Methylation deficiency disrupts biological rhythms from bacteria to humans

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The methyl cycle is a universal metabolic pathway providing methyl groups for the methylation of nucleic acids and proteins, regulating all aspects of cellular physiology. We have previously shown that methyl cycle inhibition in mammals strongly affects circadian rhythms. Since the methyl cycle and circadian clocks have evolved early during evolution and operate in organisms across the tree of life, we sought to determine whether the link between the two is also conserved. Here, we show that methyl cycle inhibition affects biological rhythms in species ranging from unicellular algae to humans, separated by more than 1 billion years of evolution. In contrast, the cyanobacterial clock is resistant to methyl cycle inhibition, although we demonstrate that methylations themselves regulate circadian rhythms in this organism. Mammalian cells with a rewired bacteria-like methyl cycle are protected, like cyanobacteria, from methyl cycle inhibition, providing interesting new possibilities for the treatment of methylation deficiencies.
Methylation reactions start with the metabolism of methionine into S-adenosylmethionine, or SAM: the universal methyl donor co-substrate in the trans-methylation of nucleic acids, proteins, carbohydrates, phospholipids and small molecules. During the methylation process, SAM is converted into adenosylhomocysteine (SAH) that is rapidly hydrolyzed into homocysteine (Hcy) and adenosine by the enzyme adenosylhomocysteinase (AHCY) to prevent competitive inhibition of methyltransferase enzymes by SAH. The ratio SAM/SAH is critical and is a measure of the methylation potential: the tendency to methylate biomolecules 1–3. Methylation deficiencies, either from poor diet or genetic polymorphisms, contribute to the etiology of many pathologies: cancer, atherosclerosis, birth defects, aging, diabetes and pancreatic toxicity, hepatotoxicity and neurological disturbances 4.

An endogenous circadian clock evolved to anticipate the daily cycles of light and darkness has been found in many organisms, from cyanobacteria to humans. Transcription-translation feedback loops of “clock genes” directly or indirectly regulating their own transcription underlie many functions of the clock, and drive oscillations of output genes controlling physiology and behavior. Some molecular components of the clock are remarkably conserved in Metazoa, notably the genes Clock and Period, coding for transcription factors, with Clock activating the transcription of Per and Per inhibiting its own transcription 5. In 2013, we reported that inhibition of the methyl cycle by AHCY inhibitors strongly affected the circadian clock in mouse and human cells 6. We now show that the link between methylation and the circadian clock we uncovered in mammals has been conserved during more than 2.5 billion years of evolution, and that circadian rhythm perturbations can be used as a quantitative gauge for the physiological consequences of methylation deficiency.

Bacterial species exist that lack AHCY but instead express an ancient SAH nucleosidase that hydrolyses SAH into S-ribosylhomocysteine and adenosine 7–8. Surprisingly, partial rewiring of the mammalian methyl cycle by ectopically expressing the E. coli SAH nucleosidase completely protected the methyl cycle from AHCY inhibition, and allowed normal circadian rhythms, even in the presence of a saturating concentration of inhibitor. These observations demonstrate the importance of methyl metabolism in the regulation of biological rhythms from cyanobacteria to humans and suggest a therapeutic application of methyl cycle reprogramming to alleviate the detrimental impact of methylation deficiencies.

**Results**

**AHCY is a highly conserved enzyme in the methyl cycle.** Methylation deficiency can be induced by carbocyclic adenosine analogs identified as AHCY inhibitors more than 30 years ago, such as 3-Deazaneplanocin A (DZnep) 9–11. This pharmacological inhibition mimics the pathological symptoms caused by AHCY deficiency 12, such as high plasma methionine, SAM and SAH; all indicators of methyl cycle aberrations. AHCY catalyzes the cleavage of SAH to adenosine and L-homocysteine, and DZnep inhibits this reaction by occupying the adenosine binding site of AHCY. The crystal structures of human 13, mouse 14 and yellow lupin (Lupinus luteus) 15 AHCY complexed with adenosine or analogs have been described, and insights into its catalytic activity have been obtained 16–19. AHCY is one of the most evolutionarily conserved proteins 20, but experimentally determined structures of AHCY complexed with DZnep remain to be described for most organisms. A full-length multiple sequence alignment (Supplementary Fig. 1) and homology modeling of AHCY from humans to cyanobacteria (Fig. 1a and Supplementary Movie 1) revealed high conservation of sequence and predicted tertiary structure. Amino acids contributing to the DZnep binding site showed at least 88% identity between all eukaryotic AHCY sequences, and 78% between human and bacterial sequences, indicating that the DZnep binding site is virtually identical across a wide range of organisms (Fig. 1b, c and Supplementary Fig. 2). Moreover, amino acids that were reported as crucial for the activity of rat AHCY (His55, Asp130, Glu155, Lys186, Asp190, and Asn191) 18 are universal (Supplementary Fig. 1). In line with the above, molecular docking simulations of AHCY with adenosine or DZnep revealed comparable ligand binding conformations and estimated binding free energies for all organisms (Fig. 1d and Supplementary Fig. 2).

DZnep is sometimes erroneously considered to be a histone methyltransferase EZH2 inhibitor, because of the misinterpretation of a report showing that it inhibits histone methylation sites targeted by EZH2 in cancer cells 21. More recent reports showed that DZnep widely inhibits histone methylation, in line with its inhibitory effect via AHCY 22-23. Together these observations strongly support the use of DZnep as a valid approach to disrupt the methyl cycle across taxa, as we next demonstrate.

**Methylation deficiency disrupts the metazoan circadian clock.** To test the effects of DZnep on the mammalian clock, we exposed a human osteosarcoma cell line (U-2 OS) and PER2::LUC mouse embryonic fibroblasts (MEFs), expressing bioluminescent clock reporters 24-25, to DZnep concentrations. In human (Fig. 2a, b) and mouse (Fig. 2c, d) cells, DZnep potently and dose-dependently increased the period of circadian oscillations, visualized by bioluminescent rhythms of Bmal1 or Per2 expression, respectively.

We next tested DZnep on the zebrafish embryonic PAC2 cell line expressing a luminescent reporter of the *Per1b* gene, a non-mammalian cell type commonly used for circadian studies, and revealed, as observed in mammalian cells, a clear effect of the drug on the circadian period (Fig. 2e, f). An effect on the entrainment to the light/dark cycles (see methods) was also observed: The *Per1b* gene normally peaks at dawn but was severely blunted and delayed in DZnep-treated cells.

To test the effects of methylation deficiency on circadian rhythms in invertebrates, we exposed *Drosophila melanogaster* haltere cultures from transgenic luciferase reporter *ptim TLM- LUC* flies to DZnep. As observed in vertebrates, DZnep caused dose-dependent period lengthening (Fig. 2g, h). In addition, an effect of 100 µM DZnep on the luminescent rhythms reporting the expression of the *sur-5* gene was also observed in freely moving *C. elegans* (Supplementary Fig. 3), a relatively new model in circadian biology 26.

**Methylation deficiency also disrupts the somite segmentation clock.** While the metazoan circadian clock is an oscillator with a period near 24 h, the somite segmentation clock in mammalian embryos cycles much faster and orchestrates the appearance of new segments or “somites” from the paraxial mesoderm (Supplementary Fig. 4a). The underlying molecular oscillator is centered on the transcription factor *Hairy & Enhancer Of Split 7* (*Hes7*), the expression of which oscillates with a period close to 2 h in mouse 27. Like circadian clock genes, *Hes7* oscillatory expression can be monitored in real-time from transgenic embryos expressing highly destabilized luciferase under the control of the *Hes7* promoter. Testing increasing concentrations of DZnep on these transgenic embryos revealed clear similarity to the effects of DZnep on circadian clock markers, resulting in the lengthening of the somite segmentation clock period (Supplementary Fig. 4b–d, Supplementary Movie 2). Together, these data demonstrate the importance of the methyl cycle in the regulation

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Fig. 1 Adenosylhomocysteinase is a highly conserved protein. a Structural superposition of AHCY from the 9 organisms investigated here, using human (1LI4), mouse (5AXA) or lupin (3OND) crystal structures as templates. The blue loop is specific to plants and green algae; DZnep is shown in yellow, NAD\(^+\) in black. See also Supplementary Movie 1. b Docking simulation of human AHCY with DZnep, based on the 1LI4 crystal structure of human AHCY complexed with Neplanocin A, an analog of DZnep. The amino acids involved in DZnep binding are indicated with their position. See Supplementary Fig. 2 for docking simulations of DZnep to AHCY from other organisms. Red and green arrows are hydrogen bonds, yellow spheres are hydrophobic effects. The estimated free energy of binding for depicted DZnep docking conformation was \(-9.87\) kcal/mol. c Discontinuous alignment of amino acids contributing to the binding of DZnep, using the human sequence as a reference and with sequence identities shown on the right. When amino acids are identical to human, a dot is shown in the alignment. The sequence logo on top is a graphical representation of the conservation of amino acids, with the consensus symbols below (* = fully conserved residue, : = conservation of strongly similar properties, . = conservation of weakly similar properties). The positions of selected conserved amino acids are given for the human sequence on top of the alignment. d Molecular docking simulations of AHCY with adenosine and DZnep showing comparable binding free energies in all organisms. Colors represent full sequence identities, relative to human.
Fig. 2 Circadian rhythms are a quantitative gauge for methylation deficiency in Metazoa. a Mean luminescence ± SEM of human Bmal1-luc U-2 OS cells treated with increasing concentrations of DZnep; b shows mean ± SEM of period, n = 3 dishes. c Mean luminescence ± SEM of PER2-LUC MEF treated with DZnep, n = 3 dishes; mean ± SEM of period shown in d. e Mean luminescence ± SEM of Per1b-luciferase PAC-2 zebrafish cells treated with DZnep, n = 4 dishes; mean ± SEM of period shown in f; note the initial 96 h of data performed under light/dark cycles. g Mean luminescence ± SEM of ptim-TIM-LUC Drosophila halteres treated with DZnep, n = 8 independent halteres, with only the upper segment of the error bars shown for clarity; mean ± SEM of period shown in h. All bar graphs analyzed by One-Way ANOVA followed by Bonferroni’s test; all indicated comparisons (a vs. b vs. c vs. d vs. e), at least p < 0.05.
of transcriptional programs orchestrating biological rhythms in Metazoa.

**Methylation deficiency disrupts the clock in plants and algae.** The most commonly used model organism to study circadian rhythms in land plants is *Arabidopsis thaliana*. We thus tested the effects of DZnep on luminescent rhythms reporting the expression of the plant evening gene TOC1 in protoplasts and extended our investigations to aquatic unicellular green algae. *Ostreococcus tauri* and *Chlamydomonas reinhardtii* represent two different classes of unicellular green algae that have successfully been used in circadian studies and constitute great models to investigate cell-autonomous metabolic processes. We tested increasing concentration of DZnep on *Arabidopsis* (Fig. 3a, b), *Ostreococcus tauri* (Fig. 3c, d) and *Chlamydomonas reinhardtii* (Fig. 3f, g), and observed an increase in period length almost identical to the results obtained in vertebrates. A plant- and green algae-specific domain has been previously described in AHCY, from amino acids 151 to 191 (Fig. 1 and Supplementary Fig. 1). Despite this insertion, however, the domains for SAH and NAD+ binding are remarkably conserved. We already showed that DZnep analogs increase SAH in mammalian cells, but to confirm that DZnep does increase SAH also in the green plant clade, we quantified SAM and SAH in DZnep-treated algae and revealed a dose-dependent increase in SAH and a decrease in methylation potential (Fig. 3e, h and Supplementary Fig. 5). In conclusion, the effects of DZnep treatment on transcriptional rhythms are also conserved in the plant kingdom. These results are especially meaningful given unicellular algae and humans are separated by more than 1 billion years of evolution.

**The methyl cycle in cyanobacteria is less sensitive to AHCY inhibition.** The circadian oscillator in cyanobacteria is composed of three proteins. Kai-A, -B and -C form a complex that regulates two key activities of KaiC: ATPase and autophosphorylation activity that result in an autonomous and self-sustained phosphorylation-based oscillator ticking with a period close to 24-h. In a cellular context, this non-transcriptional oscillator controls transcriptional outputs that in turn add robustness to the biochemical oscillator via transcription-translation feedback loops. Despite these pronounced differences in clock architecture, the circadian period in cyanobacteria lengthened in response to low concentrations of DZnep (Fig. 4a, b), but with a much lower magnitude compared to that in the eukaryotes tested here.

Inhibition of methylations by DZnep depends on the unique activity of AHCY to directly hydrolyze SAH into Hcy and adenosine. In prokaryotes, the enzyme 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) has a SAH nucleosidase activity that cleaves SAH to adenosine and S-ribosylhomocysteine. This pathway for AHCY catabolism, acting as a buffer against SAH accumulation, could explain the weaker response to DZnep compared to eukaryotes. Indeed, MTANs have been identified in some *Synechococcus* strains, such as PCC7336 and MED-G69. The lack of a strong response to DZnep may be due either to the presence of MTAN in *S. elongatus*, to DZnep being unable to bind to AHCY (this may be supported by the lowest sequence identity of AHCY in *S. elongatus* shown in Fig. 1) or having low bioavailability, or to the lack of methylation-dependent regulation of the cyanobacterial clock. Surprisingly, higher concentrations of the drug caused no significant effects on the period (Fig. 4b). Therefore, to independently verify the importance of methylations for cyanobacterial clock, we used the global methylation inhibitor sinefungin. This is a natural analog of SAM that directly binds to and inhibits methyltransferases; sinefungin was previously used in cyanobacteria to elicit methylation-dependent morphological changes. Sinefungin was more potent than DZnep, causing a dose-dependent period lengthening of a much greater magnitude (Fig. 4c, d). These data reveal an unexpected role for methylation even in the cyanobacterial circadian clock, but the methyl cycle in these cells appeared more resilient to DZnep-induced deficiency, maybe because of an alternative pathway for SAH catabolism that eukaryotes lack (Fig. 4e).

**Bacterial SAH nucleosidase rescues mammalian cells from AHCY inhibition.** AHCY mutations (R49C, D86G, Y143C, W112X and A89V) in humans lead to greatly elevated levels of S-adenosylhomocysteine (>100-fold), causing early developmental stagnation and severe pathophysiology. If the methyl cycle in these patients could be rewired to prevent S-adenosylhomocysteine build-up, it would protect them from AHCY deficiency. To test this potentially transformative hypothesis, we ectopically expressed the wild-type (WT) or the inactive D197A mutant MTAN from *E. coli* in mouse and human cells. In itself, expression of the MTAN variants, which was confirmed in mouse and human cells by immunoblotting, did not significantly change circadian parameters (Supplementary Fig. 6a, b). Cells transfected with either vectors grew well and did not display any abnormalities, in line with S-ribosylhomocysteine being a meaningless metabolite in mammalian cells. Strikingly, a complete protection from the effects of DZnep was seen in human cells expressing WT MTAN, even at the saturating concentrations of 10 and 100 μM (Fig. 5a). D197A MTAN did not provide protection, indicating the specificity of the effect (Fig. 5b). Identical results were observed in mouse cells (Fig. 5c, d). Quantification of period in human and mouse cells clearly revealed this protective effect of WT MTAN (Fig. 5e, f). Mortality and/or cell growth inhibition was observed at high DZnep concentrations on cells actively growing, but WT MTAN protected the cells against this effect whereas D197A MTAN did not (Supplementary Fig. 6c–f). Quantification of SAM and SAH confirmed the DZnep-dependent increase in SAH and resultant collapse of the methylation potential in cells transfected with mutant, but not wild-type MTAN (Fig. 5g, Supplementary Fig. 6g, h). Interestingly, in vehicle-treated cells the expression of the wild-type MTAN was sufficient to increase the methylation potential compared to cells expressing the mutant enzyme (Supplementary Fig. 6g, h).

Finally, we sought to determine the effects of DZnep on global lysine and arginine methylation, on RNA and mRNA N6-methyladenosine (m6A), and on DNA 5-methylcytosine (m5C), as well as the rescue of their potential inhibition by MTAN in mouse cells. In all cases, PER2::LUC MEFs were treated with DZnep 0, 5 or 10 μM (to obtain near-maximum period lengthening) for 48-hours. Immunoblotting using an antibody against mono- and dimethylated lysine showed that a few proteins had lower methylated levels under DZnep treatment, but only in cells transfected with the mutant inactive MTAN (Fig. 5h). Probing mono- and di-methylated arginine in the same cells showed somewhat less pronounced methylation inhibition, which was also rescued by WT MTAN (Supplementary Fig. 6i). We also checked the global levels of the specific methyl marks Histone 4 Arginine 3 symmetric demethylation (transcriptional repression) and Histone H3 Lysine 4 trimethylation (transcriptional activation) but little inhibition was seen (Supplementary Fig. 6j).

N6-methyladenosine did not significantly fluctuate between treatments when measured from total RNA samples, composed mainly (>90%) of 18S and 28S rRNA, each macromolecule...
containing only one single m6A site (Supplementary Fig. 6j). When quantified from mRNA, however, a decrease in m6A was detected under DZnep treatment, but only in cells transfected by the mutant inactive MTAN (Fig. 5i, j, k). In contrast, DNA m5C was not significantly affected (Supplementary Fig. 6k).

It is possible that a longer treatment with DZnep may have affected more stable methylations such as m6A in rRNA and m5C in DNA or caused a more widespread inhibition of histone methylation, but since the period lengthening effects of DZnep are observable by 24 h we speculate these methylations may not contribute to the period lengthening. Promoter-specific DNA or
Fig. 4 Cyanobacteria are less sensitive to AHCY inhibition. a Mean luminescence ± SEM of Synechococcus PCC 7942 kaiBC::luxAB knock-in strain treated with different concentrations of DZnep, n = 3 colonies, with only the upper section of the error bars and higher DZnep concentrations shown on a different graph for clarity; b shows mean period ± SEM, n = 3 colonies. c Mean luminescence ± SEM of Synechococcus PCC 7942 kaiBC::luxAB treated with different Sinefungin concentrations as indicated over the graphs; d shows mean period ± SEM, n = 3 colonies. e Organization of the methyl cycle, with the two-steps SAH conversion to homocysteine in bacteria, starting with MTAN. One arrow represents one enzyme, except for methyltransferases that all use SAM as a co-substrate to methylate different targets, generating SAH in the process. The enzyme AHCY, mediating SAH hydrolysis and inhibited by DZnep, is indicated on the picture. All bar graphs analyzed by One-Way ANOVA followed by Bonferroni’s test; all indicated comparisons (a vs. b vs. c vs. d) at least p < 0.05.
histone methylation, for example in the promoter of clock genes, which would not be detectable when global methylation is quantified, may also be inhibited by DZnep.

In conclusion, here we have revealed the evolutionarily conserved link between methyl metabolism and biological clocks. Moreover, we have demonstrated in mammalian cells that the period lengthening effects of DZnep exclusively depend on the inhibition of AHCY and the increase in SAH, mainly leading to protein and mRNA m6A methylation inhibition. We propose that the partial rewiring of the mammalian methyl cycle, protecting proteins and nucleic acids from the consequences of methyl cycle inhibition, might present a previously unreported strategy to treat methylation deficiencies, such as homocysteinemia or the autosomal recessive AHCY deficiency12,38–41.
Discussion

Solar ultraviolet radiation played a critical role in prebiotic chemistry, but the energy of UV photons can also degrade biologically important molecules, preventing abiogenesis. Once autocatalytic pseudo-life forms evolved with more complex chemistry, avoidance mechanisms may have provided increased fitness in such an environment. The origin of the circadian clock on the early Earth is likely to have been a simple sensing of molecules present in the milieu, increasing or decreasing under UV exposure and triggering an appropriate response. Methylene cycle metabolites are thought to have been present in the prebiotic world, since methionine can be created by a spark discharge and is proposed to be an intermediate in the prebiotic synthesis of Hcy, which was also found among organic molecules synthesized in the 1972 Miller experiment. In addition, formaldehyde, an abundant prebiotic molecule increasing during the day under the action of UV on carbon monoxide and water vapor could have affected the chemistry of early life forms by methylation and may have worked like a chemical marker of daylight. A conserved link between methyl transfer and the circadian clock may have arisen from such a scenario.

In mammalian cells we previously reported that methyl cycle inhibition decreases the methylation of internal adenoses in mRNA (m6A), as well as that of histones. While specific mRNA m6A inhibition was sufficient to elicit period elongation, histone methylation likely contributed to the period elongation obtained by DZnep, as well as that of other methylation sites in mRNA, rRNA and tRNA. Due to the considerable heterogeneity in mechanisms regulating gene expression and function in organisms tested here, identifying a single mechanism explaining period elongation in all taxa would be a difficult undertaking and is beyond the scope of the present work. Since the oldest methyltransferases are RNA methyltransferases, it is tempting to propose that inhibition of RNA methylation may at least partially contribute to the period lengthening in all species.

That mammalian cells expressing E. coli MTAN are completely protected from the period-lengthening effects of DZnep demonstrates that these effects are solely dependent upon methyl cycle inhibition and SAH accumulation. Moreover, we have shown in mouse cells that the functional consequences of methyl cycle inhibition, i.e., a decrease in the methylation of proteins and mRNA, is also rescued by WT MTAN. This confirms that MTAN, despite removing SAH from the cycle in a way that does not normally occur in mammalian cells, allows the methyl cycle to turn and drive cellular methylations when AHCY does not function. MTAN hydrolyses SAH to S-ribosylhomocysteine and adenine, not to Hcy and adenosine as AHCY does. Since Hcy is needed for the regeneration of methionine in the methyl cycle, how could the methyl cycle still maintain cellular methylations active without Hcy? It is likely that, with the abundance of free methionine in the cell, the synthesis of SAM and downstream methylations can continue unimpeded even when Hcy is low. Hcy is however also used for the synthesis of glutathione (GSH) via cystathionine and cysteine, a metabolic route called the transsulfuration pathway (Hcy → cystathionine → cysteine → GSH), which could potentially have been inhibited by MTAN expression and DZnep. To investigate potential disruption of this pathway caused by either DZnep or MTAN, we quantified these metabolites from cell extracts used in Fig. 5g. As expected, expression of WT MTAN caused a decrease in Hcy compared to cells expressing D197A (Supplementary Fig. 7). Overall, however, the transsulfuration pathway appeared more active in cells expressing WT MTAN, especially given the higher concentrations of cysteine and GSH, which were probably due to the increase in SAM observed under the same conditions (Supplementary Fig. 6g). SAM being a powerful allosteric activator of the transsulfuration pathway, a general tendency of DZnep to inhibit the transsulfuration pathway was observed, more pronounced in cells expressing the mutant MTAN (Supplementary Fig. 7). Glutathione disulfide (GSSG), generated during enzyme-catalyzed reduction reactions of GSH with peroxides or disulfide bonds, tended to decrease under DZnep treatment (Supplementary Fig. 7). The GSH/GSSH ratio, a measure of oxidative stress, was not significantly affected by either DZnep or MTAN, indicating that the mild effects of DZnep and MTAN expression on the transsulfuration pathway are unlikely to contribute to the circadian period lengthening (Supplementary Fig. 7). These data do show that WT MTAN also rescues the cells from the mild inhibition of the transsulfuration pathway by DZnep, however.

The somite segmentation clock was strongly affected by inhibition of AHCY, which is in line with the importance of 1 carbon metabolism for embryonic development. As can be seen in the Supplementary Movie 3, showing similar results to Supplementary Movie 2 but merged with brightfield images and displaying luminescence as a pseudo-color green, period lengthening of the oscillatory expression of Hes7-luciferase occurred together with a pronounced delay in the growth of the caudal tip of the pre-somatic mesoderm as well as in the appearance of new somites, demonstrating that this molecular clock as well as its output, i.e. the somitogenesis, were affected by DZnep. The mechanisms underlying this period lengthening may be distinct from those involved in the lengthening of the circadian period. Considering the short period of Hes7 oscillations—2h,— and the importance of 3′-UTR-dependent regulation of Hes7 mRNA turnover for its cyclic expression, the inhibition of mRNA methylation may at least in part contribute to the results observed.

We have presented here an identical response to methyl cycle inhibition on biological oscillators across a wide variety of species. Only a small number of examples have been reported that identified aspects of circadian timekeeping shared among...
rhythmic life, such as oxidation cycles of peroxiredoxin, 29,48,49 and regulation by oscillating intracellular magnesium concentrations. 50 Building on those early observations, this study establishes methylation as a universal regulator of rhythmicity. Importantly, to our knowledge we provide a first potential avenue of translation of fundamental understanding of methylation deficiencies to clinical applications. Biopharmaceuticals, such as recombinant MTAN or therapies using genetically engineered cells expressing MTAN, could be used to treat methylation deficiencies in the short and longer term. We consider this approach to directly targeting deficient AHCY in cases where the deficiency arises from errors of metabolism causing accumulation of metabolites that are inhibitors of AHCY; and even in cases of AHCY polymorphisms, since the endogenous inactive AHCY in patients would compete with the exogenous active AHCY.

Methods

**Molecular modeling and sequence analyses.** 3D protein structures were obtained from the Protein Data Bank (PDB) for Homo sapiens (PDB 1L14), S. adenosylhomocysteine hydrolase complexed with neplanocin, resolution 2.01 Å and Mus musculus (PDB 5AXA), S. adenosylhomocysteine hydrolase with adenosyl, resolution 1.55 Å. All PDB structures underwent pre-processing including the addition of missing amino acid atoms and hydrogens, removal of solvent atoms, and hydrogen-bond network optimization and residue protonation based on predicted pka values using the Protein Preparation Wizard 51 of Maestro Schrödinger Release 2018-1 [Maestro, Schrödinger, LLC, New York, NY, 2018]. Homology models of Dicots (template 1L14), 100% coverage, 86% sequence identity, Drosophil melanogaster (template 1L14, 100% coverage, 81% sequence identity), Caenorhabditis elegans (template 1L14, 98% coverage, 77% sequence identity), Arabidopsis thaliana (template 3OND chain A, 100% coverage, sequence 80% identity), Ostreococcus tauri (template 3OND chain A, 99% coverage, 79% sequence identity), and Synecococcus elongatus (template 1L14, 98% coverage, 41% sequence identity) were constructed via the SWISS-MODEL server. 52 Model quality was evaluated based on GMQE (global model quality estimation) ranging from zero to one with high numbers indicating good model quality and QMEAN 53, a composite and absolute measure for the quality of protein models, indicating good or poor agreement between model and template with values of around 0 or below – 4, respectively. GMQE and QMEAN values for the seven homology models were determined to be 0.98 and 0.85 for D. rerio, 0.93 and 0.82 for D. melanogaster, 0.87 and 0.35 for C. elegans, 0.99 and 0.31 for A. thaliana, 0.95 and 0.08 for C. reinhardtii, 0.91 and – 0.54 for O. tauri, and 0.75 and – 0.59 for S. elongatus, respectively. GROMACS version 2018 54 was utilized for energy minimization of all nine protein structures including oxidized cofactor NAD+ using force field AMBER99SB-ILDN 55. Cofactor parameters and topologies were obtained through ACYPE 56 using the AMI-BCC method with net charge = 1. The systems including TIP3P water were neutralized with counter ions (Cl- and Na+) to 0.1 M and minimized via steepest descent with a maximum force limit of 100 kJ/mol/nm. Molecular docking simulations were performed using the energy-minimized protein-NAD+ complexes as well as co-crystallized adenine (ADN), neplanocin (NOC), and 3-deazaneplanocan A (DZnep) (structure from co-crystal NOC in 1L14) using the Lamarckian Genetic Algorithm provided by the AutoDock2 suite. 57 Protein-ligand interactions were analyzed in LigandScout version 4. 58,59

**Assay for effect of the inhibition of the methyl cycle on vertebrate circadian clock.** Human U-2 OS cells stably transfected with a Bmal1-luciferase reporter vector and mouse PER2::LUC MEFS 60 cells were cultivated as previously described. 60 Briefly, cells were seeded into 35 mm dishes (Corning, New York, USA) and allowed to grow to confluence in DMEM/F12 medium (Nacalai, Kyoto, Japan) containing penicillin/streptomycin/amphotericin (Nacalai). Cells were then shocked by dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) 200 nM for 2 h, followed by a medium change including 15% FBS for 24 h. Next, cells were then transferred to an 8-dishes lumimeter-incubator (Kronos Dio, Atto, Tokyo, Japan). Photons were counted in bins of 2 min at a frequency of 10 min.

**Transfection of mammalian cells with expression vectors before luminometry.** The bacterial expression vector pPROEX HTa containing wild-type MTAN 61 was used as a template to amplify a long fragment of NADH, with flanking Sall/Hel restriction sites at the 5′ and 3′ end, respectively. A single 5′ HA tag 5′-gag-taccaagatGccgaagcaagacttggac-3′ was also facultatively inserted during polymerase chain reaction (PCR). The Sall/Hel digested, tagged or untagged PCR products were then ligated into a Sall/Hel-digested pSELECT-HYGR-O-MCS (Invivogen, San Diego, California, USA) using Ligation High V2 (Toyobo, Osaka, Japan). DH5apha E. coli (Takara Bio, Kusatsu, Japan) were transformed with the ligation products and streaked on a solid medium containing ampicillin. Colonies were screened by Sanger sequencing. The D197A mutation was then performed, with the wild-type vector as a template, by PCR using the primers 5′-CCATCTCCGCGCCGCGATC3′ and 5′-GATAGGCGACGCGAGATG3′ containing the mutated GCC codon, together with the 5′ and 3′ primers used for the cloning of MTAN. D197A MTAN was then cloned into pSELECT-HYGR-O-MCS as described above for the wild-type version.

**Wild-type or D197A tagged MTAN vectors were transfected into PER2::LUC MEFS or U-2 OS in 24-well plates at ~90% confluence with Lipofectamine LTX and PLUS reagent (Invitrogen, Thermo Fisher Scientific) following manufacturer’s protocol.** Cells were cultured in 12–24 (MEFS) or 6–9 (U-2 OS) hours with the transfection mixtures. After transfection, medium was changed, and cells were incubated for 24–48 hours. Antibiotic/antimycotic was omitted during transfection and in the first medium change. Expression of the HA-tagged WT and D197A MTAN was confirmed by immunoblotting using an anti-HA antibody (Roche) and β-Actin (Sigma-Aldrich). For the experiments shown in Supplementary Fig. 5c–e, transfected confluent cells were trypsinized and plated very low density and allowed to grow a few days until ~30% confluence.

Assays for the inhibition of DNA, total RNA, mRNA and protein methylation. Global DNA methylation was quantified by the Abcam 5-methylcytosine assay kit (ab233486, colorimetric) following manufacturer’s protocol. Global total RNA and mRNA mt6A was determined by the Abcam mt6A RNA Methylation Assay Kit (ab185912, colorimetric) following manufacturer’s protocol. For the blot assay, positive methylation bands were visualized in DEPC-treated water for 10 min followed by DEPC-treated 6× SSC buffer at for at least 15 min. During that time, RNA samples were denatured at 95 °C for 3 min and directly transferred on ice. Membranes were then laid on top of two flat layers of blotting paper wet but not dripping with 6× DEPC-treated SSC buffer, the reverse side of the membrane was allowed to come in contact with each RNA sample (50 ng/μl for mRNA or 400 ng/μl for totRNA) were then spotted on the membrane and allowed to seep through. RNA was crosslinked to the membrane in a FUNA-UV crosslinker (Funakoshi, Japan) equipped with 254 nm UV lights, set to “optimal crosslink” (120,000 microjoules/cm²). Membrane was rinsed charged nylon membrane was transferred to 0.45 μm syringe filters and DEPC treated water was then pipetted for 15 min in order to confirm the presence and consistent RNA amount for each dot. After several washes with DEPC-water, membrane was scanned for record. The membrane was then washed with 0.1%TBS (Nacalai, Nesque, Japan) for 10 min, then blocked for 1 h in 5% skimmed milk in 0.1%TBS. Membrane was then washed 10× overnight at 4 °C with the following antibodies: (i) anti-β-actin (ab2268, 1:1000, Sigma, St Louis, MO, USA); (ii) anti-α-tubulin (ab52806, 1:1000, Abcam, Cambridge, MA, USA). Membranes were blocked for 1 h at RT. Membranes were then washed 10× with PBS before incubation 1 h at RT with the secondary antibody (NA934, GE Life Science, USA) diluted 1:5000. For three washes of 10 min with PBS, the membrane was incubated with ECL Prime reagent (GE Life Science) and imaged with a LAS-4000 imaging system (Fujifilm, Japan). Protein methylation was measured by standard immunoblotting using the following antibodies and dilutions: Abcam ab32366, 1:500, for mono- and dibutylated lysine, Abcam ab5823, 1:500, for histone 4 Arginine 3 symmetric demethylation, Abcam ab5580, 1:1000, for Histone H3 Lysine 4 trimethylation, Synaptic System, Germany) diluted 1:1000. The next day the membrane was washed three times 10 min with PBS then incubated 1 h at RT with the secondary antibody (NA934, GE Life Science, USA) diluted 1:5000. After three washes of 10 min with PBS, the membrane was incubated with ECL Prime reagent (GE Life Science) and imaged with a LAS-4000 imaging system (Fujifilm, Japan).

Protein methylation was measured by standard immunoblotting using the following antibodies and dilutions: Abcam ab32366, 1:500, for mono- and dibutylated lysine, Abcam ab5580, 1:500, for histone 4 Arginine 3 symmetric demethylation, Abcam ab5580, 1:1000, for Histone H3 Lysine 4 trimethylation, Synaptic System, Germany) diluted 1:1000. The next day the membrane was washed three times 10 min with PBS then incubated 1 h at RT with the secondary antibody (NA934, GE Life Science, USA) diluted 1:5000. After three washes of 10 min with PBS, the membrane was incubated with ECL Prime reagent (GE Life Science) and imaged with a LAS-4000 imaging system (Fujifilm, Japan).

**Application MolView was used.**
were treated with dexamethasone 200 nM for 2 h for synchronization and, after a medium change (with 1 mM luciferin and various concentrations of DZNep), transferred to a spin-funnel/centrifuge (CFASTA-LHC, Churitus Electric Corp, Nagoya, Japan) set at 35 °C, 5% CO2. Photons were counted in bins of 10 s at a frequency of 10 min.

Preparation of PER2::LUC cells for SAM and SAH quantification. Cells plated in 10 cm culture dishes at 90% confluence were harvested (~3 × 10^7) described in the corresponding sections on methyl cycle inhibition in these cells. Dishes were treated with dexamethasone 200 nM for 2 h for synchronization and, after a medium change (with 1 mM luciferin and various concentrations of DZNep) and incubated for 48 h. Cells were washed once with 10 ml, then once with 2 ml 5% mannitol (Nacalai). Mannitol was thoroughly removed, and 0.8 ml methanol was added to the dish, rocking vigorously for 30 s for the methanol to homogenize cover the cells. Dishes were tipped and 0.55 ml ml containing 125 ng/ml BIS-TRIS (Sigma-Aldrich) was added directly to the methanol. Dishes were once more rocked by hand for 30 s, then the methanol/MilliQ mix (~0.9 ml due to methanol evaporation) was transferred to a 1.5 ml tube. Two 0.4 ml aliquots of the extracts were filtered through Vivaspin 500 KDa cut-off filters (Sartorius AG, Göttingen, Germany) then kept at −80 °C until quantification. Dishes containing cells plated simultaneously and submitted to the same treatment were used to calculate cell counts and total cell volume for normalization.

Preparation of O. tauri and C. reinhardtii for SAM and SAH quantification. O. tauri or C. reinhardtii were cultivated in their respective culture medium as described in the corresponding sections on methyl cycle inhibition in these cells. Cells were then treated with DZNep at the indicated concentration at late log phase. After 48 h, cells were harvested (~3 × 10^7) C. reinhardtii and ~10^9 O. tauri by centrifugation. Cell pellets were then resuspended with 5% mannitol (Nacalai) for C. reinhardtii or 1M sorbitol (Sigma-Aldrich) for O. tauri, which was then thoroughly removed after centrifugation; this wash was performed twice. Cell pellets were frozen in liquid nitrogen and kept at −80 °C before analysis. Cell pellets were re-suspended in 0.25 ml MilliQ containing 125 ng/ml BIS-TRIS (Sigma-Aldrich) was added to the methanol. Cell resuspensions were subjected to 10 freeze-thaw cycles (liquid nitrogen/4 °C) to break the cell membrane containing 200,000 cpm of 35S-labeled amino acids as the endogenously labeled amino acids. The extracts were filtered through Vivaspin 500 KDa cut-off filters (Sartorius AG, Göttingen, Germany) and stored at −80 °C until quantification. Dishes containing cells plated simultaneously and submitted to the same treatment were used to calculate cell counts and total cell volume for normalization.

Hcy, cystathionine, cysteine, GSH, GSSG, SAM, and SAH by LC-MS/MS. The standard stocks of SAM (Cayman Chemical, Ann Arbor, MI, USA) and SAH (Sigma-Aldrich) were dissolved in water (1 mg/mL) and stored at −80 °C before analysis. Cell pellets were then resuspended with 5% mannitol (Nacalai), then 0.25 ml MilliQ containing 125 ng/ml BIS-TRIS (Sigma-Aldrich) was added to the methanol. Cell resuspensions were subjected to 10 freeze-thaw cycles (liquid nitrogen/4 °C) to break the cell membrane. Dishes containing cells plated simultaneously and submitted to the same treatment were used to calculate cell counts and total cell volume for normalization.
For protoplast isolation and luminescent imaging, plants expressing luciferase from the CCA1 promoter in the Col-0 background (kindly provided by Karen Halliday, University of Edinburgh) were grown in sterile soil under long-day conditions (16 h light/8 h dark) at 22 °C under 60 μmol/m²/s white LED tube lights (Impact T8). Protoplasts were isolated from 3-week old leaves by mechanically stripping the lower epidermis from the underlying mesophyll cells using magic tape, then releasing the mesophyll cells by incubating the remaining leaf tissue in a solution containing cellulase and pectinase R1067. Protoplasts in a solution of 1 mM D-luciferin (Biosynth AG), 5% fetal bovine serum (Sigma-Aldrich), 50 μg/ml ampicillin, 140 mM NaCl, 115 mM CaCl2, 4.6 mM KCl, 1.86 mM MES pH 5.7 and 4.6 mM glucose were added to white, flat-bottomed 96-well plates (Luminart, Greiner Bio-one) at a concentration of 2 × 10⁶ cells/ml and entrained for 7–10 days before recording. One day before recording, 150 μL Keller medium in artificial sea water was replaced with 150 μL of Keller medium in artificial sea water containing 333 μM luciferin (Km+). Bioluminescence was measured on a TopCount (Packard) under constant darkness or constant red + blue LED light (5–12 μe/m²/s).

ASSAY FOR EFFECT OF THE INHIBITION OF THE METHYL CYCLE ON PROCARYOTIC CYCLED CLOCK

Vibrio harveyi luciferase encoded by luxA and luxB (luxAB) genes was used as a luminescence reporter in cyanobacteria Synechococcus elongatus PCC 7942. The cyanobacterial clock-regulated reporter strain luxAB::luxAB was selected for the test, expressing luxAB under the control of the promoter of the central clock genes kilB. Synechococcus strains were grown in modified BG11 media supplemented with 40 μg/ml of spectinomycin. The cultures grown on BG11 agar plates for 3 days at 30 °C under continuous cool-white illumination (LL) (50 μE/m²/s) were spiked on fresh BG11 agar plates containing 0.15% (w/v) agar, methionine and different concentrations of 3-deazaneplanocin A hydrochloride (SML0305, Sigma-Aldrich) or selenofin (Abcam, Cambridge, United Kingdom). After a single 12 h dark pulse for synchronization, vivo luminescence rhythms were captured with a charge-coupled device (CCD) camera set up within a system to accommodate the agar plates containing the colonies. Period was estimated by BioDare.

MEASUREMENT OF HES7 OSCILLATIONS IN THE MOUSE PREOSMOTIC MESODERM

Pre-osmotic mesoderm (PSM) tissues from three littermate pPax3-7-Ub:lac Tg embryos were embedded in 0.35% LMP-agarose/culture medium (10% FBS-DMEM/F12), in a silicon mold mounted on a p35mm-glass bottom dish, and luciferin-containing medium with DZnep (Sigma-Aldrich) or vehicle (MilliQ water) were added.

ASSAY FOR EFFECT OF THE INHIBITION OF THE METHYL CYCLE ON PLANT AND ALGA CIRCADIAN CLOCK

Ostrocerus tauri cells transgenically expressing a translational fusion of CCA1 to luciferase from the CCA1 promoter (CCA1-LUC)30 were grown, imaged, and analyzed as described previously. Briefly, cells were cultured under 12/12 h blue (Ocean Blue, Lee lighting filter 724) light/dark cycles (17.5 sinister/m²/s) in Keller medium in artificial sea water. Cells were then transferred to 96-well microplates (Luminart, Greiner Bio-one), at a density of ∼15 × 10⁶ cells/ml and entrained for 7–10 days before recording. One day before recording, 150 μL Keller medium in artificial sea water was replaced with 150 μL of Keller medium in artificial sea water containing 333 μM luciferin (Km+). Bioluminescence was measured on a TopCount (Packard) under constant darkness or constant red + blue LED light (5–12 μe/m²/s).

DATA AVAILABILITY

The source data underlying plots shown in main figures are provided in Supplementary Data 1. All other data shown in this work are available from J.M. Fustin upon reasonable request.

CODE AVAILABILITY

The Matlab code used for C. elegans rhythms analysis is available upon request to dgolombek@gmail.com or on Zenodo.

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**Competing interests**

The authors declare no competing interests.

**Additional information**

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