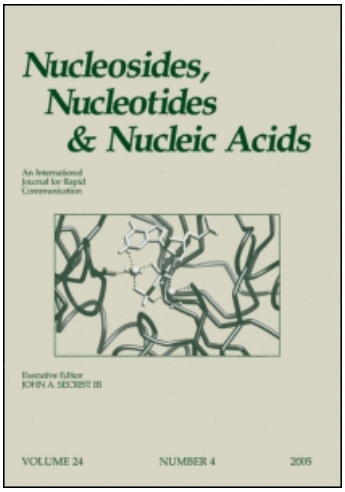


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# Deoxynucleoside Salvage Enzymes and Tissue Specific Mitochondrial DNA Depletion

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# DEOXYNUCLEOSIDE SALVAGE ENZYMES AND TISSUE SPECIFIC MITOCHONDRIAL DNA DEPLETION

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□ *Adequate mitochondrial DNA (mtDNA) copies are required for normal mitochondria function and reductions in mtDNA copy number due to genetic alterations cause tissue-specific mtDNA depletion syndrome (MDS). There are eight nuclear genes, directly or indirectly involved in mtDNA replication and mtDNA precursor synthesis, which have been identified as the cause of MDS. However, the tissue specific pathology of these nuclear gene mutations is not well understood. Here, mtDNA synthesis, mtDNA copy number control, and mtDNA turnover, as well as the synthesis of mtDNA precursors in relation to the levels of salvage enzymes are discussed. The question why MDS caused by TK2 and p53R2 mutations are predominantly muscle specific while dGK deficiency affected mainly liver will be addressed.*

**Keywords** Mitochondrial DNA depletion syndrome; mtDNA maintenance; TK2; dGK and p53R2



## INTRODUCTION

Mitochondrial DNA depletion syndrome (MDS) is a hereditary human mitochondrial disorder characterized by quantitative reduction of mtDNA copy number, with no qualitative defects (i.e., deletions or point mutations) in the mtDNA molecules. This disorder is typically tissue specific, mostly affecting muscle and liver, and in many case central nerve system. Usually, MDS begins in infancy or early childhood and is fatal.

Since the first description of MDS in the early 1990s[1] the molecular mechanism behind MDS has been elucidated in many but not all cases. Eight nuclear genes have been identified as the cause of MDS; mutations in thymidine kinase 2 (TK2), p53 inducible ribonucleotide reductase small subunit

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(p53R2) and succinyl-CoA ligase beta subunit (SUCLA2) are responsible for predominantly myopathic form of MDS while mutations in deoxyguanosine kinase (dGK), succinyl-CoA ligase alfa subunit (SUCLG1), catalytic subunit of mitochondrial DNA polymerase (polG), Twinkle gene (mitochondrial DNA helicase), and MPV17 (unknown function) are the causes of hepatocerebral forms of MDS as previously reviewed.[2] These genes are involved either in mtDNA replication or the maintenance of deoxynucleotide pools. Recent studies using mouse models have demonstrated that these genes are essential for life.[3–5,7,8] However, the tissue specific pathogenesis of these mutated genes remains unexplained.

Mitochondria are vital organelles for cellular function, not only providers of cellular energy in the form of ATP, but are also involved in multiple metabolic processes including apoptosis. Abnormalities in mitochondrial function have been implicated in many human diseases such as cancer, diabetes, Alzheimer's disease and normal aging. Human mitochondrial DNA (mtDNA) is a 16.6 kb circular double stranded DNA molecule, coding 22 tRNAs, 2 rRNAs, and 13 mRNAs. The machinery for mtDNA replication, transcription and translation are mostly encoded by nuclear genes. The 13 polypeptides coded by mtDNA are essential components of the respiratory chain complexes and the levels of these 13 proteins correlate with mtDNA copy number.

In order to understand the tissue specific pathology of MDS, we need to more clearly define the following issues: mtDNA synthesis, copy number maintenance and turnover, as well as the supply of mtdNTPs.



## mtDNA SYNTHESIS AND COPY NUMBER MAINTENANCE

Unique mitochondrial DNA replication machinery e.g. the mtDNA replisome is responsible for the replication of the mitochondrial genome.[6] mtDNA replisomes contain a number of nuclear encoded proteins including the only DNA polymerase (polG) present in mitochondria, which is a heterotrimer consisting one catalytic subunit and two identical accessory subunits. Other components necessary for mtDNA replication are mitochondrial single strand DNA binding protein (mtSSB), which stabilizes single stranded regions of mtDNA at the replication fork; the twinkle helicase which unwinds double stranded mtDNA at replication forks. mtDNA synthesis is initiated when RNA primers covalently bind to the origin of the heavy-strand and proceeds unidirectional to displace the parental heavy strand. polG and twinkle helicase together form a processive replication machinery, which uses double stranded DNA as template to synthesize single stranded DNA, the presence of mtSSB stimulates the process and thus, complete the entire synthesis cycle. (For a comprehensive review see Falkenberg.[6])

In mammals, mature oocyte contains at least 100,000 copies of mtDNA, which are found at 1–2 copies per organelle, thus, the ratio of mtDNA versus nuclear DNA (nDNA) is approximately 100,000:1 and during embryogenesis the mtDNA/nDNA ratio is drastically reduced. In somatic cells there are 5–10 copies per organelle and 1000 up to 10,000 mtDNA copies per cell.[6] mtDNA copy number is not random; it is specific for tissue type and developmental stage. Muscle, nerve, and liver contain high mtDNA copy numbers and so do mitotic cells as compared with leukocytes.

However, there is no clear molecular mechanism of mtDNA copy number control. All the components in the mtDNA replisome are important for regulating mtDNA copy number, as shown in budding yeast where deletion of the mtSSB gene resulted in the loss of mtDNA. Furthermore, polG and Twinkle gene mutations result in multiple deletions, and also depletion of mtDNA as observed in human patients and knockout mice models. However, overexpression of these two genes in mice led to increased mtDNA copy number but no pathology.[7,8] It seems that a threshold level of mtDNA copies per cell is a prerequisite for normal mitochondrial function, below this level mitochondria malfunction occur while a high mtDNA copy number is not pathogenic.



The mitochondrial transcription factor (TFAM) also serves as a key regulator of the mtDNA, since TFAM knockout in mice showed mtDNA loss and embryonic death.[9] Overexpression of TFAM increased mtDNA copy number without increasing respiratory chain capacity or mitochondrial mass.[8]

There are other factors that may also play a role in mtDNA copy number control and one of the important factors is the concentration of building blocks for mtDNA synthesis, that is, dNTPs. In yeast overexpression of ribonucleotide reductase led to increased mtDNA, indicating that the dNTP level is a key regulating factor in mtDNA copy number control.[10] Mutations in key enzymes,for example, TK2 and dGK in mtdNTP synthesis caused tissue-specific mtDNA depletion. Mouse models with TK2 knockout or mutant TK2 knockin mutations lead to mtDNA depletion in mice, but the multiorgan phenotype of knockout mice is different as compared to what is observed in human patients, who typically develop myopathy or occasionally lower motor neuron degeneration,[3,4] suggesting that there are species differences in the regulation of tissue specificity of mtDNA precursor synthesis. p53R2 has recently been shown to be responsible for ribonucleotide reduction in nonproliferating cells and mutations in the p53R2 gene caused tissue specific mtDNA depletion.[11,12] Mice lacking p53R2 also developed MDS.[5]

Recently, it was shown that loss of p53 protein led to mtDNA depletion in cultured human fibroblast cells,[13,14] indicating that p53 plays a role in mtDNA copy number maintenance. Thus, regulation of mtDNA copy

number is multi-factorial and at different level through different pathways and the precise mechanism of this control is still not known.

## mtDNA TURNOVER

Mitochondria proliferate through binary fission and their contents including mtDNA are segregated into daughter mitochondria. This process is required not only in proliferating cells where cell division requires a relatively equal distribution of cytoplasmic components into both daughter cells, but also in nonproliferating cells where mitochondria undergo degradation and regeneration. Therefore, mtDNA must replicate so that each organelle has an adequate number of mitochondrial genomes. Earlier work has shown that mtDNA continuously turns over and replicates during the entire cell cycle, without strict phase specificity as is the case with nuclear DNA.[15] In primary rat hepatocytes, which are quiescent cells, a highly active and random turnover of mtDNA was observed while no nuclear DNA replication occurred.[16]

In post-mitotic tissues there is also constitutive turnover of mtDNA.[17] Using mitochondria isolated from rat tissues it was reported that the half-life of mtDNA was highly regulated and varied from tissue to tissue. Heart, liver and kidney had the highest turnover rate (a half-life ~7 days) while brain had a significant lower turnover rate (a half-life ~30 days).[17]

We do not know why mtDNA must undergo turnover in post-mitotic cells while nuclear DNA does not. It might be due to oxidative damage of mtDNA or vulnerability of the mitochondrial genome to degradation due to the structure and/or packing of mtDNA. Nevertheless, high turnover rate or synthesis rate of mtDNA implies a high turnover rate also of the dNTP pools. Therefore, one would expect that in tissues like muscle, liver and brain mt-dNTP metabolism is different from other tissues in order to cope with rapid mtDNA turnover.

## mtDNA PRECURSOR POOL MAINTENANCE

The constant replication of mtDNA creates a demand for the building blocks, for example, dNTPs and balanced dNTP pools are vital for mtDNA synthesis and copy number control. It is well accepted that mtDNA precursors are synthesized either in situ by salvage enzymes or imported from the cytosol (Figure 1). In mitochondria TK2 and dGK catalyze the first and rate-limiting phosphorylation of deoxynucleosides, and thus determine the synthesis rate of dNTPs. The deoxynucleoside monophosphates produced are further phosphorylated by nucleoside monophosphate kinases and nucleoside diphosphate kinase to dNTPs, which can then be incorporated into mtDNA (Figure 1).

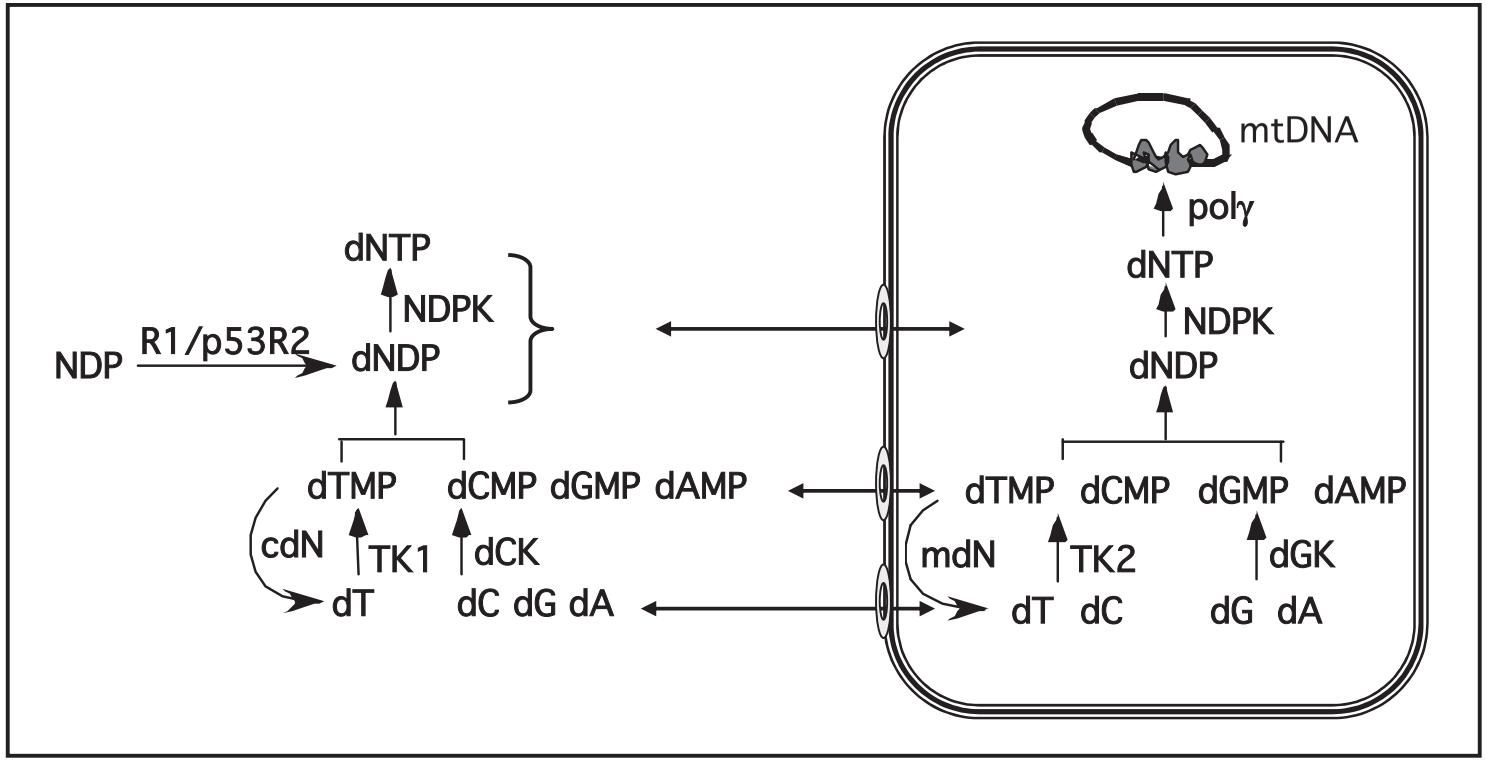


**FIGURE 1** Pathways of DNA precursor synthesis. Abbreviations: p53R2, p53 inducible ribonucleotide reductase small subunit; R1, ribonucleotide reductase large subunit; NDPK, nucleoside diphosphate kinase; TK1, cytosolic thymidine kinase; dCK, cytosolic deoxycytidine kinase; cdN, cytosolic deoxynucleotidase; TP, thymidine phosphorylase; TK2, mitochondrial thymidine kinase 2; dGK, mitochondrial deoxyguanosine kinase; mdN, mitochondrial deoxynucleotidase; pol$\gamma$, mitochondrial DNA polymerase.

## Deoxynucleoside and Deoxynucleotides Uptake and Mitochondrial Transport

In mitochondrial synthesis of dNTPs the first obstacle that has to be overcome is the uptake of deoxynucleosides, which occur through mitochondrial membrane via equilibrative nucleoside transporters (ENT) or concentative nucleoside transporters that are still not well-characterized. There are four ENTs in the human nucleoside transporter family, and hENT1 has been shown to be expressed in plasma membranes and also mitochondrial membranes.[18] Knockdown of hENT1 in cultured fibroblast cells resulted in mtDNA depletion,[19] indicating that deoxynucleoside uptake is the first step of a series of reactions in mtDNA synthesis. Recently hENT3, an intracellular nucleoside transporter, was shown to be localized to mitochondrial membrane and facilitated the import of natural deoxynucleosides and antiviral nucleoside analogs.[20] Thymidine (dT) has been shown to be taken up by isolated mitochondria, phosphorylated to triphosphates,[21] and further incorporated into mtDNA. Uptake of deoxyguanosine (dG) was coupled to ATP synthesis and dG phosphorylation.[22] Using immunocytochemical detection and tissue microarray analyses, hENT1 was detected at high levels in kidney, liver, adrenal gland, and at low levels in lung, and nerve. There was no detectable hENT1 in heart, spleen, muscle and some other tissues.[23] Further study is needed to clarify the tissue specificity in uptake of deoxynucleosides. In most studies it was presumed that the concentration of deoxynucleosides in cytosol and mitochondria is equal to extracellular concentration of these compounds.

There are experimental evidences for the transport of deoxynucleotides into mitochondria and this process could occur at mono-, di-, and triphosphate levels.[24,25] However, the molecular nature of these transporter proteins is not known. The previously described mitochondrial deoxynucleotide carrier protein has turned out be a transporter for thiamine.[24]



## Deoxynucleoside Salvage Enzymes

The next regulatory level is the activity level and tissue specific distribution of salvage enzymes. Mitochondrial TK2 and dGK are constitutively expressed at low level while the cytosolic enzymes TK1 and dCK are mainly expressed in lymphoid tissues or proliferating cells at relatively high levels. In post-mitotic tissues the activity levels of TK1, TK2, dCK, and dGK, measured in whole tissue extracts from mouse, showed a tissue-specific distribution and as did the catabolic enzymes, for example, nucleotidases.[26] However, in this study there was no distinction between TK1 and TK2 activity and TK2 and dCK activity since these three enzymes have overlapping activity.

Recently, we characterized pyrimidine salvage enzymes in isolated mitochondria and cytosol fractions from various rat tissues using ionic exchange

chromatography and taking the advantage of substrate specificity difference of these three enzymes.[27] We found that TK2 is the major pyrimidine salvage enzymes in most tissues both in mitochondria and cytosol. The levels of mitochondrial TK2 activity were highest in spleen, brain and lung, intermediate in liver, heart, and kidney, and lowest in skeletal muscle. In all tested tissues, cytosolic TK2 level was lower compared with mitochondrial TK2. The two cytosolic enzymes, TK1 and dCK, were found only in the cytosolic extracts of spleen and liver, both TK1 and dCK activity was low in liver cytosolic extracts but high in spleen cytosolic extracts.

### Deoxynucleoside Catabolic Enzymes

The catabolic enzymes, for example, pyrimidine deoxynucleotidases and thymidine phosphorylase, were also studied.[27] Cytosolic dNT1 and mitochondrial dNT2 showed similar tissue distribution pattern. dNT1 activity was high in spleen, lung, kidney extracts while dNT2 activity was high in liver, spleen and kidney extracts. Thymidine phosphorylase (TP) activity was detected in both mitochondrial and cytosolic extracts of all tissues, and the cytosolic TP activity was high in liver, heart, spleen and lung but low in brain and skeletal muscle, mitochondrial TP activity was low in all tissues.



### Correlation of dNTP Pool Size and Salvage Enzyme Activity

There are a few studies measuring mitochondrial dNTP pools in postmitotic animal tissues as compared with dNTP pools studies using cultured cells. With mitochondria isolated from rat tissues Song et al. have measured dNTP pools in different organs from rat at different ages.[28] There was no significant difference in dNTP pools between young and old rats, but rather significant differences in different organs was observed. An asymmetry of dNTP pools in rat tissue mitochondria was observed with dGTP as a much larger component (>85%) of the total dNTP pools with the exception that the liver dGTP pool was ~35% of total dNTPs.[24,28] Comparing the size of the four dNTP pools the dCTP/dTTP ratio was high (~20) in heart, liver, and skeletal muscle, but low (~5) in brain; the ratio of dGTP/dATP was high (~30) in heart and skeletal muscle, but low (~ 5) in liver and brain. This difference may be explained by the different levels of anabolic and catabolic enzyme activities in the tissues; TP competes with TK2 for the dT substrate and dNT1/dNT2 competes with TMPK for the dTMP substrate and the out come may be reduced dTTP level in mitochondria, which have high levels of catabolic enzymes. However, there is no known catabolic enzyme that competes with TK2 for dC, and therefore, dCTP can be accumulated at higher levels than dTTP in the mitochondria.

dGTP asymmetry was also observed in mitochondrial dNTP pools measured in cultured primary skin fibroblast cells from normal individuals and

from a patient with dGK deficiency[29] although it was not as substantial as was observed in rat tissues.[24,28] However, dGTP asymmetry was not observed in dNTP pools measured in mouse liver mitochondria.[25] Technically, it is more difficult to measure mitochondrial dNTP pools due to the small size of mtdNTP pools and degradation of dNTP during mitochondria isolation procedure. Ferraro et al. have found large variations in mtDNA pools in relation to ATP pool and the length of mitochondria isolation procedure.[25] The high mitochondrial dGTP pool reported by Song et al. may be due to the assay system used.

TK2 activity with dT as substrate measured in rat tissue mitochondrial extracts plotted against the size of mitochondrial dTTP pool in the corresponding tissue demonstrated a linear correlation of TK2 activity to dTTP pools (Figure 2A). This result suggests that TK2 is the rate-limiting enzyme in the synthesis of mitochondrial dTTP. When TK2 activity measured with dC as substrate was plotted against the dCTP pool no correlation was observed (Figure 2B). Especially, brain mitochondrial dCTP pool is much lower than expected as compared to the TK2 activity. This could be due to the fact that dCTP is involved in lipid metabolism in brain tissue, which resulted in lower concentration of free dCTP.



With isolated rat heart mitochondria McKee et al.[21] studied phosphorylation of dT and the kinetics of the total phosphorylated products e.g. the sum of dTMP, dTDP, and dTTP versus dT concentrations showed negative cooperativity and the $K_m$ values were in the same range as in studies with purified human TK2.[30] Recently, Morris et al. showed that dTTP and dCTP are solely synthesized from dT and dC via phosphorylation by TK2 in perfused rat heart and there was no detectable de novo pyrimidine synthesis activity.[31] All these results indicated that TK2 is essential in mitochondrial dTTP synthesis in post-mitotic tissues.

The high levels of dCTP and dGTP pools in rat mitochondria reported by Song et al.[24,28] are unexpected but the dTTP and dATP pools were in similar range to the values reported by others,[25,29,31,32] and dTTP pools correlated to mitochondrial TK2 activity. Therefore, it is important to perform further studies on the level of enzymes and mtdNTP pools in animal tissues in order to understand how mtDNA synthesis and turnover is controlled.

dGK activity reported in whole mouse tissue extracts was high[26] and since we know that there are many other interfering enzymes in the extracts, and dGK enzyme activity is sensitive to many factor in the assays, therefore, dGK levels in mitochondria needs to be determined with a more specific assay.

## De Novo DNTP Synthesis Activity in Post-Mitotic Tissue

One of characteristics of de novo synthesis of dNTPs is cell cycle regulation of ribonucleotide reductase, TK1 and thymidylate synthase (TS) activity.

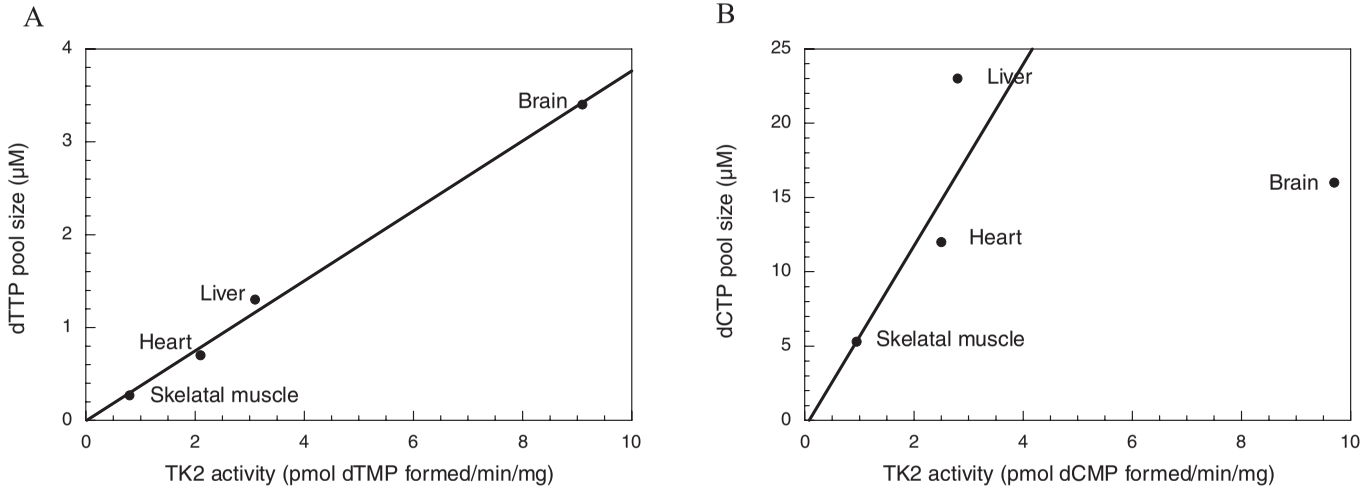


**FIGURE 2** Correlation of TK2 activity to dTTP and dCTP pools in rat mitochondria. A) TK2 activity measured with dT as substrate versus dTTP pool in the same tissue. B) TK2 activity measured with dC as substrate versus dCTP pools. The dTTP pool and dCTP pool data were from Song et al.[28]

These enzymes have the highest activity in S-phase cell in order to provide dNTPs for nuclear DNA replication. After mitosis, their activities decrease drastically due to proteolytic degradation. In nonproliferating cells, de novo synthesis of dNTP is minimal. However, the discovery of p53R2 suggests that de novo synthesis activity occurs in nonproliferating cells[11] and mutations in p53R2 caused MDS.[12] However, TS activity is required for de novo synthesis of dTTP, and therefore, until TS activity, especially in muscle, liver and brain, is determined the capacity for de novo dTTP synthesis is not known.

## WHY IS MDS TISSUE SPECIFIC?

As mentioned earlier, mutations in several nuclear genes resulted in tissue specific mtDNA depletion although in some cases multiple organs are involved. Three genes involved in dNTP synthesis, TK2, dGK, and p53R2, give different phenotypes, that is, TK2 and p53R2 mutations caused myopathic form of MDS while dGK mutation affected mainly the liver. Liver and muscle tissues have high-energy demand, which is provided mainly by oxidative phosphorylation. Mitochondrial respiratory chain activity requires the expression of all mtDNA encoded proteins and is directly correlated to mtDNA copy number. Reduced mtDNA content result in less mtDNA encoded proteins produced and low respiratory activity. Defect TK2, dGK, or p53R2 will impair synthesis of dNTPs, which lead to imbalanced dNTP pools and reduced mtDNA replication and mtDNA depletion. Both muscle and liver have high mtDNA turnover, and the dTTP pool is lowest in muscle mitochondria while the dGTP pool is lowest in liver mitochondria.[28] This could explain why muscle mitochondria are sensitive to fluctuation in the dTTP pool while liver mitochondria are more sensitive to changes in the dGTP pools. Thus, TK2 defects preferentially affect muscle tissue and dGK deficiency affects liver. The p53R2 dependent cytosolic synthesis apparently can not compensate the defect in the salvage pathway, most likely because of limited TS activity for dTTP synthesis and/or limiting transport of deoxynucleotides across mitochondrial membrane in these tissues. Still many other factors may contribute to tissue specificity of MDS, and further studies are needed in order to elucidate the tissue specific pattern of dNTP metabolism in human and in other animal model systems.



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