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# Progressive loss of mitochondrial DNA in thymidine kinase 2-deficient mice

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**Deficient enzymatic activity of the mitochondrial deoxyribonucleoside kinases deoxyguanosine kinase (DGUOK) or thymidine kinase 2 (TK2) cause mitochondrial DNA (mtDNA)-depletion syndromes in humans. Here we report the generation of a Tk2-deficient mouse strain and show that the mice develop essentially normally for the first week but from then on exhibit growth retardation and die within 2–4 weeks of life. Several organs including skeletal muscle, heart, liver and spleen showed progressive loss of mtDNA without increased mtDNA mutations or structural alterations. There were no major histological changes in skeletal muscle, but heart muscle showed disorganized and damaged muscle fibers. Electron microscopy showed mitochondria with distorted cristae. The Tk2-deficient mice exhibited pronounced hypothermia and showed loss of hypodermal fat and abnormal brown adipose tissue. We conclude that Tk2 has a major role in supplying deoxyribonucleotides for mtDNA replication and that other pathways of deoxyribonucleotide synthesis cannot compensate for loss of this enzyme.**

## INTRODUCTION

The mitochondrial genome encodes several components of the respiratory chain complexes that are essential for normal cellular metabolism. Replication of mitochondrial DNA (mtDNA) is not linked to nuclear DNA replication but occurs throughout the cell cycle in both proliferating and non-proliferating cells (1). The dNTP substrates required for mtDNA replication are either imported into the mitochondria from the cytosol where *de novo* dNTPs synthesis occurs or generated by phosphorylation of deoxyribonucleosides in the mitochondrial matrix (2). The first and rate-limiting step in the intramitochondrial phosphorylation of deoxyribonucleosides is catalyzed by the mitochondrial deoxyribonucleoside kinases thymidine kinase 2 (TK2) or deoxyguanosine kinase (DGUOK). TK2 is a pyrimidine deoxyribonucleoside kinase that phosphorylates deoxythymidine and deoxycytidine whereas DGUOK is a purine deoxyribonucleoside kinase that phosphorylates deoxyadenosine and deoxyguanosine (3). Although the two deoxyribonucleoside kinases together phosphorylate all four different deoxyribonucleosides that are the building blocks for mtDNA, this pathway for dNTP synthesis is not sufficient to sustain mtDNA replication, and mitochondria are also dependent on dNTPs imported from the cytosol (4,5).

MtDNA-depletion syndromes (MDS) constitute a heterogeneous group of diseases that usually present with signs of severe mitochondrial dysfunction in early childhood (6,7). Several mutations in the *TK2* and *DGUOK* genes have been linked to inherited-MDS in humans (8–11). The mtDNA-depletion phenotype in patients with TK2 or DGUOK deficiency shows that the mitochondrial nucleoside kinases are required for mitochondrial dNTP synthesis and mtDNA replication. Although TK2 and DGUOK catalyze similar reactions of deoxyribonucleoside phosphorylation, the tissues affected and the symptoms of the patients differ depending on which enzyme is affected. TK2 deficiency causes predominantly severe myopathy whereas DGUOK deficiency causes liver failure and encephalopathy (8–11). We decided to investigate the molecular mechanism of mtDNA depletion due to Tk2 deficiency and we report in the present study the generation of a Tk2-deficient mouse strain that we use as a model for the disease.

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## RESULTS

### Growth retardation, hypothermia and early mortality in Tk2-deficient mice

The *Tk2* gene was disrupted in embryonic stem cells by homologous recombination using a targeting vector in which exon IV and part of exon V of the *Tk2* gene were replaced with a neomycin resistance cassette (Fig. 1A and B). The genomic region deleted by insertion of the resistance cassette encodes the substrate-binding region of the Tk2 active site (amino acids 83–116) that is essential for enzymatic activity. Heterozygote $Tk2^{+/-}$ mice appeared normal and these mice were intercrossed to generate homozygous $Tk2^{-/-}$ mice. The genotypes of the mice were confirmed by Southern blot analysis (Fig. 1C).

dThd phosphorylation in brain-protein extracts from newborn mice was decreased in the $Tk2^{-/-}$ mice (0.33 pmol/mg/min) compared with the $Tk2^{+/+}$ mice (4.5 pmol/mg/min). The dThd kinase activity is the result of both Tk1 and Tk2 activity and we used BVDU phosphorylation, a selective substrate for Tk2 (12), to specifically assay the activity of this enzyme. The BVDU phosphorylation rate was 2.1 pmol/mg/min in extracts from $Tk2^{+/+}$ mice, whereas $Tk2^{-/-}$ mice phosphorylated BVDU at a rate of 0.019 pmol/mg/min. The >100-fold decrease in BVDU phosphorylation confirmed that the $Tk2^{-/-}$ mice had lost Tk2 enzymatic activity. We used dCyd to inhibit Tk2 activity in the dThd phosphorylation assay and these experiments showed that the Tk1 activity in brain from newborn mice was also decreased in the $Tk2^{-/-}$ mice (0.51 pmol/mg/min) compared with the $Tk2^{+/+}$ mice (2.7 pmol/mg/min).

Homozygous $Tk2^{-/-}$ mice appeared normal at birth and were initially indistinguishable from their heterozygous or wild-type littermates (Fig. 1D–G). However, from 7 days of age the $Tk2^{-/-}$ mice showed growth retardation compared with the wild-type animals (Fig. 2A). The relative organ weights (i.e. organ weight correlated to total body weight) of heart, liver, spleen and kidney at birth were normal in the $Tk2^{-/-}$ mice and remained similar to the $Tk2^{+/+}$ mice at day 12 (Fig. 2B). The $Tk2^{-/-}$ mice exhibited a high rate of early mortality and several animals died by the second week of life with no animal surviving more than 30 days (Fig. 2C). The $Tk2^{-/-}$ mice were notably colder than their $Tk2^{+/+}$ littermates and also exhibited shivering. We determined the surface body temperature of these mice and showed that the Tk2-deficient mice were progressively and severely hypothermic from 10 days of age (Fig. 2D).

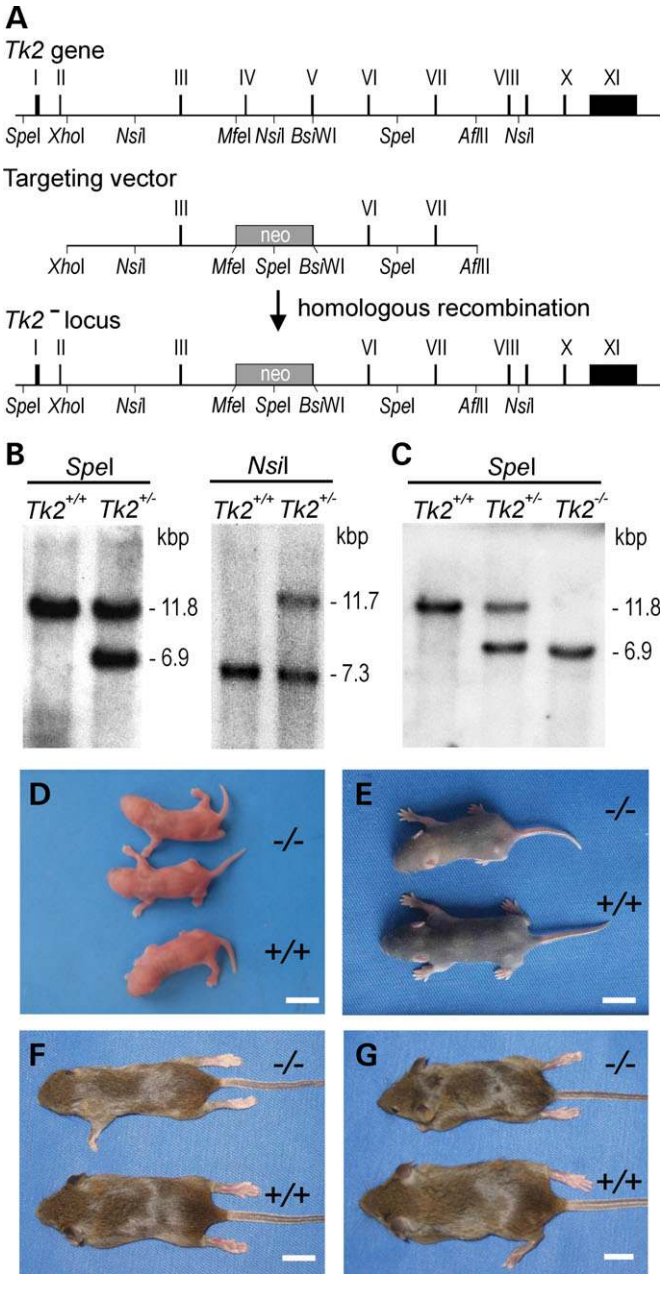


**Figure 1.** Targeted disruption of the *Tk2* gene and generation of $Tk2^{-/-}$ mice. (**A**) Homologous recombination of the *Tk2* gene with the targeting vector generated a $Tk2^{-}$ locus with replacement of exons IV and V (exons indicated with roman numbers) with a neomycin (neo) resistance cassette. (**B**) Genotyping of the *Tk2* allele in the targeted embryonic stem cells. The DNA was digested with *Spe*I or *Nsi*I that generated fragment of 6.9 or 11.7 kb in cells that had correctly incorporated the targeting vector. (**C**) Genotyping of mice by analysis of *Spe*I-digested DNA. Detection of a 6.9 kb DNA fragment indicated correct incorporation of the targeting vector. Photographs of genotyped $Tk2^{+/+}$ (+/+) and $Tk2^{-/-}$ (−/−) littermates at 0 (**D**), 7 (**E**), 12 (**F**) and 17 (**G**) days of age. White bars indicate 10 mm distance.

### Progressive depletion of structurally intact mtDNA in Tk2-deficient mice

We determined mtDNA levels in skeletal muscle, heart muscle, liver, adipose tissue, brain and spleen at multiple ages (Fig. 3A–C). All investigated organs from $Tk2^{-/-}$ mice showed 40–80% reduced mtDNA levels in 14 days-old animals. Skeletal muscle, heart, liver and spleen from $Tk2^{-/-}$ mice had normal mtDNA levels at birth but showed progressive loss of mtDNA during the first 2 weeks of life. In contrast, brain and adipose tissue exhibited mtDNA depletion already in newborn mice. Altered dNTP pools have been shown to cause structural alterations and deletions in mtDNA as well as induce point mutation (13,14). Southern blot analysis of *Sac*I-digested mtDNA from $Tk2^{-/-}$ mice showed a single band of the expected size and no evidence of deletions or other structural alterations. Long template PCR amplification of liver mtDNA did not show any evidence of truncated mtDNA fragments in the $Tk2^{-/-}$ mice (data not shown).

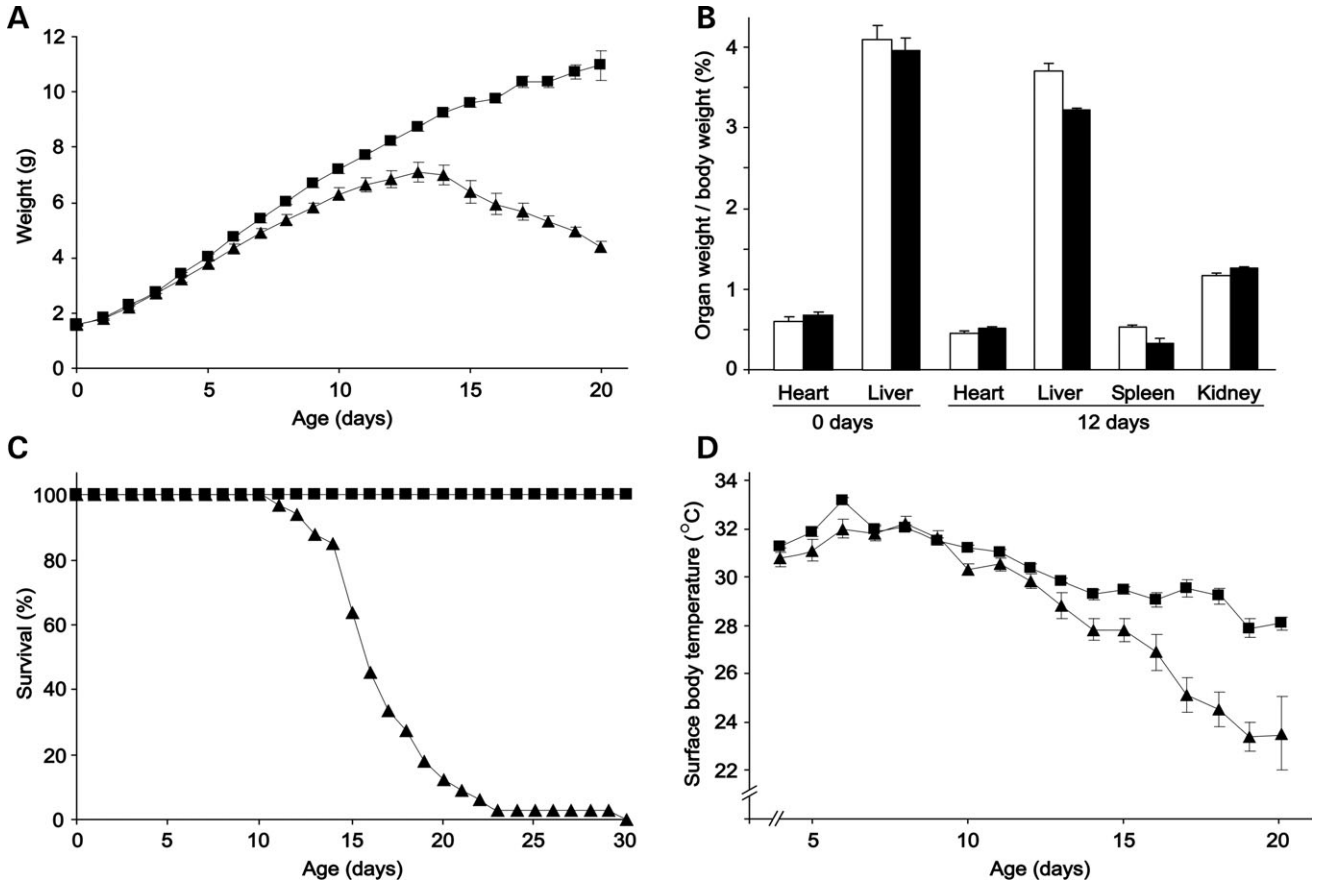


**Figure 2.** Growth retardation, early mortality and hypothermia of $Tk2^{-/-}$ mice. (**A**) Body weight of $Tk2^{+/+}$ (■) (mean ± SD, $n = 27$) and $Tk2^{-/-}$ (▲) (mean ± SD, $n = 5$) mice. (**B**) Relative organ weight (organ weight/body weight, mean ± SD) of $Tk2^{+/+}$ (white bars) and $Tk2^{-/-}$ (black bars). (**C**) Survival of $Tk2^{+/+}$ (■) and $Tk2^{-/-}$ (▲) mice. (**D**) Surface body temperature of $Tk2^{+/+}$ (■) ($n = 27$, mean ± SD) and $Tk2^{-/-}$ (▲) ($n = 10$, mean ± SD) mice.

We determined the presence of mtDNA mutations by sequencing multiple cloned mtDNA fragments. MtDNA clones from $Tk2^{-/-}$ and wild-type mice were derived by PCR amplification of total DNA extracted from liver of 7 and 12 day-old animals. Sequencing of the mtDNA clones revealed <1.3-point mutations per 10 kb mtDNA in both wild-type and $Tk2^{-/-}$ clones. Accordingly no increase in mtDNA mutation rate was found in the $Tk2^{-/-}$ mice. Thus the mtDNA depletion in the $Tk2^{-/-}$ mice was not associated with either increased mtDNA mutation rate or deletions in the mtDNA genome.

## Cardiomyopathy and lipodystrophy in Tk2-deficient mice

Histological analysis of tissues from 7 days-old $Tk2^{-/-}$ animals showed no gross abnormalities in heart muscle, skeletal muscle, brain, spleen or kidney. While no major histological changes were found in skeletal muscle from 14 days-old animals, heart muscle displayed disorganized muscle fibers with decreased stainability suggesting major damage to the integrity of individual cardiac muscle cells (Fig. 4A). Electron microscopy analysis of heart mitochondria from 14 days-old $Tk2^{-/-}$ mice showed pronounced disruption of cristae structure, whereas mitochondria from 7 days-old mice showed only minor changes (Fig. 5). Liver structure was found to be similar in both wild-type and *Tk2*-null animals, although the latter showed an increased number of intracellular small lipid vesicles in sections from 14 days-old mice (Fig. 4A). In the skin, hair follicle development and epidermal maturation proceeded without major disturbances, but at 14 days of age $Tk2^{-/-}$ animals showed near complete absence of the hypodermal fat layer that is normally well developed at the end of the anagen part of the hair cycle (Fig. 4B). We also observed changes in the structure of interscapular brown adipose tissue. At 14 days, the adipocytes displayed heterogeneity in size and accumulation of lipid vesicles in contrast to the homogenous pattern seen in the wild-type mice (Fig. 4C).

## No compensatory increase in mRNA expression of dNTP synthesizing enzymes

DNA replication is dependent on a balanced supply of dNTPs, and several enzymes involved in deoxyribonucleotide synthesis are extensively regulated (2). We compared the mRNA expression of several deoxyribonucleotide metabolizing enzymes in liver from 7 days-old $Tk2^{+/+}$ and $Tk2^{-/-}$ mice to study how the loss of Tk2 affected the expression pattern of these enzymes (Table 1). Several deoxyribonucleoside and nucleotide kinases had decreased expression levels, and decreased mRNA expression was also observed for

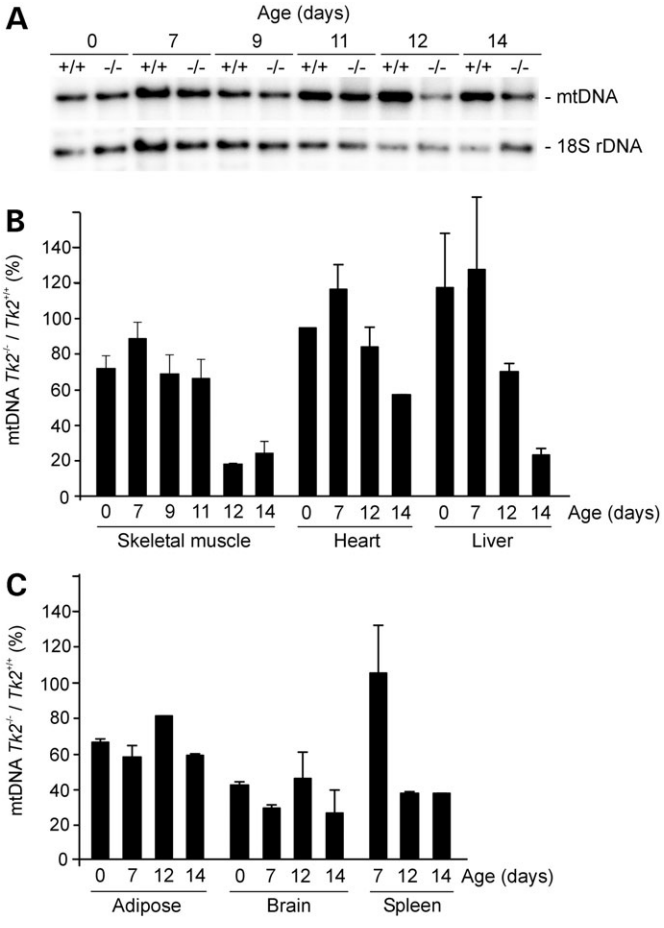


**Figure 3.** Depletion of mtDNA in Tk2-deficient mice. (**A**) Quantification of skeletal muscle mtDNA levels in $Tk2^{+/+}$ (+/+) and $Tk2^{-/-}$ (−/−) mice by Southern blot analysis. The mtDNA levels were correlated to nuclear genomic 18S rDNA. (**B, C**) mtDNA levels in $Tk2^{-/-}$ mice relative to mtDNA levels in $Tk2^{+/+}$ mice (mean ± SD) in skeletal muscle, heart, liver, adipocytes, brain and spleen at different ages.

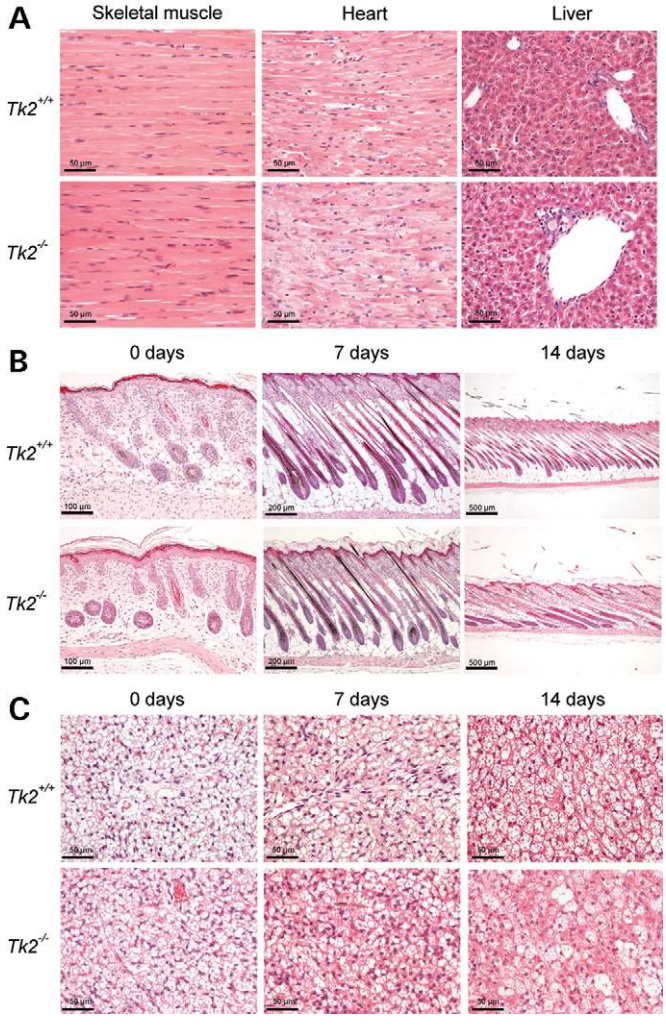


**Figure 4.** Histological analysis of tissues from Tk2-deficient mice. (**A**) Skeletal muscle, heart and liver from 14 days-old $Tk2^{+/+}$ and $Tk2^{-/-}$ mice. Section from skin (**B**) and brown adipose tissue (**C**) from 0, 7 and 14 days-old mice.

several enzymes involved in *de novo* pyrimidine deoxyribonucleotide synthesis. There was no compensatory increase in any enzyme involved in pyrimidine deoxyribonucleotides synthesis. There was neither any change in expression of 5′,3′-nucleotidases or thymidine phosphorylase that are important for the catabolism of dThd and dTMP.

## DISCUSSION

We have generated a Tk2-deficient mouse strain and showed that these animals exhibit mtDNA depletion in multiple organs. Although the $Tk2^{-/-}$ mice appeared normal at birth they rapidly developed a severe and fatal phenotype with signs of structural damage to cardiac myocytes, loss of hypodermal fat and abnormal brown adipose tissue. The mtDNA depletion in all investigated organs from $Tk2^{-/-}$ mice is a major difference compared with the phenotype of Tk2 deficiency in humans where the disease usually presents with severe but isolated myopathy with normal mtDNA levels in other organs including brain and liver (9–10). One possible explanation for the more severe phenotype in our mouse model is that humans with TK2 deficiency have point mutations in the *TK2* gene that results in defective TK2 enzymes with some degree of residual enzymatic activity (9–11). The Tk2 gene-targeting construct used to generate the $Tk2^{-/-}$ mice truncates the protein and removes a large part of the active site. Accordingly, these mice have a total lack of TK2 without any residual enzymatic activity and the total loss of TK2 activity may explain the more severe phenotype in mice compared with humans. No humans with *TK2* non-sense mutations and total lack of TK2 protein have been identified. This suggests that total loss of TK2 expression in humans also causes a more severe phenotype that may not be compatible with life.

Alterations in the mitochondrial dNTP pools have been associated with both deletions and point-mutations in mtDNA (2,13,14). The effects of alterations in the dTTP pool have been studied specifically as mutations in the thymidine phosphorylase genes are associated with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (13–15). We hypothesized that the $Tk2^{-/-}$ mice would exhibit mtDNA

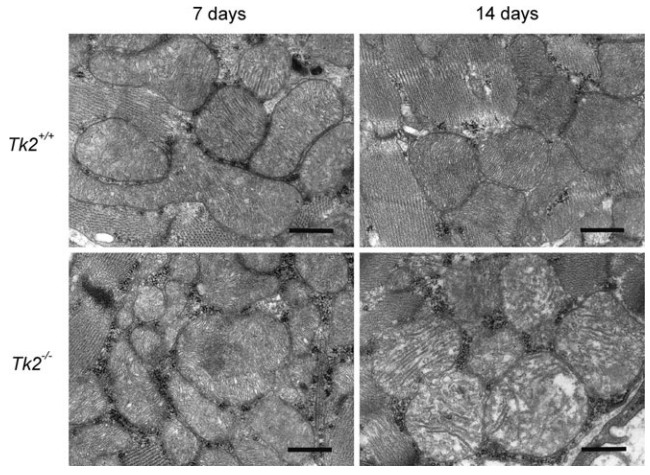


**Figure 5.** Electron microscopy images of heart mitochondria from 7 and 14 days-old $Tk2^{+/+}$ and $Tk2^{-/-}$ mice. Black bars indicate 0.5 μm distance.

**Table 1.** Alterations in mRNA expression of enzymes involved in pyrimidine deoxyribonucleotide metabolism. The RNA was extracted from livers of 7 days-old mice. mRNA expression in $Tk2^{-/-}$ versus $Tk2^{+/+}$ mice was compared and the fold increase (+), fold decrease (−) or no change (nc) in expression is indicated

| Gene name | Gene symbol | $Tk2^{-/-}$ versus $Tk2^{+/+}$ mRNA expression (fold change) |
|---|---|---|
| Nucleoside kinases | | |
| Thymidine kinase 1 | *Tk1* | −2.8 |
| Deoxycytidine kinase | *Dck* | −2.8 |
| Deoxyguanosine kinase | *Dguok* | nc |
| Uridine cytidine kinase 1 | *Uck1* | nc |
| Uridine cytidine kinase 2 | *Uck2* | nc |
| Nucleotide kinases | | |
| Thymidylate kinase | *Dtymk* | −1.9 |
| UMP-CMP kinase | *Cmpk* | nc |
| Nucleoside diphosphate kinase-1 | *Nme1* | −1.3 |
| De novo deoxypyrimidine synthesis | | |
| Ribonucleotide reductase R1 | *Rrm1* | −1.9 |
| Ribonucleotide reductase R2 | *Rrm2* | −2.5 |
| p53-ribonucleotide reductase R2 | *Rrm2b* | nc |
| Thymidylate synthase | *Tyms* | nc |
| dThd/dTMP catabolism | | |
| Cytosolic 5′,3′-nucleotidase | *Nt5c* | nc |
| Mitochondrial 5′,3′-nucleotidase | *Nt5m* | nc |
| Thymidine phosphorylase | *Ecgf1* | nc |

mutations. However, there was no increase in mtDNA point mutations in the $Tk2^{-/-}$ mice nor did we observe any deletions or other structural alterations in mtDNA. These findings suggest that loss of Tk2 expression causes a deficiency of pyrimidine dNTPs within the mitochondria that is so severe that mtDNA replication cannot occur due to lack of dNTP substrate.

The mtDNA depletion in the $Tk2^{-/-}$ mice shows the essential role of intramitochondrial deoxyribonucleoside phosphorylation to generate pyrimidine dNTPs for mtDNA replication. However, large proportions of the dNTPs required for mtDNA replication are synthesized *de novo* in the cytosol and imported into mitochondria (4,5). The major dNTP synthesis occurs during the S-phase of the cell cycle, but studies show that the cytosolic p53 inducible ribonucleotide reductase subunit 2 (p53R2) also is active in resting cells and contributes to dNTP synthesis (16). Patients with p53R2 deficiency exhibit mtDNA depletion and this provides evidence that cytosolic *de novo* dNTP synthesis also is required for mtDNA replication (17). Tk2 is accordingly required for deoxyribonucleoside phosphorylation in the mitochondria, but this pathway is not sufficient to supply all pyrimidine dNTPs for mtDNA replication.

Multiple tissues showed progressive loss of mtDNA in the $Tk2^{-/-}$ mice during the first 2 weeks of life. However, mtDNA levels were normal at birth in several tissues including heart, liver and spleen. These findings suggest that mtDNA replication is normal during fetal development although the animals lack Tk2 expression. dNTP supply to mitochondria in the fetal tissues is likely to be derived from *de novo* cytosolic dNTP synthesis. A recent study shows that the relative importance of the cytosolic *de novo* dTTP synthesis versus the intramitochondrial phosphorylation of dThd by Tk2 is different in proliferating compared with non-proliferating cells (5). In proliferating cells the mitochondrial dTTP pool is synthesized *de novo* via the S-phase specific ribonucleotide reductase and TK2 is dispensable in such cells. However, in non-proliferating cells the dTTP pool depends on both the enzymatic activities of TK2 and *de novo* synthesis by the p53R2-R1 complex. Accordingly, the dependence on Tk2 for mtDNA replication increases as tissues mature and cells enter a resting state. During embryogenesis there is a high fraction of proliferating cells that are less dependent on Tk2 activity to maintain mtDNA replication. However, even in organs that have a high fraction of proliferating cells such as spleen, the $Tk2^{-/-}$ mice showed mtDNA depletion with increasing age of the animals. These findings show that Tk2 activity is required also in mature tissues with proliferating cells and that the cytosolic enzymes involved in deoxyribonucleotide synthesis cannot fully compensate for the loss of Tk2 activity.

The $Tk2^{-/-}$ mice exhibited shivering and showed severe hypothermia compared with the wild-type mice. The hypothermia and shivering can be explained by defective non-shivering thermogenesis caused by the abnormal brown adipose tissue together with loss of isolating hypodermal fat that we observed in the $Tk2^{-/-}$ mice. However, we cannot exclude that the shivering is due to a neurological disorder in the mice as brain shows a marked reduction in mtDNA levels and mitochondrial dysfunction has been associated with both encephalopathy and neuropathy.

Nucleoside analogs used in HIV therapy, such as AZT, are phosphorylated by TK2 in the mitochondria. These nucleoside analogs interfere with mtDNA replication and cause mtDNA depletion (18). Adverse effects caused by mtDNA depletion include lipodystrophy and cardiomyopathy, pathological findings also were observed in these Tk2-deficient mice. It is likely that the threshold level of mtDNA that is required for normal tissue function differs between different organs. The Tk2-deficient mice showed lipodystrophy when the mtDNA levels in adipocytes were decreased by 40%, whereas mtDNA levels in spleen were depleted by 60% without any major pathological findings. Our data show that although

Tk2 is absent in all tissues in our mouse model, the phenotypic alterations differ in a similar pattern as drug induced mtDNA deficiency. Differentiated organs have thus evolved with specific requirements of the dNTP producing pathways and our study concludes that these pathways are complementary, and not alternative, for mtDNA synthesis and the vital functions of mitochondria.

## MATERIALS AND METHODS

### Construction of a Tk2 targeting vector and generation of Tk2-deficient mice

A bacterial artificial chromosome library with genomic DNA from 129S6/SvEvTac mouse strain (RPCI-22, BACPAC resource at the Children's Hospital Oakland Research Institute, CA, USA) was screened with a ≈1 kb probe derived from the *Tk2* gene (bp 5087–6151). A clone (RPCI22-61H9) that contained a large part of the *Tk2* gene was identified and a 15 kb fragment containing exons III–VI of the gene was isolated by digestion with *Xho*I and *Afl*II and cloned into a pBluescript plasmid vector. To construct the targeting vector, we deleted and replaced a *Mfe*I-*Bsi*W1 fragment that contained exon IV and part of exon V (bp 7066–9753) with a neomycin resistance cassette derived from a pPNT plasmid vector (Fig. 1A). The targeting construct was sequenced in all junctions to verify correct positions of the DNA fragments.

129/SVJ embryonic stem cells were electroporated with linearized targeting vector and geneticin-resistant cell colonies were selected. The colonies were analyzed by Southern blot analysis to identify colonies where homologous recombination had occurred. C57BL mouse blastocysts were microinjected with the ES cells and implanted into pseudopregnant mouse females. Male chimeras selected by agouti coat color were crossed with C57BL/6 females. We tested the mice by Southern blot analysis for the presence of the targeting construct. Heterozygous $Tk2^{+/-}$ mice were intercrossed to generate homozygous $Tk2^{-/-}$ mice. Genomic DNA of homozygous $Tk2^{-/-}$ mice was analyzed by Southern blot analysis to confirm that the animals lacked the wild-type *Tk2* alleles.

### Tk1 and Tk2 enzymatic activity

Total dThd kinase activity assays in crude protein extracts of brain from newborn mice were performed as described (19). The Tk2 enzymatic activity was determined with $^{3}$H-bromovinyl-deoxyuridine as a substrate (12).

### Histological analysis of mouse tissues

Mouse organs were dissected and their weight determined. The organs were fixed overnight in neutral buffered formalin, dehydrated and infiltrated with paraffin according to standard procedures. Sections were stained with hematoxylin and eosin.

### Transmission electron microscopy of heart mitochondria

Sections of hearts from 7 and 14 days-old mice were prepared and examined by transmission electron microscopy as described (20).

### Quantification, sequencing and analysis of mtDNA

MtDNA levels were quantified relative to nuclear DNA levels using Southern blot analysis as described (21). MtDNA for cloning was amplified by standard PCR methods. The region amplified spanned nucleotides 4773–5497 of the C57Bl/6 mtDNA sequence (NC_005089), which is outside the 4.6 kb mtDNA pseudogene copy found in chromosome 1 of the mouse complete genome sequence (NT_039170.7). The PCR fragments were cloned and sequenced in two directions using BigDye 3.1 chemistry, and was resolved using a 3130xl sequencer (ABI). A long template PCR with 200 ng mouse liver total DNA (oligonucleotide primers 5′- AACGG CTAAACGAGGG and 5′-TGCGCCACATAGACGAGT) was used to detect mtDNA deletions as described (22).

### Determination of surface body temperature

The surface body temperature was determined using a non-contact Infrared Pocket Thermometer directed to the neck and shoulder of the mice (Ken Scientific Corporation, Torrington, CT, USA).

### Expression analysis of deoxyribonucleotide metabolizing enzymes

Total RNA was isolated from livers of 7 days-old mice using RNeasy kit (Qiagen). The RNA was reverse transcribed, labeled and hybridized to GeneChips Mouse Genome 430 2.0 Arrays (Affymetrix). The arrays were analyzed at the Bioinformatics and Expression Analysis core facility at the Karolinska Institute (Novum, Sweden). Gene Chip Operating Software version 1.4 was used to analyze data from two separate experiments and the data was compared between RNA extracted from $Tk2^{+/+}$ and $Tk2^{-/-}$ mice.

*Conflict of Interest statement*. None declared.

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