Proofreading deficiency in mitochondrial DNA polymerase does not affect total dNTP pools in mouse embryos

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Mitochondrial DNA (mtDNA) mutator mice express proofreading-deficient mtDNA polymerase gamma (PolgD257A) and age prematurely1,2, whereas mice with other mitochondrial defects do not show global signs of early ageing. The reason for this discrepancy is not completely understood. Hämäläinen et al.3 recently reported that both induced pluripotent stem cells (iPSCs) and primary embryonic fibroblasts harbouring PolgD257A demonstrate increased mtDNA replication frequency, leading to depletion of the total cellular deoxyxynucleoside 5′-triphosphate (dNTP) pool. The authors proposed that the decreased availability of cellular dNTPs for nuclear-genome replication leads to compromised nuclear-genome maintenance and premature ageing. Here, we report that total cellular dNTP pools are normal in mtDNA mutator mouse embryos (genotype PolgD257A/D257A), which shows that a living organism exclusively expressing PolgD257A has normal total dNTP pools despite ubiquitous rapid cell division.

In actively proliferating eukaryotic cells, dNTPs are produced primarily de novo in the cytosol and are transported into the nucleus and mitochondria for DNA synthesis. The dNTP-pool balance in dividing cells is tightly controlled by the allosterically regulated enzymes ribonucleotide reductase and dCMP deaminase, and even minor imbalances in the dNTP pool are mutagenic.4,5 dNTPs can also be catabolized in the cytosol to corresponding deoxynucleosides (dNs) either by stepwise dephosphorylation, in which the last step of conversion of dNMPs to dNs is carried out by 5′-nucleotidases, or by one-step dephosphorylation by the dNTP triphosphohydrolase sterile alpha-motif (SAM) and histidine–aspartte (HD) domain-containing protein 1 (SAMHD1), another enzyme that is allosterically controlled by dNTPs.6 dNs can be transported into the mitochondria, where dedicated mitochondrial deoxyribonucleoside kinases phosphorylate them to form dNMPs that in turn are phosphorylated to form dNTPs. dNs can also be transported between cells and can similarly be phosphorylated to form dNTPs through salvage pathways.7 Salvage pathways play an important role in non-dividing cells in which de novo dNTP production is very low.

dNTP pools can be measured by enzymatic assays, as in Hämäläinen et al., which use the incorporation of radiolabelled nucleotides by DNA polymerases, or by high-performance liquid chromatography (HPLC)-based separation coupled with ultraviolet (UV) or mass-spectrometry detection. The enzymatic assays have several drawbacks. First, metabolites present in cell extracts might affect DNA-polymerase activity. In particular, this concerns NTPs that are present in logarithmically growing cells in concentrations exceeding those of dNTPs by ~30- to ~170-fold depending on the NTP–dNTP pair.8 NTPs are to a certain extent misincorporated by DNA polymerases, and thus compete with dNTPs in the assay.9 The use of certain DNA polymerases minimizes interference from NTPs, but probably does not completely eliminate it. Second, the concentrations of the four dNTPs are measured in four separate assays, which adds variability to the results. Third, the enzymatic assays lack an internal standard that can be measured in the same reaction.

Using an HPLC-based method coupled with UV detection, we measured dNTP and NTP levels in embryonic day 13.5 (E13.5) mouse PolgD257A/D257A embryos. At this mid-gestation time point, there is massive growth and the embryo consists primarily of actively dividing cells in their natural milieu. We found no decrease in the total dNTP pools in the homozygous PolgD257A/D257A embryos compared with those in wild-type and heterozygous embryos (Fig. 1a). Furthermore, in contrast to the results of Hämäläinen et al., there were no differential decreases in dATP and dTTP. Our representative HPLC chromatograms show nearly identical levels of dNTPs and NTPs in embryos of all three genotypes (Fig. 1b,c). As expected, dNTP pools were elevated in PolgD257A/D257A embryos. At this mid-gestation time point, there is massive growth and the embryo consists primarily of actively dividing cells in their natural milieu. We found no decrease in the total dNTP pools in the homozygous PolgD257A/D257A embryos compared with those in wild-type and heterozygous embryos (Fig. 1a). Furthermore, in contrast to the results of Hämäläinen et al., there were no differential decreases in dATP and dTTP. Our representative HPLC chromatograms show nearly identical levels of dNTPs and NTPs in embryos of all three genotypes (Fig. 1b,c). As a positive control to show the sensitivity of our method for measuring nucleotide-pool balances, we measured dNTP and NTP pools in Samhd1-knockout E13.5 embryos, which are known to have increased dNTP pools.10 As expected, dNTP pools were elevated in Samhd1-knockout E13.5 embryos by 1.5-fold to 4.5-fold, compared with those in wild-type embryos (Fig. 1a,c,d). The greatest increase was in the dGTP pool, which is in agreement with previously published data.11,12

In their reply, Hämäläinen et al. provide arguments for why their dNTP measurement method should be considered state of the art. We agree that DNA-polymerase-based methods can be very accurate, but we note that the method developed by the authors gives highly variable results. In the original description of the method, dNTP levels were measured in the same sample on 2 different days, and the levels of dATP, dGTP, dTTP and dCTP varied by 1.1-, 1.2-, 1.3- and 2.9-fold, respectively, in HEK293 cells (see Fig. 2b in ref. 12).
HPLC-based assays using strong anion-exchange separation coupled with UV detection avoid these drawbacks. First, NTPs do not interfere with the detection of dNTPs because they are removed by a boronate affinity step, and most other metabolites in the cell extracts are eluted early during the HPLC separation. Second, all four dNTPs are measured during the same HPLC separation, eliminating possible variations due to sample handling. Third, an aliquot of the cell extract prior to NTP removal can be used as an internal standard for correction of possible losses during the extraction procedure and for normalization of the amount of material used for the preparation of extracts.

In their reply, Hämäläinen et al. corrected labelling errors, which cleared up some of our concerns. We find it, however, surprising that the dNTP-pool balances are so different between actively proliferating iPSCs and mouse embryonic fibroblasts (MEFs). For example, the dCTP pool is by far the largest pool in wild-type MEFs, whereas it is one of the lowest in the wild-type iPSCs (Fig. 2b and Extended Data Fig. 3b in ref. 1). The dNTP-pool balance is normally under strict allosteric control, and it is therefore not easy to explain how the induction of pluripotency in rapidly proliferating cells can change the cellular dNTP-pool balance so dramatically. Such marked dNTP-pool imbalances would be highly mutagenic, which raises questions as to whether they are present in vivo or are caused by the in vitro culture conditions of the cells and/or problems with the dNTP-pool measurements.

We conclude that mtDNA mutator embryos with rapid ubiquitous cell division have normal dNTP pools, and changes in dNTP pools are therefore unlikely to explain the premature-ageing phenotype.

Methods
To isolate wild-type, heterozygous PolgD257A (Polg+/+), homozygous PolgD257A (Polg+/mut) and Samhd1 homozygous knockout (Samhd1−/−) embryos, female mice were paired individually overnight with males and inspected for vaginal plugs the next morning. At E13.5, the pregnant females were euthanized by cervical dislocation. The embryos were immediately dissected out in ice-cold PBS, and the tails were taken for genotyping. The embryos were thereafter quickly snap-frozen in Eppendorf tubes in liquid nitrogen and stored at −80 °C. After the addition of ice-cold 12% (wt/vol) trichloroacetic acid, 15 mM MgCl2 solution and glass beads (1 mm zirconia/silica beads, BioSpec), the embryos were thawed on ice and homogenized on a BeadBeater (BioSpec) for 2 × 30 s at 4 °C in a cold room. The supernatant was collected by centrifugation at 20,000g for 5 min at 4 °C and neutralized with an ice-cold mixture of 98% triocylamine and 10 ml Freon as described in ref. 13. Aliquots (30 μl) were saved for analyses of NTPs, and the rest of the aqueous phase was pH adjusted with 1 M ammonium carbonate (pH 8.9), loaded onto a boronate column (Affi-Gel Boronate Gel, Bio-Rad) and eluted with 50 mM ammonium carbonate (pH 8.9) and 15 mM MgCl2 to separate dNTPs from NTPs. The eluates containing dNTPs were adjusted to pH 3.4 and analysed on a LaChrom Elite HPLC system (Hitachi) with a Partisphere SAX HPLC column (Iichrome). NTPs were analysed on the same column using 30-μl aliquots of the aqueous phase adjusted to pH 3.4. Animal studies were approved by the animal welfare ethics committees (Stockholms djurförsöksstätska nämnd and Uméa djurförsöksstätska nämnd) and performed in compliance with European law.

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References
1. Trifunovic, A. et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429, 417–423 (2004).
2. Kujoth, G. C. et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science 309, 481–484 (2005).
3. Hämäläinen, R. H. et al. Defects in mtDNA replication challenge nuclear genome stability through nucleotide depletion and provide a unifying mechanism for mouse progeria. Nat. Metab. 1, 958–965 (2019).
4. Mathews, C. K. Deoxyribonucleotide metabolism, mutagenesis and cancer. Nat. Rev. Cancer 15, 528–539 (2015).
5. Ahluwalia, D. & Schaaper, R. M. Hypermutability and error catastrophe due to defects in ribonucleotide reductase. Proc. Natl Acad. Sci. USA 110, 18596–18601 (2013).
6. Kumar, D. et al. Mechanisms of mutagenesis in vivo due to imbalanced dNTP pools. Nucleic Acids Res. 39, 1360–1371 (2011).

Fig. 1 | dNTP pools in E13.5 mouse embryos. a, Quantification of dNTP pools in wild-type (Polg+ +), heterozygous PolgD257A (Polg+mut) and homozygous PolgD257A (Polgmutmut) E13.5 mouse embryos show no difference among the three genotypes. Quantification of dNTP pools in wild-type Samhd1 (Samhd1+ +) and Samhd1 homozygous knockout (Samhd1−/−) E13.5 mouse embryos provide positive controls (n denotes number of embryos). Data are presented as mean ± s.d. b, An overlay of representative HPLC chromatograms of the optical density at 260 nm (OD260) of dNTPs in the Polg+ +, Polg+mut and Polgmutmut embryos. c, An overlay of the corresponding HPLC chromatograms of the OD260 of NTPs in the Polg+ +, Polg+mut and Polgmutmut embryos used in b. d, An overlay of representative HPLC chromatograms of the OD260 of dNTPs in the wild-type and Samhd1−/− embryos. e, An overlay of the corresponding HPLC chromatograms of the OD260 of NTPs in the wild-type and Samhd1−/− embryos used in d. The dNTP and NTP standards (std) are shown in red.
7. Kong, Z. et al. Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in biological samples by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry. *Nucleic Acids Res.* 46, e66 (2018).

8. Nick McElhinny, S. A. et al. Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. *Proc. Natl Acad. Sci. USA* 107, 4949–4954 (2010).

9. Ferraro, P., Franzolin, E., Pontarin, G., Reichard, P. & Bianchi, V. Quantitation of cellular deoxynucleoside triphosphates. *Nucleic Acids Res.* 38, e85 (2010).

10. Rentoft, M. et al. Heterozygous colon cancer-associated mutations of SAMHD1 have functional significance. *Proc. Natl Acad. Sci. USA* 113, 4723–4728 (2016).

11. Rehwinkel, J. et al. SAMHD1-dependent retroviral control and escape in mice. *EMBO J.* 32, 2454–2462 (2013).

12. Landoni, J. C., Wang, L. & Suomalainen, A. Quantitative solid-phase assay to measure deoxynucleoside triphosphate pools. *Biol. Methods Protoc.* 3, bpy011 (2018).

13. Jia, S., Marjavaara, L., Buckland, R., Sharma, S. & Chabes, A. Determination of deoxyribonucleoside triphosphate concentrations in yeast cells by strong anion-exchange high-performance liquid chromatography coupled with ultraviolet detection. *Methods Mol. Biol.* 1300, 113–121 (2015).

**Author contributions**

C.K. isolated and genotyped Polg mouse embryos and P.T. and A.K.N. isolated and genotyped Samhd1 mouse embryos. S.S. performed the dNTP-pool measurements. S.S., N.-G.L. and A.C. analysed the data. A.C. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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