studies have found that inhibition or genetic deletion of SHMT enzymes in cancer cell lines are associated with decreased formaldehyde (Vekariya et al., 2023; Tenney et al., 2024), it remains unclear if this is caused by depletion of the unstable folate metabolites. The uncertainty arises because disrupting folate metabolism affects multiple cellular pathways, any of which could indirectly influence formaldehyde levels. For example, the enzymatic demethylation of nucleic acids and proteins have been shown to generate formaldehyde in vitro (Porter et al., 1985; Hopkinson et al., 2010; Duncan et al., 2002; Shi et al., 2004; Gerken et al., 2007). Since folate deficiency leads to cellular hypomethylation, this would reduce substrate availability for demethylation reactions and consequently decrease formaldehyde production through this alternative pathway. Another important intersection between folate and formaldehyde biology is the ability of THF to directly bind

formaldehyde to form 5,10-methyleneTHF (Fig. 2). This mechanism is utilized by methylotrophic bacteria to capture formaldehyde derived from methanol as a carbon source to support growth (Crowther et al., 2008; Müller et al., 2015). However, it is not clear whether THF assimilation of formaldehyde is a significant sequestration mechanism in mammals. Several mammalian enzymes do indeed utilize THF as a cofactor to capture formaldehyde generated as metabolic byproducts during glycine cleavage and demethylation reactions (Luka et al., 2011; Porter et al., 1985; Kikuchi et al., 2008). Therefore, it is hypothetically plausible that depletion of THF in mammalian cells can increase endogenous formaldehyde (Garcia et al., 2016). However, current studies have not addressed how much formaldehyde can be generated by these enzymatic reactions in vivo. Furthermore, it is not clear if the extra formaldehyde generated by these enzymes could overwhelm the robust formaldehyde detoxification enzymes Alcohol dehydrogenase 5 (ADH5) and Acetaldehyde dehydrogenase 2 (ALDH2) (Dingler et al., 2020; Pontel et al., 2015) and millimolar concentration of intracellular glutathione that can sequester formaldehyde (Umansky et al., 2022; Rosado et al., 2011).

Folate deficiency impairs the response to DNA damage

Folate deficiency can indirectly increase the burden of DNA damage by sensitizing cells to DNA alkylation (Duthie et al., 2000b; Branda et al., 2001), oxidation (Duthie and Hawdon, 1998; Duthie et al., 2000b; Wang et al., 2021), and damage from ionizing radiation (Beetstra et al., 2005). A mechanism by which folate deficiency increases the toxicity of DNA-damaging agents is through the inhibition of DNA repair. For example, Pol β , the DNA polymerase that mediates the terminal step of BER, is upregulated in response to oxidative stress, but this upregulation is not activated during folate deficiency (Cabelof et al., 2004; Unnikrishnan et al., 2011). Folate-depleted cells exhibit altered binding of regulatory factors to the Pol β promoter, thus resulting in decreased Pol β gene transcription. Another DNA repair protein, Rad54, a key player in homologous recombination, is reduced in folate-deficient mice due to increased Rad54 promoter methylation (Wang et al., 2021). Other studies have linked folate deficiency with induction of DNA strand breaks in a conserved region of the P53 gene, thus inhibiting the activation of DNA damage response (Kim et al., 2000; Crott et al., 2004; Kim et al., 1997). Folate deficiency does not globally impair all DNA repair pathways. Several DNA repair enzymes are upregulated following folate deficiency. For example, the expression of UDG (Cabelof et al., 2004), OGG1, and methylguanine methyltransferase (Duthie et al., 2010) with respective roles in the repair of uracil, 8-oxoguanine, and O⁶-methyl-guanine lesions have been observed to increase in folate-deficient rodent tissues. However, it is worth noting that regulation of DNA repair typically occurs at the level of posttranslational modification of DNA repair proteins rather than at the level of gene transcription. In fact, multiple whole-transcriptome analyses by RNA sequencing in folate-depleted human hematopoietic cell lines (Maynard et al., 2024), murine embryonic stem cells (Pei et al., 2019; Wang et al., 2022a), and murine neural stem cells (Xu et al., 2022) do not reveal

significant changes in the expression of DNA repair genes. Therefore, it would be interesting to leverage recent advancements in phosphoproteomic mass spectrometry to characterize the protein signaling events in DNA damage response upon folate deficiency (Faca et al., 2020).

Consequences of folate-induced DNA damage

Following on from our discussion of the different mechanisms of DNA damage that arise from folate deficiency, the following section focuses on the consequences of such DNA damage on genome biology (Fig. 3).

Replication stress and folate-sensitive fragile sites

A major consequence of folate deficiency, which includes unbalanced deoxynucleotide triphosphate pools characterized by an elevated Deoxyuridine triphosphate/Deoxythymidine triphosphate (dUTP/dTTP) ratio (James et al., 1994; van der Weyden et al., 1991; James et al., 1993; Oliver et al., 1997; James et al., 1992; James et al., 1997), is the induction of replication stress (Lamm et al., 2015; Saxena et al., 2024; Bonagas et al., 2022), defined as the slowing of DNA replication and the stalling of replication forks (Gaillard et al., 2015). This section will focus on how replication stress impacts specific genomic loci, which are intrinsically vulnerable to chromosomal instability upon exposure to folate stress.

A small proportion of the population (<2%) harbor heritable polymorphisms at specific genomic loci that exhibit elevated chromosomal instability in response to folate depletion (Kähkönen et al., 1989). Collectively, there are currently 24 identified unstable genomic loci in the human genome known as folate-sensitive fragile sites (Durkin and Glover, 2007; Lokanga et al., 2021). The fragility of the chromosomes from these individuals can be observed by culturing their lymphocytes in folate-depleted media, which causes dramatic cytogenetic abnormalities affecting chromosomes that carry the fragile site, including in chromosomes 2, 10, 11, 16, 20, and X (Sutherland, 1977; Sutherland, 1979a; Sutherland, 1979b). The most widely studied folate-sensitive fragile site is the Fragile site, X chromosome, A (FRAXA) locus located in the 5'-untranslated region of the Fragile X mental retardation 1 (*FMRI*) gene on the long arm of the X chromosome. The FRAXA locus is characterized by repetitive CGG sequences that are prone to expansion or shrinkage of the trinucleotide repeats due to inherent slippages by DNA polymerase during DNA replication. Hyperexpansion of CGG repeats at the FRAXA locus is known for causing fragile X syndrome (Lubs, 1969; Hagerman et al., 2017), an inherited form of intellectual disability arising from hypermethylation and transcriptional silencing of the *FMRI* gene (Pieretti et al., 1991; Santoro et al., 2012). However, independently from its role in fragile X syndrome, the hyperexpanded FRAXA locus also has the propensity to form stable DNA secondary structures including hairpins and G-quadruplexes (Usdin and Woodford, 1995; Fry and Loeb, 1994; Chen et al., 1995). In common with other structure-forming repetitive DNA sequences (Murat et al., 2020; Mellor et al., 2022), the DNA secondary structure-forming sequences at the FRAXA locus can impede DNA replication of the *FMRI* gene (Yudkin et al., 2014; Gerhardt et al., 2014). Replication

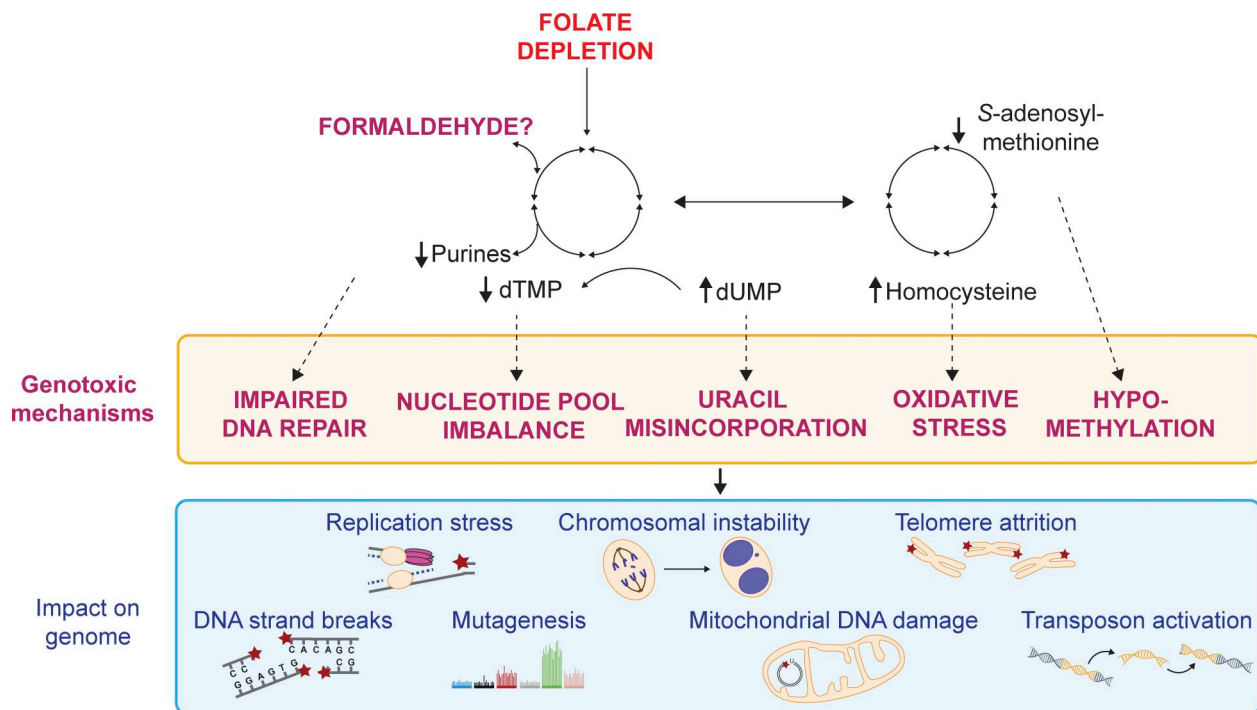


Figure 3. **Mechanisms of genotoxicity and impact on genome arising from folate depletion.** The disruption in the folate one-carbon cycle alters the abundance of nucleotides (purines, dTMP, and dUMP) and metabolites necessary to catalyze methylation reactions (SAM, homocysteine). This triggers different mechanisms of DNA damage that impact both nuclear and mitochondrial genome biology to induce genomic instability. The link between folate depletion and formaldehyde biology remains speculative in mammalian cells, so it is not highlighted as a genotoxic mechanism. dUMP, deoxyuridine monophosphate.

stress induced by folate deficiency further delays DNA replication, causing cells to enter mitosis with incompletely replicated FRAXA locus (Garribba et al., 2020; Lokanga et al., 2021). To compensate, cells with expanded FRAXA repeats activate mitotic DNA synthesis (MiDAS), a rescue mechanism that attempts to complete replication during mitosis (Garribba et al., 2020). In cells that fail to initiate MiDAS, the under-replicated FRAXA locus manifest as a site of mitotic fragility, forming single-stranded anaphase bridges between sister chromatids. These anaphase bridges lead to a cascade of chromosomal defects: aberrant chromatid segregation, fusion events, and chromosome breakage. Ultimately, these mitotic errors produce daughter cells with aneuploidy and micronuclei (Bjerregaard et al., 2018; Garribba et al., 2020). Folate deficiency can lead to chromosomal instability at other genomic loci containing CG-rich trinucleotide repeat sequences. A region at chromosome 2p11.2 that contains more than 300 CG-rich trinucleotide repeats, termed folate deficiency-induced bending 1 (FOLD1), exhibits characteristic “bending” and “kinked” chromosome morphologies and mis-segregation when human cell lines are subjected to folate stress (Garribba et al., 2021). Interestingly, unlike the FRAXA locus, the FOLD1 locus does not undergo MiDAS during folate depletion, indicating variable mechanisms by which folate-dependent replication stress translates to chromosomal instability at different susceptible loci.

Mutagenesis arising from folate deficiency

In this section, we will review how folate deficiency triggers mutagenesis at the nucleotide level, which includes single-

nucleotide substitutions and small insertion/deletions. Due to the inherent technical challenge of detecting rare single-nucleotide mutations, a widely used method to detect elevated mutagenesis in mammalian cells is to assay gene mutations that confer a selectable phenotype. One such in vitro mutation assay performed in cultured cells selects for loss-of-function mutations at the Hypoxanthine phosphoribosyltransferase (HPRT) gene on the X chromosome that confers resistance to 6-thioguanine toxicity. Chinese hamster ovary cells cultured in folate-deficient media for 6–8 days exhibited a 65% increase in the frequency of 6-thioguanine-resistant mutants (Branda et al., 1997). Combining folate deficiency with exogenous alkylating mutagens resulted in synergistic increase in HPRT mutation frequency, with mutation spectra reflecting increased C to T transitions and T to A transversions (Branda et al., 1997; Branda et al., 1999). HPRT mutation assay of lymphocytes isolated from women with breast cancer revealed that women with low serum folate harbored an increased frequency of mutant lymphocytes (Branda et al., 1991; Branda et al., 1992). Mutation assays can also be performed in vivo using rodent models. Deleterious mutations in the Phosphatidylinositol Glycan Anchor Biosynthesis Class A (*Pig-a*) gene on the X chromosome disrupts the encoded glycosyl transferase enzyme essential for synthesizing glycosylphosphatidylinositol anchors that attach several proteins to the surface of red blood cells (as well as other hematopoietic cells). Quantification of the number of blood cells that exhibit loss of glycosylphosphatidylinositol-anchored surface proteins provides an estimate for the frequency of *Pig-a* mutant cells (Dertinger et al., 2011). BALB/c mice fed a folate-deficient diet

exhibited mild elevation in the frequency of *Pig-a* mutant blood cells (MacFarlane et al., 2015), but it was not reproduced in mice with a mixed genetic background, suggesting potential differences in sensitivity of different mouse strains to folate depletion (Diaz et al., 2021).

Both *HPRT* and *Pig-a* mutation assays suffer from low recovery of mutations due to readout using a single gene. Another approach is to introduce multiple copies of non-murine transgenes into the mouse genome to function as transgenic rodent reporter (TGR) models. The Big Blue Mouse (Kohler et al., 1991) and MutaMouse (Gossen et al., 1989; Boerrigter et al., 1995) contain multiple copies of the bacterial *lacI* (Big Blue) and *lacZ* (MutaMouse) gene as mutational targets. These transgenes can be recovered from the murine tissue as a bacteriophage and scored for loss-of-function mutations in the *lacI/lacZ* gene by blue/white 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) stain in *Escherichia coli* host. The major advantages are the increased sensitivity for mutations afforded by the multiple copy number of transgenes; mutations can be assayed from any tissue, and mutant transgenes do not disrupt normal physiology. While single-nucleotide base substitutions at all four bases can be detected robustly, the disadvantages of TGR models are that other mutation types are less well represented, including dinucleotide substitutions and small insertion/deletions (Beal et al., 2020). Furthermore, TGR models are unable to detect “silent” mutations in the reporter gene that do not confer the quantified phenotypic change (Salk and Kennedy, 2020). Both Big Blue Mouse and MutaMouse have been subjected to folate-deficient diet, resulting in 30–50% increase in mutation frequencies in the bone marrow and colon. Mutations were preferentially found at A:T basepairs, which could reflect uracil misincorporation with subsequent error-prone DNA repair that introduces mutations opposite the adenine (LeBlanc et al., 2018; Diaz et al., 2021; Linhart et al., 2009). *Ung*^{−/−} murine embryonic fibroblasts cultured in folate-deficient media had an elevated mutation rate as measured using the Big Blue Mouse (Kronenberg et al., 2008). Notably, the same Big Blue mutation assay did not detect increased mutation rate *in vivo* from brain or colon tissue of *Ung*^{−/−} mice fed 3–8 mo of folate-deficient diet (Kronenberg et al., 2008; Linhart et al., 2009). This discrepancy could be related to faster and continuous cell division in cultured fibroblasts compared with slower cell division in mouse tissues, particularly neurons that are mostly non-dividing.

Mutation rate varies across the genome, being influenced by factors including (but not limited to) local sequence context, transcription, chromatin accessibility, and DNA replication kinetics (Hodgkinson and Eyre-Walker, 2011). The increasing use of improved sequencing technologies has provided opportunities to perform unbiased assessment of how folate deficiency impacts mutagenesis across the entire genome (Salk and Kennedy, 2020). Notably, two studies utilized whole-genome sequencing to study the transgenerational impact of parental folate deficiency on the mutation frequency of progeny offsprings (Zhao et al., 2022; Cao et al., 2023). After maintaining two generations of mice (F0 mice and their F1 offsprings) on a folate-deficient diet, folate-deficient F1 males crossed with folate replete F1 females produced F2 embryos that harbored threefold

increased *de novo* mutation rate (Zhao et al., 2022). Furthermore, mutations at A:T basepairs were increased in the embryos (Cao et al., 2023; Zhao et al., 2022), similar to previous findings using the TGR mice (Diaz et al., 2021; Linhart et al., 2009). These studies highlight the importance of folate sufficiency in both mother and father in protecting the progeny from genomic instability and raises indication to treat paternal folate deficiency prior to conception. It should be noted that increased mutation rates have not been universally observed across all folate-deficient models, with no change in the frequency of single-nucleotide polymorphism (SNPs) or structural variants (SVs) between control and folate-deficient 5-Methyltetrahydrofolate-homocysteine methyltransferase reductase (*Mtrr*) hypomorph embryos (Blake et al., 2021). Interestingly, one study showed that folate deficiency in Hela cell line and mouse lung tissues increased the expression of Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3B (APOBEC3B) and its murine ortholog APOBEC3 (Wu et al., 2023). APOBEC enzymes are cytidine deaminases that generate uracil, which results in a characteristic pattern of C to T transitions based on the local sequence preceding the deaminated cytosine (Beale et al., 2004; Wang et al., 2010a). This results in a well-defined mutational signature that was initially identified in breast cancer (Nik-Zainal et al., 2012; Alexandrov et al., 2013; Taylor et al., 2013). At present, it is unknown if a similar mutation signature correlates with folate deficiency. With further improvements in sequencing technologies, particularly the emergence of various error-corrected sequencing methodologies (Salk and Kennedy, 2020), future studies will have the opportunity to perform unbiased assessment of how folate deficiency impacts mutagenesis in different somatic tissues.

Telomere attrition

Due to the inherent inability of DNA polymerase to completely replicate the 3′ ends of linear DNA strand, chromosomes are capped with telomeres—nucleoprotein structures formed from repetitive DNA sequences bound and protected by complexes of telomere-binding proteins (Smith et al., 2020). Telomeres serve as sacrificial protection from the iterative attrition of DNA following DNA replication (Chakravarti et al., 2021). Telomeres progressively shorten with each cell division, but importantly, they protect the loss of genomic sequences within the chromosome. Critically short telomeres activate DNA damage signaling pathways to trigger senescence and prevent subsequent reentry into cell division. Therefore, telomeres act as a natural “molecular clock” to impose a finite capacity in the ability of somatic cells to undergo cell division (Hayflick and Moorhead, 1961; Hayflick, 1965). An important cause of accelerated telomere shortening is DNA damage that results in the error-prone repair or incomplete DNA replication of telomeres (Von Zglinicki, 2002). The sequence composition of telomeres, specifically the “5′-TTAGGG-3′” motif, makes them inherently vulnerable to DNA damage generated by folate deficiency. The high thymine content creates ample opportunities for uracil misincorporation, while the tandem guanine bases are ideal substrates for oxidative modification to 8-oxoguanine. Indeed, both uracil and 8-oxoguanine have been detected in telomeres from folate-

deficient cell lines and rodent tissues (Bull et al., 2014; Li et al., 2019; Zhou et al., 2022).

Studies in healthy human populations show an association between lower serum folate level and decreased telomere length in peripheral blood leukocytes that is more significant with age (Bull et al., 2009; Richards et al., 2008; Praveen et al., 2020; Paul et al., 2009; Nomura et al., 2017; Tucker, 2019). Interestingly, a study in children found shorter telomeres in individuals carrying a natural polymorphism in the reduced folate carrier (RFC G80A rs1051266) that increases susceptibility to folate deficiency due to reduced cellular uptake of 5-methylTHF (Milne et al., 2015). The impact of maternal folate status on the telomere length of their newborn offspring has also been studied. A rodent model of gestational folate deficiency induced significantly shorter telomeres in the neonatal brain tissue (Ren et al., 2023). In humans, there is a correlation between lower maternal serum folate and shorter telomeres from newborn cord blood lymphocytes (Entringer et al., 2015). Regarding the role of dietary folate intake and telomere length, a large (422,693 enrolled participants) study using the UK Biobank showed a small but statistically significant correlation between self-reported regular dietary folate intake and shortened leukocyte telomere length (Spinou et al., 2024). In addition to DNA damage, it is important to highlight that folate deficiency can also impact telomere biology by altering DNA methylation patterns. In human cell lines, short-term folate deficiency induces telomere lengthening associated with global DNA hypomethylation (Bull et al., 2014). A more in-depth review of DNA methylation and telomere biology can be found elsewhere (Moores et al., 2011). Despite the observational data that link folate deficiency with shorter telomeres, questions remain regarding the genotoxic mechanisms inflicted at the telomere secondary to folate stress and whether folate deficiency triggers a telomere-specific DNA damage response (Barnes et al., 2022).

Mitochondrial DNA damage

Mitochondria support the replication and maintenance of their own genome consisting of 16.6 kilobase of circular double-stranded DNA. While most mitochondrial proteins are encoded in the nuclear genome, the mitochondrial genome encodes 13 oxidative phosphorylation proteins and 24 RNAs that are essential for mitochondrial function. Therefore, DNA damage to the mitochondrial genome is detrimental to mitochondrial integrity and has been associated with neurodegenerative diseases and metabolic disorders (Sharma and Sampath, 2019). The best characterized folate-dependent mitochondrial DNA lesion arises from uracil misincorporation during replication of the mitochondrial genome. Several studies have shown that the depletion of cellular folate results in greater uracil misincorporation into the mitochondrial genome compared with nuclear DNA, indicating that the mitochondrial genome is more sensitive to disrupted one-carbon metabolism (Fiddler et al., 2021; Heyden et al., 2023). In addition to uracil misincorporation, folate deficiency has been associated with increased oxidative stress and oxidative DNA damage in mitochondria (Chang et al., 2007; Lv et al., 2019, also see Elevation of oxidative DNA damage).

In response to mitochondrial DNA damage, mitochondria deploy limited DNA repair pathways. DNA glycosylases, including UNG1 and OGG1, can localize to mitochondria to remove uracil and 8-oxoguanine lesions from the mitochondrial genome (Prakash and Doublé, 2015). The failure to adequately repair such DNA damage can lead to deletions within the mitochondrial genome. Several studies in rodents and humans have observed that folate deficiency in different tissue compartments is associated with increased deletions in the mitochondrial genome (Fiddler et al., 2021; Wu et al., 2009; Lv et al., 2019; Chou et al., 2007; Chou and Huang, 2009; Kronenberg et al., 2011). Unlike the nuclear genome, multiple copies of mitochondrial genome can be supported within the mitochondria. This allows normal and damaged mitochondrial genomes to coexist in a state called heteroplasmy (Stewart and Chinnery, 2015). The degree of heteroplasmy can be quantified by sequencing the mitochondrial genome to measure the frequency of mutated mitochondrial genomes. This method has revealed that vitamin B12 deficiency in a mouse model results in elevated heteroplasmy that correlated with increased uracil content in mitochondrial DNA (Walsh et al., 2024). Imbalances in heteroplasmy that result in the accumulation of mutations in the pool of mitochondrial genome have been attributed as a causal factor for age-related neurodegenerative disease (Stewart and Chinnery, 2015). The role of folate-deficient induced mitochondrial DNA damage in neurodegeneration has been studied in mice carrying genetic deletion of UNG (Kronenberg et al., 2011). Compared with wild-type mice, *Ung*^{-/-} mice on folate-deficient diet exhibited increased markers of neurodegeneration in the hippocampus, altered monoamine metabolism, and cognitive and behavior deficits. Overall, there is now compelling evidence that the mitochondrial genome is susceptible to genotoxic mechanisms induced by folate deficiency.

Folate deficiency, genomic instability, and impact on health

How does DNA damage arising from folate deficiency impact health? While it is beyond the scope of this review to discuss folate-dependent health disorders in detail, this section will highlight the association between DNA damage during folate deficiency and associated health disorders. The causal link between maternal folate deficiency and neural tube defects is unequivocal. A clear etiology arises from nucleotide pool insufficiency that disrupts cell proliferation during neural development; however, studies also implicate DNA damage during folate deficiency as a contributory factor to the increased risk of neural tube defects in the children of folate-deficient mothers (Wang et al., 2023b). Folate deficiency-induced genomic and mitochondrial DNA damage in the brain has also been attributed in the pathology of neurodegenerative diseases (Fenech, 2010; Kronenberg et al., 2011).

Paternal folate deficiency is associated with increased levels of markers of DNA damage and decreased DNA integrity in the sperm of folate-deficient male mice (Swayne et al., 2012; Lambrot et al., 2013) and increased mutation frequency in their offsprings (Zhao et al., 2022; Cao et al., 2023). Given the known negative impact of DNA damage from other sources, including chemotherapies on germ cell development and fertility, it is

plausible that increased germ cell genomic instability arising from folate deficiency could contribute to decrease in sperm quantity and quality in mice and humans (Hoek et al., 2020). This has been difficult to dissect, in part because of the parallel large scale epigenetic changes found within the sperm, which are also induced by folate deficiency (Lambrot et al., 2013). By extrapolating the impact of the mutation frequency observed in the offsprings from folate-deficient mice, it was estimated that such folate deficiency in human germ cells could result in the transmission of 6–15 deleterious mutations to the next generation (Zhao et al., 2022).

Genetic instability and mutagenesis are key drivers of carcinogenesis (Hanahan and Weinberg, 2011). Folate deficiency is associated with an increased risk of developing certain cancers, such as colon cancer (Pieroth et al., 2018). The mechanisms by which folate deficiency promotes cancer formation has been extensively reviewed and have been proposed to involve multiple factors, including increased DNA damage, impaired DNA repair, and aberrant DNA methylation (Duthie, 2011; Kim, 2003; Mason and Tang, 2017). To specifically study the role of DNA damage from folate deficiency as a causal driver of carcinogenesis, several studies have utilized mice carrying genetic inactivation of DNA repair pathways, the prediction being that enhancing the genomic instability induced by folate deficiency should further accelerate carcinogenesis. Surprisingly, the findings from such studies failed to demonstrate increased tumors or tissue dysplasia in DNA repair mutant mice subjected to folate deficiency. Mice carrying deletion of UNG failed to exhibit increased tumors when fed a folate-deficient diet (Linhart et al., 2009). Mice with BER deficiency fed a folate-deficient diet were protected from malignant changes in the colon (Ventrella-Lucente et al., 2010). Furthermore, folate-deficient diet did further increase tumor development in *Apc^{+/+} Msh2^{-/-}* mice that are highly predisposed to neoplasms of the small intestinal and colon (Song et al., 2000). These studies highlight the complexity in modelling folate deficiency as a carcinogenic metabolic state. One possibility could be that the DNA damage induced by folate deficiency plays a role beyond cancer initiation. For example, chromosomal instability has been shown to trigger cytoplasmic DNA-sensing pathways as a mechanism of promoting cancer progression and metastasis (Bakhroum et al., 2018; Li et al., 2023). Studying this will require more sophisticated cancer models that more faithfully recapitulate advanced stages of cancer biology.

Conclusion and future outlook

Based on the existing body of research over the last half-century, folate deficiency has emerged as a nutritional and metabolic state that both generates and induces vulnerability to DNA damage. Multiple mechanisms of genotoxicity have been observed during folate depletion in vivo, ranging from nucleotide pool imbalance, uracil misincorporation, DNA oxidation, and genotoxic consequences of DNA hypomethylation. The impact of this genotoxic stress disrupts genome function and introduces mutations at both the chromosomal and nucleotide level. Furthermore, both the nuclear and the mitochondrial genome are vulnerable to genotoxicity from folate deficiency with clear negative health consequences. We envisage several compelling

opportunities by leveraging recent novel technologies to advance our understanding into the mechanisms of DNA damage in folate deficiency. Firstly, it will be interesting to assess the spectra of chemically modified DNA adducts produced during folate deficiency using unbiased liquid chromatography–mass spectrometry (La Barbera et al., 2025; Chang et al., 2018; Carrà et al., 2019; Hurben et al., 2024) and nanopore sequencing (Xu and Seki, 2020) that can simultaneously identify and quantify multiple types of DNA lesions. This information will inform on the relative significance and contribution from different mechanisms of DNA damage and potentially highlight novel DNA lesions not previously associated with folate deficiency. Secondly, advances in high-resolution sequencing provide an opportunity to map in vivo mutational hotspots and extract mutational signatures in folate-deficient tissues and cells directly from animal models and human individuals. Such methods include end sequencing (END-seq) that maps DNA strand breaks across the genome (Canela et al., 2016; Wong et al., 2021) and ultralow error rate sequencing, including nanorate sequencing (Abascal et al., 2021) and PacBio single-molecule, real-time sequencing (Revollo et al., 2021; Miranda and Revollo, 2024), to detect nucleotide mutations in post-mitotic tissue. With different tissues exhibiting variations in uracil misincorporation and DNA repair impairment in response to disrupted folate metabolism (Heyden et al., 2024; Duthie et al., 2010), it will be interesting to compare mutagenesis across different somatic tissues during folate deficiency. Lastly, cancer cells can exhibit localized folate deficiency due to increased cell division and metabolic demand (Piyathilake et al., 2000). If we can identify specific types of DNA damage that are elevated in folate-deficient cancer cells, this could be exploited as a novel therapeutic target with antifolates in combination with inhibitors of specific DNA repair mechanisms to generate cancer-localized genotoxicity. Overall, the availability of these new technologies will ensure novel discoveries will continue to be made in understanding how folate deficiency impacts genome integrity.

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