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Novel Insight into Thymidylate Kinase: Biochemical Characterization of the Human and Zebrafish Enzymes

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Thymidylate Kinase in Human and Zebrafish

Abstract

Deoxythymidine triphosphate (dTTP or T) is one of the four building blocks for DNA. In dTTP synthesis, the catalysis of dTMP to dTDP, is mediated by one known enzyme only: Thymidylate kinase (TMPK, EC 2.7.4.9). Two clinical reports on TMPK mutations indicate that mutations in TMPK cause neurological disorders in humans. To increase our understanding of TMPK, we performed extensive investigations using human fibroblasts and zebrafish embryos.

In paper I, the main purpose was to characterize TMPK mutations and their effects on dTTP synthesis with human fibroblast cells. TMPK mutations dramatically decreased the activity of the enzyme; intriguingly dTMP could still be incorporated into DNA without affecting cell proliferation rates. This implied that an alternative TMPK may exist in fibroblasts. By combining western blot and enzyme activity measurements in subcellular protein fractions, a new TMPK activity in the outer membrane preparations of mitochondrial protein fraction was detected (paper II). Isolation of this alternative TMPK enzyme from fibroblasts was partially performed.

Different human TMPK isoforms were studied in paper III. Isoforms 2, 3, 4, and 5 were not functional, due to the lack of important structural elements. Recombinant isoform 6 showed one thousand times lower activity than isoform 1. Thus, none of the isoforms 2-6 are likely to be the alternative TMPK.

In study IV, we used zebrafish embryos to further study TMPK. The canonical TMPK showed a dynamic expression pattern. The protein levels were the highest at 24 hours post fertilization, which is the neurulation period. Our result indicated that there was a correlation between the canonical TMPK expression and neuronal development.

In summary, our studies expand our understanding of the TMPK enzyme, and provides a correlation between TMPK and neuronal pathology during early development. They further suggest that a novel form of TMPK enzyme exists and is important during embryonic development.

Keywords: (Nucleotides synthesis, dTMP kinase, Thymidylate kinase, TMPK, Deoxythymidine monophosphate kinase)

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Tymidylatkinas hos människa och Zebrafisk

Abstrakt

Deoxitymidintrifosfat (dTTP eller T) är en av de fyra byggstenarna för DNA. I dTTP-syntes förmedlas katalysen av dTMP till dTDP av endast ett känt enzym: tymidylatkinas (TMPK, EC 2.7.4.9). Två kliniska rapporter om TMPK-mutationer indikerar att dessa mutationer orsakar neurologiska störningar hos människor. För att öka vår kunskap om TMPK utförde vi omfattande undersökningar med mänskliga fibroblaster och zebrafiskembryon.

I delstudie I var huvudsyftet att karakterisera TMPK-mutationer och deras effekter på dTTP-syntes med mänskliga fibroblaster. Mutationerna orsakade en dramatisk minskning av enzymets aktivitet; intressant nog kunde dTMP fortfarande inkorporeras i DNA utan att påverka hastigheten av cellväxt. Detta antydde att ett alternativt TMPK kan finnas i fibroblasterna. Genom att kombinera western blot-analys och enzymaktivitetsmätningar i subcellulära proteinfraktioner detekterades en ny TMPK-aktivitet i yttermembranpreparaten av den mitokondriella proteinfraktionen (delstudie II). Isolering av detta alternativa TMPK-enzym från fibroblaster kunde endast delvis fullföljas.

Olika mänskliga TMPK-isoformer studerades i delstudie III. Isoformerna 2, 3, 4 och 5 var inte funktionella på grund av bristen på viktiga strukturella element. En rekombinerad isoform 6 visade tusen gånger lägre aktivitet än isoform 1. Därför kunde ingen av isoformerna 2-6 sannolikt vara det sökta alternativa TMPK-enzymet.

I delstudie IV använde vi embryon från zebrafisk för att ytterligare undersöka TMPK. Kanonisk TMPK visade ett dynamiskt uttrycksmönster. Proteinnivåerna var högst 24 timmar efter befruktning, vilket är neurulationsperioden. Vårt resultat indikerade att det fanns en korrelation mellan det kanoniska TMPK-uttrycket och den neurala utvecklingen.

Sammanfattningsvis utökar våra studier förståelsen av TMPK-enzymet och visar en korrelation mellan TMPK och neuronal patologi under tidig utveckling i zebrafiskar. Våra resultat föreslår också att en ny form av TMPK-enzym existerar och är viktig under embryonal utveckling.

Keywords: (Nucleotides synthesis, dTMP kinase, Thymidylate kinase, TMPK, Deoxythymidine monophosphate kinase)

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Dedication

To my dear family and friends.

Loving life

GZ, Wang

I do not want to think over

that I´ll be able to succeed.

Since I´ve decided to go to a distant place,

I´ll try my best to make the trip.

…

I do not want to think over

that I´ll meet with the fierce wind and cold rain

Since my destination is the horizon,

I´ll leave a deep impression on the Earth.

I do not want to think over

If my future will be smooth or bumpy.

Since I enjoy my life,

Everything is to be expected.

热爱生命

江国真

我不去想是否能够成功

既然选择了远方

便只顾风雨兼程

我不去想身后会不会袭来寒风冷雨

既然目标是地平线

留给世界的只能是背影

我不去想未来是平坦还是泥泞

只要热爱生命

一切， 都在意料之中

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. JunMei Hu Frisk, Jo M.Vanoevelen, Jörgen Bierau, Gunnar Pejler, Staffan Eriksson, and Liya Wang (2021). Biochemical Characterizations of Human TMPK Mutations Identified in Patients with Severe Microcephaly: Single Amino Acid Substitutions Impair Dimerization and Abolish Their Catalytic Activity. ACS Omega, 6, 33943-33952. https://doi.org/10.1021/acsomega.1c05288

II. JunMei Hu Frisk, Staffan Eriksson, Gunnar Pejler, and Liya Wang (2020). Identification of a novel thymidylate kinase activity. Nucleosides, Nucleotides and Nucleic acids, 39 (10-12), 1359-1368. https://doi.org/10.1080/15257770.2020.1755043

III. JunMei Hu Frisk, Gunnar Pejler, Staffan Eriksson, and Liya Wang (2022). Structural and functional analysis of human thymidylate kinase isoforms. Nucleosides, Nucleotides and Nucleic acids. https://doi.org/10.1080/15257770.2021.2023748

IV. JunMei Hu Frisk, Stefan Örn, Gunnar Pejler, Staffan Eriksson, and Liya Wang (2022). Differential Expression of Enzymes in Thymidylate Biosynthesis in Zebrafish at Different Developmental Stages: Implications for *dtymk* Mutation-caused Neurodegenerative Disorders. BMC Neuroscience. https://doi.org/10.1186/s12868-022-00704-0.

Papers not included in this thesis:

I. Jun Mei Hu Frisk, Lena Kjellén, Stephen G.Kaler, Gunnar Pejler and Helena Öhrvik (2017).Copper Regulates Maturation and Expression of an MITF: Tryptase Axis in Mast Cells. The Journal of Immunology, 199 (12), 4132-4141. https://doi.org/10.4049/jimmunol.1700786

II. Jun Mei Hu Frisk, Lena Kjellén, Fabio R. Melo, Helena Öhrvik and Gunnar Pejler (2018). Mitogen-Activated Protein Kinase Signaling Regulates Proteoglycan Composition of Mast Cell Secretory Granules. Frontiers in Immunology, (9), 1670. https://doi.org/10.3389/fimmu.2018.01670

III. Gunnar Pejler, Jun Mei Hu Frisk, Daniel Sjöström, Aida Paivandy & Helena Öhrvik (2017). Acidic pH is essential for maintaining mast cell secretory granule homeostasis. Cell Death & Disease (8) e2785.

IV. Jo M.Vanoeveln, Jörgen Bierau, Janine C. Grashorn, Ellen Lambrichs, Erik-Jan Kamsteeg, Levinus A.Bok, Ron A, Wevers, Marjo S.van der Knaap, Marianne Bugiani, JunMei Hu Frisk, rita Colnaghi, Mark O´Driscoll, Debby M.E.I.Hellebrekers, Richard Rodenburg, Carlos R.Ferreira, Han G. Brunner, Arthur van den Wijngaard, Ghada M.H. Abdel-Salam, Liya Wang & Constance T.R.M. Stumpel. (2022). *DTYMK* is essential for genome integrity and neuronal survival. Acta Neuropathologica, 143, 245-262. https://doi.org/10.1007/s00401-021-02394-0.

V. JunMei Hu Frisk, Staffan Eriksson, Gunnar Pejler, and Liya Wang (2022). Heavy metal tolerance of *Mesorhizobium delmotii* thymidylate kinase. Nucleosides, Nucleotides and Nucleic acids. http://dx.doi.org/10.1080/15257770.2055059

VI. JunMei Hu Frisk, WeiHua Ye, Stefan Knight, Staffan Eriksson, Gunnar Pejler, and Liya Wang (2022). Structural and functional studies of thymidylate kinase from *Mesorhizobium delmotii* (manuscript).

The contribution of JunMei Hu Frisk to the papers included in this thesis was as follows:

I. Planned the studies with help from supervisors, performed most laboratory work and data analysis and writing the first version of the manuscript.

II. Planned the studies with help from supervisors, performed most laboratory work and data analysis and writing the first version of the manuscript.

III. Planned the studies, performed most laboratory work and data analysis and writing the first version of the manuscript.

IV. Planned the studies with consultation/guidance from supervisors and Stefan Örn, performed most laboratory work & data analysis and writing the first version of the manuscript.

Abbreviations

DNA	deoxyribonucleic acid
dThd	deoxythymidine / thymidine
dTMP	deoxythymidine monophosphate
dTDP	deoxythymidine diphosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUDP	deoxyuridine diphosphate
dUTP	deoxyuridine triphosphate
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
ATP	Adenosine triphosphate
dAdo	deoxyadenosine
dAMP	deoxyadenosine monophosphate
dADP	deoxyadenosine diphosphate
dATP	deoxyadenosine triphosphate
dCyd	deoxycytidine
dCMP	deoxycytidine monophosphate
dCDP	deoxycytidine diphosphate
dCTP	deoxycytidine triphosphate
dNMP	deoxynucleoside monophosphate
dNDP	deoxynucleoside diphosphate

dNTP	deoxynucleoside triphosphate
dGMP	deoxyguanosine monophosphate
dGDP	deoxyguanosine diphosphate
dGTP	deoxyguanosine triphosphate
IMP	Inosine 5-monophosphate
MTHF	N^5,N^{10}-methylenetetrahydrofolate
dGK	deoxyguanosine kinase
dCK	deoxycytidine kinase
NDPK	Nucleoside diphosphate kinase
dNK	deoxynucleoside kinase
pol	DNA polymerase
TMPK	Thymidylate kinase, thymidine monophosphate kinase
TK1	Thymidine kinase 1
TK2	Thymidine kinase 2
TYMS	Thymidylate synthase
RNR	Ribonucleotide reductase
hpf	hours post fertilization in zebrafish embryos

1. Introduction

1.1 DNA polymerase and deoxyribonucleoside triphosphates (dNTPs)

1.1.1 DNA polymerase

DNA polymerase (pol) exists in life forms, which have DNA as genetic material. Pol is a general term for enzymes that catalyze the elongation of DNA polymers in the direction of 5´ to 3´ of a growing chain in a template-directed manner. In mammalian cells, there are ~16 reported DNA polymerases, marked with letters from the Greek alphabet: α, β, ɣ, δ, ε, ζ, η, etc. Based on sequence homology and structural similarities, pols have been grouped in five different families: A, B, C, X and Y (Braithwaite DK & J Ito 1993; Ohmori H et al., 2001). Each pol has roles in one or more pathways of DNA synthesis and processing. For example, pol α is mainly responsible for nuclear DNA replication, pol β for DNA repair, and pol ɣ for mitochondrial DNA replication (Hubscher U et al., 2002, Rood RD & Doublie S 2016). One conserved feature of all pols is that they recognize dNTPs and hydrolyze the dNTPs to pyrophosphate and deoxyribonucleoside monophosphates **(**dNMPs) during the polymerization reaction.

On the template-directed DNA strand, pol α binds incoming complementary dNTP and cleaves the bond between α-, and β- phosphates of dNTP. The cleavage of the phosphoanhydrider bond renders a large negative Gibbs free energy (ΔG) ~7 kcal/mol (Kottur J & Nair DT 2018). This energy is further used as an energy source to form a phosphodiester bond (ΔG_{inc}) between the new incorporated dNMP and the growing DNA chain.

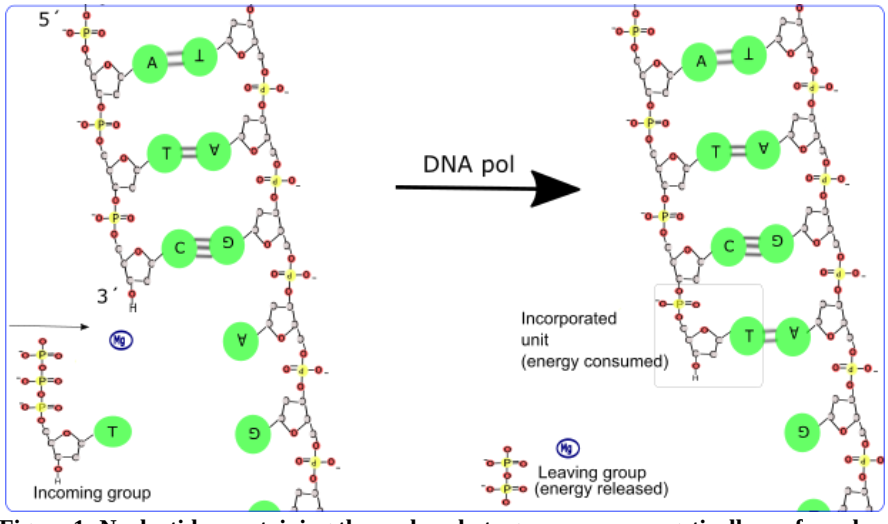


Figure 1: Nucleotides containing three phosphate groups are energetically preferred substrates for DNA polymerases. The pol mediated catalytic reaction contains several critical steps: pols recognize the "right" dNTP, hydrolyze the dNTP to dNMP, release energy which facilitates the α-phosphate of the new dNMP to form a phosphodiester bond with the 3´-hydroxyl group of the growing DNA chain. The released high energy ($\Delta G < 0$) from the cleavage of phosphate bonds is used as an energy source for the addition of nucleotides to the backbone of DNA, thus achieving energy balance. The outcome is DNA chain elongation by one unit at the 3´ end of the growing strand and a released pyrophosphate group.[1]

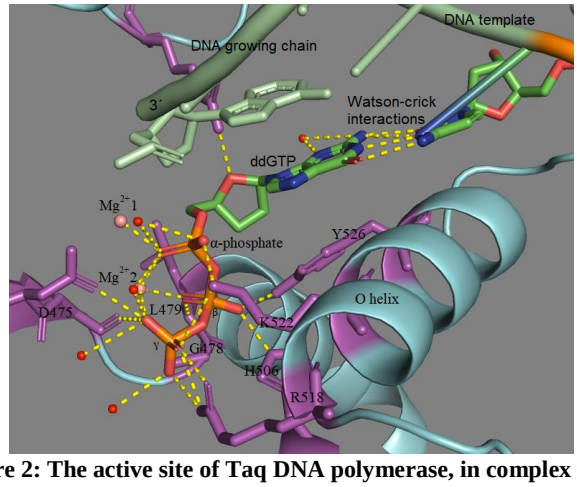


Figure 2: The active site of Taq DNA polymerase, in complex with DNA, ddGTP, and Mg^{2+}. The three phosphate groups of ddGTP, especially β- and ɣ- phosphate, induce a closed conformation of the active site. As shown by Tap DNA polymerase, the ɣ- phosphate interacts with residues of R518, K522 on O helix (causing rotation of the O-helix to the palm domain of pol), of D475 (β-strand pointing to the O helix), and of G478 connecting

1 The figure was created by program InkScape.

loop, and Mg^{2+}. Therefore, ɣ- phosphate from ddGTP has structural importance for the catalysis activity. For clarity, only the structures having direct interaction with the active site are shown. Water are in red color and Mg^{2+} in salmon, the interacting residues (one letter) to phosphate groups are in magenta. The structure was downloaded from the Protein Data Bank with PDB code: 1F7P, and visualized in program PyMol.

In my very early stage as a PhD student, a naive question arose in my mind. Why only dNTPs? Can deoxyribonucleoside diphosphates (dNDP) or dNMP be utilized by pols for polymerization? The answer came from the energy laws of thermodynamics (Figure 1) and structural analysis of pols (Figure 2).

As mentioned earlier, dNTPs are energetically favorable for catalysis. The energy required for each dNMP incorporation varies between 3.5-7 kcal/mol. The energy gained from pyrophosphorolysis is consumed by polymerization and thus reaches energetic equilibrium in the long term. dNDPs have much higher K_m values and thus much lower DNA synthesis efficiency (Bruker CR & Luptak A, 2017). dNMPs have no high energetic bonds as dNTPs. Thus, dNTPs are the energetically preferred substrates for pols.

In the crystal structure of Taq DNA polymerase (PDB code: 1F7P), the three phosphate groups of ddGTP (analog to dGTP) stack between the DNA base at the primer 3´-end and the O helix of pol I, producing a pronounced tilt between the O helix and the DNA template. The binding from ddGTP to O helix of pol are strongly facilitated by interactions between β- and α-phosphates (Doublie S et al., 1998). The ɣ- phosphate is directly interacting with D475, G478, R518, K522 and the second Mg^{2+}. These interactions are essential to the pol´s active site. Therefore, the ɣ-phosphate has a significant importance for the conformational change and catalysis.

1.1.2 dNTPs pool

dNTP pools contain four structurally similar deoxynucleotide triphosphates: deoxythymidine triphosphate (dTTP or T), deoxycytidine triphosphate (dCTP or C), deoxyguanosine triphosphate (dGTP or G) and deoxyadenosine triphosphate (dATP or A). The four nucleotides are composed of a single ring (pyrimidine) or a pair of fused rings (purine), a pentose (a five-carbon sugar moiety) and phosphate group(s). Nucleosides contains no phosphate group. The number of phosphate groups in nucleotides determines the prefix of the compound; they are called deoxyribonucleoside monophosphate

(dNMP), deoxyribonucleoside diphosphate (dNDP) or deoxyribonucleoside triphosphate (dNTP), respectively. Due to the presence of phosphate groups, the nucleotides are acidic and negatively charged. The phosphate group positioned nearest the carbon of the sugar are named α-, β-, and ɣ-phosphates (Figure 3). A balanced dNTP pool is the result of strict regulation at multiple steps.

Pols have faithful processivity and the fidelity of pols processivity is a key determinant of genome stability. From the structural and biochemical aspects, the determining factors affecting DNA replication fidelity are that: geometrical constraints imposed in the active site of pols strongly favor Watson-Crick over non-Watson-Crick interactions; two hydrogen bonds are preferred over one in nucleotide selectivity (Kool ET 2001, Kunkel TA, Bebenek K 2000, Ludmann S & Marx A 2016). Except for this biochemical feature, mammalian cell culture studies provided another important aspect at DNA replication fidelity: a balanced supply of dNTP pool is essential for the accuracy of DNA replication. An imbalanced dNTP pool triggers nuclear DNA fragmentation and cell apoptosis as found in different cell culture studies (Oliver FJ et al., 1996; Yoshioka A et al., 1987; Meuth M 1989; Pai CC & Kearsey SE 2017). This is not only applicable to nuclear DNA, as Nikkanen et al., 2016 also demonstrated that perturbation of the dNTP pool in mitochondria is consistent with mtDNA replication disorders in mice.

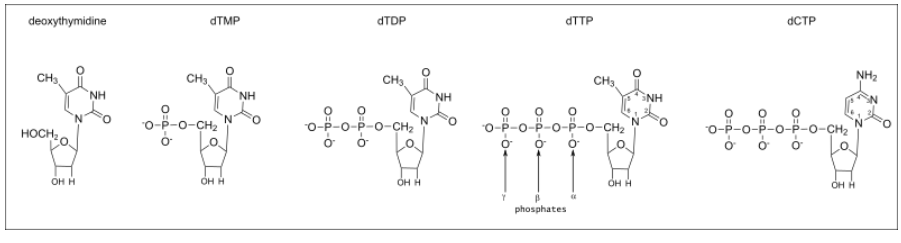


Figure 3: Nomenclature and structure of the pyrimidine nucleoside and nucleotides. As examples, deoxythymidine and its phosphorylated forms are shown. Deoxythymidine can be converted to dTTP by three consecutive phosphorylation steps. dTTP and dCTP differ mainly in the C4 and N5 positions of the base. This feature determines enzyme specificity, which will be explained in a later section (TMPK structure).

During the different phases of the cell cycle, cytosolic dNTPs varies greatly in concentration. In general, four dNTPs are arranged in descending order according to their concentrations: dTTP > dCTP > dATP > dGTP in humans. The same order is also seen in the mitochondrial dNTP pool. dTTP is always in greater abundance compared to the other three dNTPs (Kornberg et al

1992; Rampazzo et al.,2004). Furthermore, the dNTP pools are much lower in resting cells than in proliferating cells.

1.2 Nucleotide metabolism

A balanced dNTP pool is depending on three forms of biochemical regulation: synthesis, consumption and degradation. These steps are strictly regulated under normal physiological conditions. Many enzymes play a role in maintaining dNTPs in their normal range. An unbalanced dNTP pool has been recognized as a factor reducing genome integrity, and causing diverse genetic disorders as demonstrated in many clinical studies. In the coming section, a brief description of nucleotide synthesis and degradation will be introduced to gain an overview of the field, with a particular focus on TMPK.

1.2.1 The *de novo* pathway

Biosynthesis of dTTP, dCTP, dATP and dGTP are under tight regulatory control in cells. There are two principal pathways for generation of nucleotides: the *de novo* pathway and the salvage pathway. The synthesis of dNTP involves many specific and few unspecific enzymes (Figure 4).

In the *de novo* pathway, both pyrimidines and purines are generated from amino acids, CO_2, and phosphoribosyl pyrophosphate (PRPP), with high ATP consumption. The procedure requires many enzymatic reactions. Inosine 5-monophosphate (IMP) is the central intermediate of purine synthesis, and is converted to AMP or GMP via distinct two-step reactions. For AMP synthesis, adenylosuccinate synthetase and adenylosuccinase are two main players. For GMP production, IMP dehydrogenase and GMP synthetase catalyze the reaction.

The next phosphorylation step of dCMP, dTMP, dAMP, dGMP, and UMP to their corresponding diphosphates is catalyzed by specific nucleoside monophosphate kinases: CMP kinase, dTMP kinase, AMP kinase, GMP kinase, and UMP kinase, respectively.

Ribonucleotide reductase (RNR EC 1.17.4.1), or ribonucleoside diphosphate reductase, catalyzes the formation of dUDP, dADP, dGDP and dCDP from the substrates UDP, ADP, GDP and CDP, respectively. This reaction is

involved in reducing the 2´-hydroxyl group of the pentose ring of the nucleoside diphosphates to a hydrogen group. Importantly, dTDP, different from all other dNDPs, is not a direct product of RNR-mediated reactions, but requires further steps invovling thymidylate synthase and TMPK.

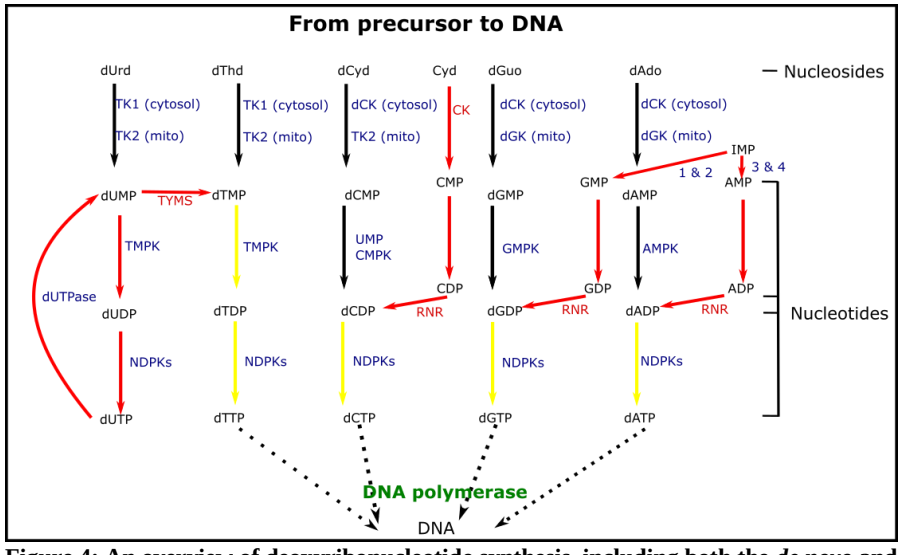


Figure 4: An overview of deoxyribonucleotide synthesis, including both the *de novo* and the salvage pathways. The overview summarized key enzymes (presented with abbreviation or number) in dNTP synthesis. Syntheses of dCDP, dGDP and dADP have similar pathways; while dTDP synthesis has a separate pathway. The figure is simplified and does not include all the metabolic steps. The salvage pathway is labelled with a black pil, the *de novo* pathway in red and the converged pathway shown in yellow. Enzymes are in blue and compounds in black. Enzymes with numbers are adenylosuccinate synthetase (1), Adenylosuccinase (2), IMP dehydrogenase (3), GMP synthetase (4).

1.2.2 The salvage pathway

The salvage pathway of dNTP synthesis is more energetically favorable since it utilizes preexisting deoxyribonucleosides from extracellular sources, typically from degraded nutrients or recycled DNA.

Deoxyribonucleoside kinases (dNKs) are a family of enzymes that phosphorylate deoxynucleosides to dNMPs. The four dNKs in mammalian cells are deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK), thymidine kinase 1 (TK1) and thymidine kinase 2 (TK2). The latter two enzymes are involved in dTTP synthesis and will be described in the section of dTTP synthesis.

Human dCK (EC 2.7.1.74), located in cytosol, is encoded by a gene on chromosome 4. It has a molecular weight of 30 kDa, presenting as a homodimer in the crystal structure. Human dCK has a relative broad substrate specificity and it can catalyze phosphorylation of the natural substrates deoxycytidine (dCyd), deoxyadenosine (dAdo), deoxyguansine (dGuo) and some chemotherapeutic agents, such as AraC (anti-cancer compound), CdA (anti-leukemic compound) and ddC (anti-HIV compound) (Eriksson et al., 1991). ATP and UTP can both be phosphate donors.

Human dGK (EC 2.7.1.113) has a molecular weight of 29 kDa and exists as a dimer. dGK is a mitochondrial enzyme and phosphorylates dGuo, dIno and dAdo. Expression of dGK is low in many cells and tissues, including spleen, skin, liver, placenta and neoplastic tissue. dGK expression is not correlated to cell cycles.

NDPKs catalyze the transfer of ɣ- phosphate from ATP (or other nucleoside triphosphate) to different nucleoside diphosphates (NDPs or dNDPs). All dNDPs can serve as phosphate acceptor. NDPKs are ubiquitous and unspecific enzymes, located in different subcellular locations. NDPKs are encoded by *NM23* (also named *NME*). In mammals, *NME* encodes the NM23H family, including 10 identified proteins: NM23 H1, H2, H3, H4, H5, H6, H7, H8, H9 and H10 (Kim B & Lee KJ. 2021). Due to the abundance of NDPKs, they may be considered to be on a free passage for dNDPs. The dNDPs thus can be converted to dNTPs quickly; the rate-limiting step of dNTPs synthesis is instead the dNDP synthesizing enzymes, i.e., nucleoside monophosphate kinases.

1.2.3 Nucleotide degradation

Purines and pyrimidines can be either recycled via the salvage pathway or be degraded, to maintain homeostasis of the dNTP pools. Key enzymes responsible for dNTPs degradation are 5´-nucleotidase (5´-NT), thymidine phosphorylase, cytidine deaminase and uridine phosphorylase.

5´-NT hydrolyzes the phosphodiester bond between the 5´-carbon and the phosphate group in a nucleoside monophosphate. 5´-NT is a collective name, with three major classes of enzymes, namely serum 5´-NT, ecto-5´-NT and cytosolic 5´-NT. They are classified by their subcellular localization and

substrate preferences. Thymidine phosphorylase (EC 2.4.2.4) is a major dThd degrading enzyme in humans. It cleaves off the sugar moiety from dThd that converts dThd to thymine and deoxyribose-1-phosphate. Cytidine deaminase (EC 3.5.4.5) catalyzes the deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively. Uridine phosphorylase (EC 2.4.2.3) converts uridine to uracil and ribose-1-phosphate.

1.3 dTTP synthesis

The biosynthesis of dTTP shares similarities and differences with the biosynthesis of the other three dNTPs. RNR plays an indirect role in the upstream of *de novo* synthesis of dTTP, by converting UDP to dUDP. dTDP is distinct from other dNDPs since it cannot be generated directly by RNR (Figure 5). Thus, dTDP synthesis by TMPK is the only route, and is thereby the rate-determining step of dTTP synthesis.

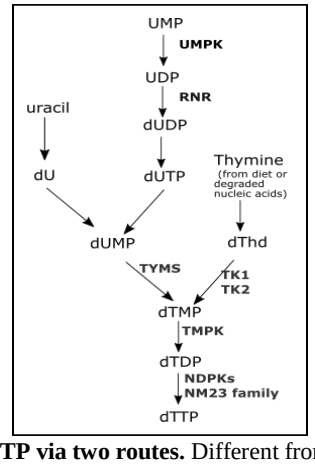


Figure 5: Biosynthesis of dTTP via two routes. Different from other dNDPs, RNR in dTTP synthesis cannot generate dTDP directly from dUDP. dTMP can be synthesized by either TYMS (the *De novo* pathway) or TK1/TK2 (the salvage pathway). Therefore, TMPK is a rate-limiting step in both *De novo* and the salvage pathways. Enzyme are marked with bold text, and compounds in regular style.

1.3.1 Thymidylate synthase

Thymidylate synthase (TYMS EC 2.1.1.45), having a molecular weight of 37 kDa, is responsible for dTMP synthesis by transferring a methyl group from N5, N10-methylenetetrahydrofolate (MTHF) to the pyrimidine base of dUMP. The reaction gives the products: dTMP and dihydrofolate (DHF). TYMS provides the *de novo* pathway for production of dTMP.

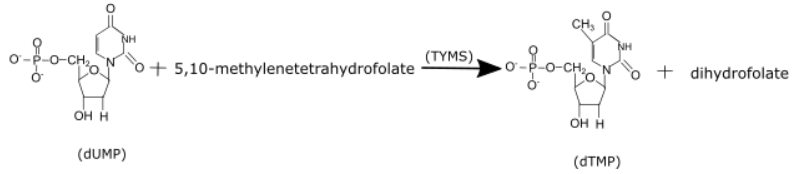


Figure 6. TYMS-mediated synthesis of dUMP to dTMP. TYMS catalyzes the reductive methylation of dUMP at position of C5, and the product is dTMP.

1.3.2 TK1

TK1 and TK2 are two enzymes of dTMP synthesis in the salvage pathway; they have different intracellular locations and somewhat overlapping substrates. TK1 is identified as a cytosolic enzyme. TK1 expression is tightly regulated throughout the cell cycle, with the highest level in the S phase and is reduced to a minimal level during the late G2 and M phases in different cell culture studies. Ubiquitin-proteasome degradation is the main mechanism for regulation of the TK1 levels under normal conditions during the G2/M phases.

TK1 phosphorylates thymidine to dTMP, with very high affinity (K_m: 0.5-3 µM); it can also accept deoxyuridine but not dCyd as substrate. TK1 exists both as dimer, tetramer and polymer forms. Polymerization has considerable significance for the enzyme activity. TK1 as a tetramer has 30 times higher activity than its dimer (Jensen & Munch-Petersen 1994; Munch-Petersen et al., 1995). Different cofactors affect TK1 activity. ATP induces TK1 from a closed dimer to an open active tetramer (Segura-Pena et al., 2007); Zn^{2+} stabilizes the enzyme´s globular structure and forms part of the substrate binding site (Welin et al., 2004); and Mg^{2+} plays a vital role in the active site by bridging the ATP and dThd.

TK1 is the most intensively studied among the salvage enzymes from different organisms but more specifically in companion animals and humans. One of the most pronounced characteristics of TK1 is that serum TK1 level is correlated with malignancies in human patients, dogs, cats and horses (Taylor SS et al., 2012, Wang L et al., 2021, Saellström S et al., 2022). Therefore, TK1 has significant clinical importance, for screening malignant diseases and predicting the re-occurrence after cancer treatment (Bitter EE

et al., 2020). Applications of serum TK1 as biomarker in proliferative diseases started in the early 1980s. The first generation of TK1 assay that measured serum TK1 was focused on TK1 activity measurement by using ^{125}I or ^{3}H-dThd as substrate (Gronowits & Kallander 1980 & Gronowitz et al., 1983). The next generation of the TK1 assay was non-radiometric assay (developed in the late 1990s), using the substrate analogue zidovudine (AZT) to replace radiolabeled dThd as substrate in the measurement. Antibodies against AZT-monophosphate was used to measure the TK1 levels (Öhrvik et al., 2004). 5-bromo-deoxyuridine (BrdU) is another alternative substrate used in the DiviTum TK1 assay. The product BrdU-monophosphate could be incorporated into a poly-A oligonucleotide in a microtitre plate (Nishman et al., 2013). The TK1 ELISA produced by Arocell AB, utilizing C-terminal specific monoclonal antibodies for detection of the TK1 protein complex in serum. It is a robust and sensitive assay; that can easily be adopted to routine clinical practice for diagnostic and prognostic purposes. Pets, like dogs and cats, have acquired increased status and value in our modern society but also have increased prevalence and treatment options for cancer diseases. A TK1 sandwich ELISA with dog-specific antibodies has been developed and studied. It has been clearly shown to be overall more sensitive than TK1 activity assays, especially with serum samples from solid tumors (Jagarlamudi KK et al., 2015). Now, a TK1 ELISA kit for dogs (Alertix Veterinary Diagnostic AB) is under clinical investigation. It is a long and remarkable journey for TK1 biochemistry leading to practical contributions to human and veterinary medicine.

1.3.3 TK2

TK2 has a broader substrate range than TK1, and it can phosphorylate several natural compounds, i.e., dThd, dCyd and dUrd. Both ATP and CTP can serve as phosphate donors, with ATP being somewhat more efficient. TK2, different from TK1, is constitutively expressed, mainly in mitochondria, playing an essential role in the constant and balanced supply of dNTPs for mtDNA (Wang L et al., 2003). TK2 activity has a tissue-specific distribution, with high expression in brain, lung and spleen, and low in skeletal muscle (Wang L, 2010)

TK2 mutations are correlated to pathophysiological conditions, of which the most prevalent one is the mitochondrial DNA depletion syndrome (MDS), which results in myopathy, hepato/neuropathy and progressive external ophthalmoplegia, often in early childhood (Wang L 2010; Eriksson et al., 2002).

1.4 Thymidylate kinase

1.4.1 TMPK in dTTP synthesis

dTMP from the *de novo* and the salvage pathway converges in the requirement for Thymidylate kinase (TMPK). It is thus an essential intermediate enzyme in dTTP synthesis, since there is no known mechanism to bypass this step (Figure 5). The TMPK-mediated reaction utilizes ATP as phosphate donor and Mg^{2+} as cofactor, giving dTDP and ADP as products.

Human TMPK is encoded by the gene *DTYMK* (gene synonyms: *CDC8*), located on chromosome 2 (www.ensembl.org). In GenBank, it is deposited with seven predicted exons giving rise to 6 putative protein isoforms (GenBank NP_036277.2 for isoform 1, NP_001158503.1 isoform 2, NP_001307831 isoform 3, NP_001307832.1 isoform 4, NP_001307833.1 isoform 5 and NP_001307834.1 isoform 6). Only isoform 1 has been studied in different species, and it is considered as the canonical isoform. In the subsequent text of this thesis, the gene of TMPK will be referred as *DTYMK* and TMPK refers to the canonical human TMPK isoform 1 protein if no other information is stated.

TMPK contains 212 amino acids, with an estimated molecular weight of 23.8 kDa and a pI value of 8.4 (calculated by online source: web.expasy.org). The mRNA level and enzyme activity of TMPK fluctuate during the cell cycle, peaking during the S phase as shown in the human leukemia cell line HL-60 (Huang SH et al., 1994). In yeast, TMPK is also reported as a cell cycle-regulated enzyme (White et al., 1987).

1.4.2 The structure of TMPK

The TMPK structures in several species have been solved, including *Thermus thermophilus* (5X8B), *Staphylococcus aureus* (2CCG), *Ehrlichia chaffeensis* (3LD9), *Candida albicans* (5UIV), *Escherichia coli* (5X8B), *Saccharomyces cerevisiae* (2TMK), *Mycobacterium tuberculosis* (1N5J), *Vaccinia virus* (2V54), *Vibrio cholerae* (3N2I), *Aquifex aeolicus* (2PBR), *Thermotoga maritima* (3HJN), *Pseudomonas aeruginosa* (3UWK), *Sulfurisphaera tokodaii* (2PLR), *Homo sapiens* (1E2F). All these structures are in homodimer, or homo-oligomer forms, usually in ligand bound state,

with closed conformations. Their active centers (dTMP binding pocket and Lid region) are in an ordered structure. The amino acid sequences from these species share low homology (<20%). However, all TMPK structures deposited in Protein Data Bank share high similarity in overall architecture (Figure 7), containing in principle 8/9 helices as outer shell and 5 β-strands forming a slightly twisted β-sheet as a core of the protein. The main catalytic domains of TMPKs are the dTMP-binding pocket, the Lid region, P-loop, and DRx motif.

A typical example of TMPK structure in Protein Data Bank is 1E2F from human (Human TMPK). This structure depicts the interactions of TMPK with two ligands (dTMP and ATP analog) and one cofactor (Mg^{2+}). dTMP consists of a nucleobase thymine, a pentose sugar moiety and a phosphate group (Figure 8). The thymine base is placed deep inside the pocket and the phosphate is on the protein surface. α3, α4 and residue Y151 in the P- loop interact with the thymine base by a combination of hydrophobic and H-bonds. α4 close to thymine is a hydrophobic spine, with residues Y98, A99, F100, G102, V103, A104 and F105. Furthermore, F105 in α4, F72 in α3 and Y151 in the loop region contribute three pairs of π-π interactions from three directions (left side, right side and top), at distance ~3.6 Å. A H-bond is formed from C4 of thymine to amino group of the side chain of R76 at α3. Together, thymine is placed in a complicated geometrical coordination deep inside the binding pocket.

This geometric placement explains the substrate specificity of Human TMPK. Purine nucleoside monophosphate cannot be placed in the dTMP pocket due to the geometrical constraints and hydrophobic interaction. dCMP is similar to TMP (figure 3), but it has an amino group at C4 of the thymine. The interacting residue (R76) from the protein matrix provides two amino groups. Therefore, dCMP can not bind to the dTMP pocket due to the side chain of R76. Evolutionary, the arginine is preserved at this position in TMPKs from *H.sapien*, *V.virus*, *S.cerevisiae*, *P.falciparm*, and *M.tuberculosis*. In the bacteria *S.aureus*, *E.coli*, *V.cholerae*, *T.thermophilus*, the interacting residue of R76 is replaced by a glutamine, which has both a carbonyl group and amino group in the side chain. This implies that TMPKs in these bacteria can probably bind CMP as substrate and thus has less selectivity.

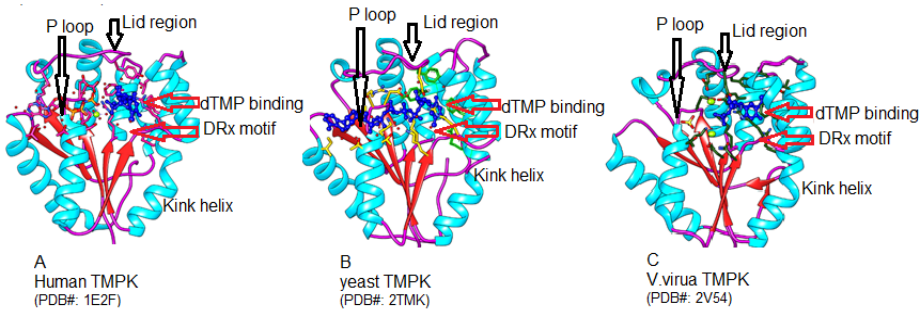


Figure 7: Structure of TMPKs. Three examples of TMPKs were extracted from the Protein Data Bank. All the TMPKs share similarity in the conserved p-loop, DRx motif, dTMP binding pocket. Five beta-strands makes up the core structure with surrounding helices. ATP and Mg^{2+} binding sites are located on the surface. A long helix with a kink in the middle is optimal for dimer interacting surfaces.

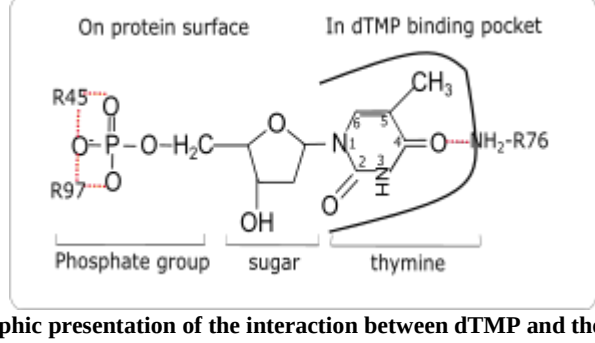


Figure 8: Graphic presentation of the interaction between dTMP and the protein matrix. dTMP is composed of thymine, sugar and a phosphate group. The whole dTMP is stretched out as a twisted line in the enzyme and ligand complex. The thymine is placed deep inside of the dTMP binding pocket, and its dominant interactions are a hydrophobic force by three π-π stackings; the phosphate group is binding to the protein surface, and the main interactions are H-bonds. The red dotted line represents the H-bonds from dTMP to protein matrix. The black line represents the geometry of dTMP binding pocket and the size is approximate. π-π stackings to thymine are not shown. The C4 of thymine binds to side chain of R76, which is a contributing factor to the selectivity of substrate, since dCMP has a NH_2 in the binding position. dTMP interactions with Mg^{2+} and ATP analog are not shown here.

Hydrophobic interactions in the form of three pairs of π-π are a driving force in protein and thymine interactions in human TMPK, which are also preserved in TMPKs from *V.virus* (PDB# 2W0S), *S.cerevisiae* (PDB# 3TMK) and *M. tuberculosis* (PDB# 1N5I). Other bacterial TMPKs (PDB#: 2Y0G, 4DWJ, 4TMK, 3N2I and 5X8B) have only one or two pairs of π-π stackings, which indicates that these TMPKs show less discrimination against substrates, which could be of importance in drug development.

The phosphate group in dTMP is placed close to the entrance of the binding pocket, in a hydrophilic environment. It has a high coordinate number (CN) with complicated interactions by H-bonds to 45R, 97 R (from DRx motif), Mg^{2+} and ATP analog from all directions (Figure 8).

Mg^{2+} plays an important role as cofactor for the catalytic activity of TMPK. Mg^{2+} is located in the opening of the dTMP binding pocket, being close to the protein surface. Mg^{2+} stacks with a hydrophilic residue (S20), ATP analog and water molecules in human TMPK. Mg^{2+} has a high affinity for water and prefers oxygen-containing ligands / residues because of a favorable electrostatic interaction energy (Dudev T and Nikolova V., 2016). The Mg^{2+}-H_2O interactions act as a space filler in the transition state. The six bonds from Mg^{2+} form a octahedron, with Mg^{2+} in the middle, and all bonds have an ideal distance of 2.1 Å.

It is worth mentioning the importance of the α3 helix (kink-helix), which is 25 residues long and thus the longest helix in the protein. It shows a 30° bend at the residue P81. Proline, with a rigid ring structure as side chain, cannot rotate in a helix. Thus, the helix may form a kink if proline is in the middle of helix. This feature has a great significance for TMPK. Firstly, the kink creates a curved helix, which facilitates the dimer interface in the TMPK dimer. Secondly, this kink makes a turn of α3, toward the dTMP binding pocket, which facilitates the interaction of F72 and R76 with thymine. Mutation of P81 in an infant has been demonstrated to give catastrophic consequences (Vanoevelen et al., 2022).

1.4.3 The pathophysiological role of TMPK mutation

In the recent decades, studies on TMPK have mostly focused on isolation and identification of TMPK in cancer tissue, TMPK structure determinations from different species, TMPK´s role in the activation of drugs, and inhibition of TMPKs in different pathogenic species. Two recent publications depict TMPK mutations in humans, providing insight on their pathogenic impact.

Lam et al (2019) reported a clinical case where two children at the ages of 1 and 6 years suffered from microcephaly, hypotonia and severe intellectual disabilities. Whole exome sequencing revealed two genetic alternations (a frameshift and a missense mutation A99T) in the *DTYMK* gene. The paper presented an *in silico* study and the study suggests that the TMPK mutations are correlated with mitochondrial DNA depletion.

Another detailed study of pathogenic consequences of *DTYMK* mutations both in humans and in zebrafish was published by Vanoevelen et al., 2022, in collaboration from several universities in the Netherlands, UK, USA, Egypt and Sweden. Our group contributed to TMPK enzyme related studies in this work, including measurements of dNTP pools, TMPK and TK activity and functional analysis of mitochondrial complexes (I-V). The two children ($TMPK^{mut}$) suffering from microcephaly and hypotonia; had a missense mutation in the *DTYMK*. $TMPK^{mut}$ zebrafish was created by crisper/Cas9 genome editing. The $TMPK^{mut}$ zebrafish replicated mostly the phenotypes of microcephaly and neuronal death as the children. dNTP pools in fibroblasts derived from one of the children did not show abnormalities, but decreased TMPK activity and increased TK activity was seen. Since the TK2 mutations lead to mtDNA depletion, we hypothesized that the *DTYMK* mutation may affect mitochondrial function. Studies of mitochondrial complexes I to V could not demonstrate significant differences comparing extracts from $TMPK^{mut}$ and $TMPK^{wt}$ in zebrafish. However, DNA replication was partly impaired and neuronal cell death was prominent. In conclusion, the similarities of TMPK pathologies in humans and zebrafish implicates neurodevelopmental disorders as one of the dominant hallmarks of loss-of-function of *DTYMK*. This is an unexpected result because no animal cells should be able to synthesize DNA without functional TMPK. It is the only known enzyme responsible for biosynthesis of dTDP. Why is *dTYMK* deficiency leading to neurodegenerative disease? With this in mind, I continued my studies looking for a possible explanation.

1.5 Zebrafish

1.5.1 The Zebrafish model

Zebrafish, *Danio rerio*, has been a popular tool in biomedical and genetic studies. Zebrafish has many advantages over mice and rats, i.e., zebrafish is a highly social animal, can produce a large amount eggs, has high reproduction frequency and fast embryonic development with a high level of optical transparency, and is economic and is easy to maintain. Howe et al 2013 carried out a hybrid of high-quality finished clone sequencing (83%) and whole-genome shotgun (WGS) sequencing (17%) of the zebrafish genome. The project revealed that 71.4% of the human genes have at least one zebrafish orthologue; and 69% of zebrafish genes have at least one human orthologue. Zebrafish research thus provides great opportunities to enhance our understanding of the mechanisms of protein function/regulation and its correlation to pathology.

Classification of developmental stages of zebrafish embryo is of great importance for the establishing of this animal model. Kimmel CB et al., (1994) have defined the embryonic developmental stages in zebrafish. The stages are defined by hours post fertilization (hpf) and they are 8 stages: (1) Zygote period, (2) cleavage period, (3) blastula period, (4) gastrula period, (5) segmentation period, (6) pharyngula period; (7) hatching period and (8) early larval period. The zygote period is defined at 0- ¾ hpf, when the produced eggs complete the first zygotic cell cycle. The cleavage stage occurs during ¾- 2¼ hpf, when the 2 to 7 cell cycles process rapidly and synchronously, 3 blastomere tiers become visible in stereo microscopy. The next stage is the blastula period, at 2¼ - 5¼ hpf, when 8 and 9 cell cycles occur, blastomere tiers (5 > 11 tiers) of the cell develop quickly and yolk sacs become engulfed completely. During the gastrula period, at 5¼ -10 hpf, primary germ layer is gradually formed and then an embryonic shield is produced. From 10 to 24 hpf, the embryos enter the segmentation period, structures of brain, eye, notochord, myotomes and tail bud become distinct. One prominent feature of this period is that neurulation and segmentation overlap extensively. At 24-48 hpf, it is defined as the pharyngula period. The typical features of this period are pigmentation in eyes and skin, appearance of red blood cells on yolk and prominent heartbeat. The hatching period occurs at 48 -72 hpf, when the zebrafishes break chorions and become freely swimming. From 72 hpf, the zebrafishes reach the early larval period.

It is worth mentioning that during the segmentation period, neurulation is in its prime, at approximately 10-24 hpf. Harrington et al., 2009, performed a comprehensive analysis of neural development in the 24 hpf of zebrafish model by combing imaging of neural tissue and tracking the behavior of cells in real time. During neural development, neurulation morphogenetic movements shape the posterior neural tube (PNT). This process occurs in a stepwise manner, including (a) neural cells populated in the anterior tail bud region undergo neural tube morphogenesis, (b) neural rod is formed by intercalation and delamination of the neural cells and (c) neural convergence and infolding contribute to successive elongation and progressive thickening of PNT. In neurogenesis, epidermis originates from outer ectoderm, neural tube gives rise to the central nervous system (cns) and neural crest to the peripheral nervous system.

Understanding the timing of neuronal development is needed for defining neuropathology observed in zebrafish. In paper IV, our investigation covered all embryonic developmental stages as mentioned above and strongly suggested a correlation between TMPK expression and neurodevelopment disorders.

1.5.2 dNTPs synthesis enzymes in zebrafish

During the last two decades, several genetic methods have been applied in zebrafish including: retrovirus-induced insertional mutations, morpholino antisense oligonucleotides approach and CRISPR/Cas9 gene editing. The Hopkins Nancy´s group in MIT carried out insertional mutagenesis (IM) screening of 390 zebrafish genes and classified the morphological pathologies related to the mutations (Golling G et al., 2002 & Amsterdam A et al., 2004). The IM screen covers several nucleotides synthesis enzymes i e., thymidylate synthase (TYMS), RNR subunit R2 (RR2) and cad (Carbamoyl-phosphate synthetase2-aspartate transcarbamy lase-dihydroorotase). Based on the results from the IM screening, RR2 (hi^{318}) mutant zebrafishes present with central nervous system (cns) necrosis, smaller eyes and dorsally bent body; a TYMS mutation (hi^{3510}) in zebrafishes lead to tiny eyes and smaller heads; and cad (hi^{2694}) mutant zebrafishes have small heads and eyes, underdeveloped livers / guts and pericardial edema. All these mutant fishes survived at least 5 days after fertilization. A Cad

mutation was also investigated in zebrafish by morpholino antisense oligonucleotides knockdown (Willer GB et al., 2005). Cad mutant zebrafishes show reduced eye and fins, malformed jaw structure, lacking retinal cell morphogenesis and display increased cell apoptosis.

I applied the zebrafish model in my PhD projects from 2018 to 2021. Zebrafish embryos, often from 0 to 120 hpf, were used in my studies. The short developmental stages in the zebrafish model facilitated the research and shortened the project time.

2. Present Investigations

2.1 Outline

Based on earlier investigations of TMPK mutations, the mutations do not cause dTTP depletion in fibroblasts. We continued studies of TMPK in human fibroblasts and zebrafish models with the following specific objectives:

- To study cell growth of $TMPK^{mut}$ human fibroblasts and whether the dTTP synthesis pathway is impaired due to TMPK mutations (paper I)
- How single amino acid mutations in the canonical TMPK protein lead to loss of protein function (paper I)
- To explore possible alternative pathways for dTDP synthesis in human fibroblasts (paper II)
- To assess the isoforms of human TMPK for possible compensatory activity (paper III)
- To study TMPK dynamics during earlier developmental stages in the zebrafish model (paper IV)
- To study the correlation between TMPK and neuronal development in the zebrafish model (paper IV)

2.2 Methodology

2.2.1 Cell Cultures

$TMPK^{mut}$ fibroblasts were derived from a patient with compound heterozygous mutations (P81L and D128N, one mutation in each allele); a fibroblast cell line as control (Cont) was derived from a healthy human of adult age. Written informed consents were obtained according to the declaration of Helsinki by our collaborator. A cancer cell line, BJ cells immortalized with hTERT (a gift from Prof. Staffan Johansson at Uppsala University, Sweden) was also cultured at the same condition. Cells were grown in complete DMEM media containing 10% heat-inactivated FBS, 1 mM of sodium pyruvate, and 1% of penicillin−streptomycin. All cell cultures were maintained at 37 °C in a humidified incubator, supplied with 5% CO_2. Cell growth varied and cells were harvested or reseeded once it reached > 80% confluence. The cell pellets were washed with ice-cold PBS buffer three times and stored at -20°C.

2.2.2 Zebrafish embryos

Zebrafishes were maintained in the zebrafish facility at VHC, Swedish University of Agricultural Sciences, Uppsala, Sweden. The animals' maintenance was approved by regional ethical committee for animal experimentation (Dnr.5.8.18-008/2017). Zebrafish embryos at developmental stages earlier than free-feeding are considered as cell model study according to Swedish research animal regulations (SJVFS 2019:9). The zebrafishes were kept at 27.5 °C with 12 h light and 12 h dark. Groups of ten adult zebrafishes were placed in a stainless steel mesh spawning cages one night before experiments. Spawning was started when the light was turned on. Success of fertilization was assessed by microscopic observations. Eggs were maintained in petri dishes with filtered and oxygenated water. Water change occurred daily; and died or malformed embryos were removed as soon as possible. The embryos were collected at 0, 6, 24, 48, 72, 96, 120 and 144 hours post fertilization (hpf). The collected embryos were rinsed with filtered water three to five times before protein isolation. Zebrafishes > 3 years with mixed gender were euthanized and collected for dissection.

2.2.3 Protein expression and purification

Mutant TMPK protein constructs were cloned into the pET-14b vector with an N-terminal fusion 6xHis tag (Genscript Inc). The constructs were then transformed into *E. coli* strain BL21 (DE3) pLysS; The *E.coli* strain was cultured in LB media, supplemented with 0.4% glucose, antibiotics, 1 mM $MgSO_4$ and M9 salts (1 g NH_4Cl, 3 g KH_2PO_4, 6 g Na_2HPO_4*7 H_2O per liter). Once the OD at 600 nm of the culture reached 0.6, 0.1 mM of IPTG was added to induce protein expression at 28 °C for 22 hrs. The culture was collected in a buffer (Tris 7.6, NaCl 100 mM, $MgCl_2$ 5 mM and DNAase) and lysed using a cell disruptor. Cell debris was removed by centrifugation at 14 500 x g for 45 min. The protein was loaded into a Ni_2 column, pre-equilibrated with buffer (Tris 7.6, NaCl 100 mM, $MgCl_2$ 5 mM and 2 mM imidazole). The column was washed with excessive buffer (Tris 7.6, NaCl 100 mM, $MgCl_2$ 5 mM and 40 mM imidazole) overnight in a cold room. Then imidazole concentration in the buffer was stepwise increased from 50 mM to 300 mM, 20 ml elution for each increased KCl concentration. The protein was further purified with size exclusion chromatography if necessary. The purity of protein was > 95% as judged by SDS-PAGE analysis. The proteins were dialyzed immediately to reduce the imidazole concentration.

2.2.4 The radiolabeled TMPK assays

The TMPK reaction mixture contained 10 mM Tris/HCl pH 7.6, 5 mM $MgCl_2$, 2.3 μM ^{3}H-dTMP, 2 mM ATP, 4 mM DTT and an appropriate amount of protein preparation. The reaction mixtures were incubated at 37 °C for a total of 30 min. After incubation, the reaction mixture was spotted onto DEAE filter paper (DEAE filtermat PerkinElmer). After 20 minutes of drying, the unreacted ^{3}H-dTMP was washed away with 50 mM ammonium format, 5 minutes each time, and repeated 4 times. In this washing step, the products ^{3}H-dTDP and ^{3}H-dTTP bound to DE 81 DEAE paper due to their extra phosphate group, and ^{3}H-dTMP can be washed away due to weaker binding. The products were eluted with 0.5 mL of HCl (0.1 M) and KCl (0.2 M) in a 6 ml tube and elution was performed on a shaker for 20 minutes at room temperature. Two milliliter of scintillation liquid was added into each tube and mixed for 10 minutes. The radioactivity was detected in a Tri-Carb, PerkinElmer instrument.

2.2.5 The coupled spectrophotometric TMPK assay

Steady-state kinetic studies were carried out using a coupled spectrophotometric method. Each reaction mixture contained: phosphoenolpyruvate (PEP, 0.5 mM), pyruvate kinase (4 units/ml), lactate dehydrogenase (4 units /ml), NADH (0.1 mM), $MgCl_2$ (5 mM), DTT (5 mM), Tris/HCl (pH 7.6, 10 mM), substrates ATP (1 mM or at variable concentrations) plus dTMP (0.1 mM or variable concentrations). The TMPK enzyme was added to start the reactions before the measurement. The duration of the measurement was 120 s at 21°C. The collected data was converted to initial velocity data (V_0), thus kinetic parameters of the TMPK could be determined using the Michaelis−Menten equation.

This method was developed by Blondin C et al., 1994. The principle of the method is: products from reaction (1) work as substrates to reaction (2), pyruvate from reaction (2) drives reaction (3), as shown in Figure 9. The rate of the reactions was monitored by a decrease of absorbance at 340 nm using a kinetic model in the spectrophotometer.

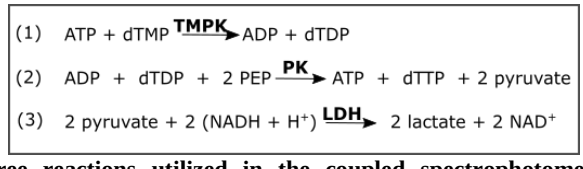


Figure 9: Three reactions utilized in the coupled spectrophotometric assay. By combination of three reactions, phosphorylation of dTMP in reaction (1) is related to the concentration changes of NADH in reaction (3), which can be measured by the absorbance at 340 nm. In the reactions, PK is pyruvate kinase and LDH is lactate dehydrogenase.

2.2.6 DNA synthesis measured by incorporation of radiolabeled dThd in cultured fibroblasts

Cells were incubated with fresh media, with or without the presence of 0.5 μM ^{3}H-dThd. The cell culture was incubated for 10 h before harvest; cell culture media was also collected. Cells were washed three times with ice-cold PBS. Cell pellets were suspended in 0.5 ml of extraction buffer, containing 0.25 mg/ml proteinase K, 100 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.1% SDS, and 200 mM NaCl. Incubation was performed overnight at 50 °C. This step used high temperature and proteinase enzyme to digest proteins. At higher temperature (50°C), protein unfolds, making it easier for proteinase K to degrade them. Proteinase K cleaves also nuclease, which protects DNA from nuclease attack and leaves DNA intact. After

incubation, the mixture was centrifuged at 16 000 x g for 20 min at 4 °C was performed with the DNA-isopropanol mixture. The supernatant was collected into a new tube and 0.3 ml of ice-cold isopropanol was added to precipitate DNA on ice for 60 min. Another centrifugation with 20 min at speed 16 000 x g at 4 °C for the DNA-isopropanol mixture. This time the supernatant was discarded and the pellet was saved. Ice-cold ethanol (70%) was used to wash the pellets, twice. The pellets were dried at 37 °C. RNAse free water (100 μL) was added to dissolve the DNA. Ten μL of DNA was mixed with scintillation fluid for 10 minutes, and the radioactivity was measured.

2.2.7 Thin layer chromatography (TLC) to separate the metabolites

TLC was employed to analyze the labeled metabolites, i.e., ^{3}H-dThd, ^{3}H-dTMP and ^{3}H-dTDP/^{3}H-dTTP. Due to the different charges and sizes of these compounds, the speed of migration varied, ^{3}H-dThd migrates quickest, ^{3}H-dTMP slower and ^{3}H-dTDP/^{3}H-dTTP slowest. The measured radioactivity comes from ^{3}H-dTMP, ^{3}H-dTDP and ^{3}H-dTTP depending on the migration distances from the starting point on the plate.

Cell pellets were washed three times with ice-cold PBS. The nucleotides were extracted with 10% TCA on ice. One microliter sample was loaded on the membrane and left 10 minutes to dry. The membrane was developed in a tightly closed glass chamber that contained 60 ml of 0.2 M sodium dihydrogen phosphate solution. When the front line of the solution reached the end of the membrane, the development was terminated. The membrane was cut to 18 pcs from the bottom to top and eluted in a 6 ml plastics tube with 0.5 ml elution buffer (0.1 M HCl and 0.2 M KCl) for 20 minutes at room temperature. Then scintillation liquid was added into each tube and incubated further 10 minutes before counting.

2.2.8 Antibody production and verification

Polyclonal TMPK antibodies were designed and produced in rabbits by GenScript. Both antigenic peptides are C +13 amino acids long and located on the surface of the proteins, and have high hydrophilicity. The antibodies were purified with affinity chromatography on an antigen-containing matrix and then assessed by ELISA procedures at GenScript. The specificities of the antibodies were further analyzed by immunoprecipitation or peptide

blocking. Two of the four designed antibodies showed high sensitivity and low background, and were thus used in papers I to IV.

2.3 Results and discussions

2.3.1 Paper I: *DTYMK* mutations in Patient-derived fibroblasts

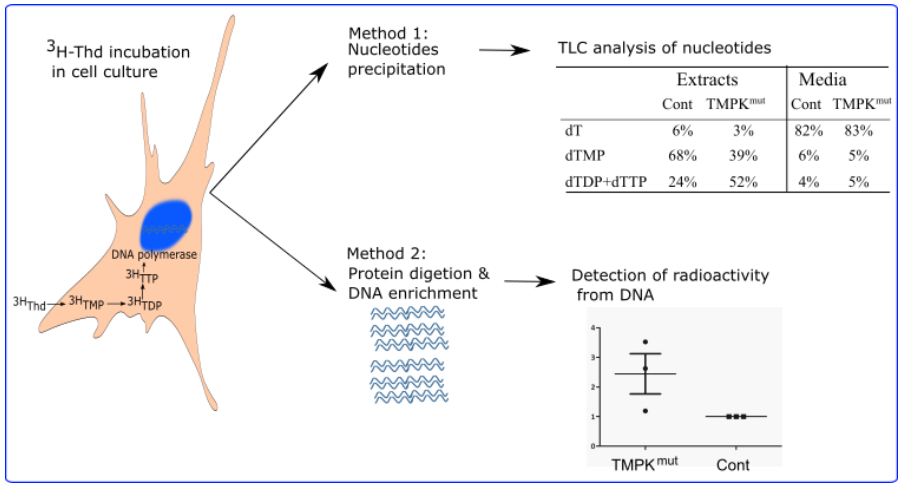
Aim of the study

TMPK is responsible for the only known dTTP synthesis pathway. dTTP, together with dGTP (G), dATP (A), and dCTP (C) constitute the dNTP pool, which provides the building blocks for DNA. Our aim was to investigate whether TMPK mutations could block or impair cell growth in fibroblasts and how single amino acid mutations (P81L, D128N, A99T) could affect the enzyme activity.

Result and discussion

Fibroblasts derived from a patient with two compound heterozygous mutations (one mutation in each allele, P81L and D128N, named $TMPK^{mut}$), Controls (Cont) derived from a healthy adult and immortal fibroblasts were also studied. The proliferation of $TMPK^{mut}$ fibroblasts was not impaired, compared with Cont during a two-month period. A radiolabeled enzyme assay was applied to test TMPK catalytic activity in extracts from the cell cultures; and $TMPK^{mut}$ showed much lower catalytic activity.

We investigated the dTTP synthesis pathway in $TMPK^{mut}$ fibroblasts compared to Cont by using the TLC assay with radiolabelled cell culture. After 10 h incubation, ^{3}H-dTDP and ^{3}H-dTTP were the dominant nucleotides (52%) in the cell cultures, indicating that the $TMPK^{mut}$ fibroblasts maintained its capacity to convert dTMP to dTDP/dTTP (Figure 10). Since the result was not expected, we verified the TLC result using a second method, i.e., to analyze the radioactivity incorporated into the isolated DNA. Three experiments showed variation, but all results with isolated DNA from $TMPK^{mut}$ showed higher radioactivity than found in Cont (Figure 10). These results suggest that an alternative enzyme to the canonical TMPK exists in $TMPK^{mut}$ cells.



	Extracts		Media	
	Cont	TMPK^{mut}	Cont	TMPK^{mut}
dT	6%	3%	82%	83%
dTMP	68%	39%	6%	5%
dTDP+dTTP	24%	52%	4%	5%

Figure 10: dThd radiolabeled experiment with Cont and mutant cell cultures. TMPK^{mut} and Cont fibroblasts were incubated with ^{3}H-dThd. Two methods were applied to assess the dTTP synthesis pathways with ^{3}H-dThd. Method 1: nucleotides were extracted with TCA (10%) and then analyzed by TLC. The result revealed that 52% of ^{3}H-dTDP/dTTP in TMPK^{mut} and 24% in Cont. Method 2: protein digestion and DNA isolation followed by radioactivity detection. TMPK^{mut} cells demonstrated higher radioactivity than Cont.

To study the impact of single amino acid mutations in TMPKs, WT TMPK and three reported mutations (P81L, A99T and D128N) were expressed and purified. P81L and A99T mutations substantially reduced enzyme activity. Steady-state kinetic analysis showed that these mutations lead to remarkable low binding affinity for these substrates ATP and dTMP. The D128N mutant had too low activity to allow kinetic analysis.

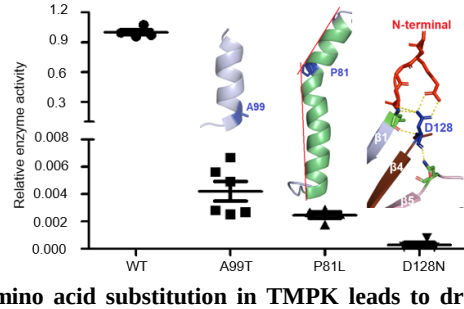


Figure 11. Single amino acid substitution in TMPK leads to dramatically decreased activity. Recombinant WT and TMPK mutants (A99T, P81L and D128N) were compared for their catalytic activity. Three mutations poses different alterations to TMPK structures, which affected the catalytic activity dramatically.

The TMPK mutations led to different responses to metal ions. The P81L mutation led to increased catalytic activity when Mn^{2+}, instead of Mg^{2+}, was present. The mutant TMPKs had reduced activity with increased NaCl concentrations.

Human TMPK is a dimer in the crystal structure. We tested the purified protein by size-exclusion chromatography. The canonical TMPK was eluted predominantly in the dimer form, while mutants were eluted mostly as monomers. Apparently, all three mutations changes TMPK from dimers to momomers which may explain the reduced catalytic activity.

TMPK is highly conserved in mammals, especially the functional domains: the dTMP pocket, the p-loop, the DRX motif and the Lid region. In the human TMPK structure, 1E2F was visualized and the effects of mutations were analyzed. The A99 mutation is localized in α4, in the dTMP binding pocket. The contact face of α4 toward thymine is dominated by hydrophobic amino acids (Y105, A104, V103, G102, F100, A99 and Y98). The A99T mutation influenced the hydrophobicity in the thymine binding pocket and increased the propensity of the α4. In the case of D128N mutation, D128 stabilizes protein folding by interacting with β1 and β4. The D128N mutation led to the loss of interactions and destabilized the core β sheet, which could impair the protein structure. The P81L mutation has a catastrophic effect on the protein structure. P81 induces the 30°C kink in the α3 helix. The kink makes the interactions between F72 and R76 in α3 to thymine possible, and enables a curved structure for α3, which is important for dimerization (Figure 11).

In conclusion, this study demonstrates that fibroblasts with loss of canonical TMPK activity could grow at normal rate as dTTP synthesis was not impaired in cell culture. Still the single mutation of amino acids in the three reported cases had profound effects on protein structure and catalytic activity.

2.3.2 Paper II: Identification of a novel thymidylate kinase activity

Aim of the study

TMPK, a required enzyme in both the *de novo* and the salvage pathways of dTTP synthesis, has a crucial role in cell growth. Two recent research projects with TMPK deletions / missense mutations in children showed that children survived albeit with a reduced life expectancy. In paper I, a cell culture study demonstrated that TMPK loss of activity did not lead to cell growth impairment. The aim of this paper was to attempt to isolate an alternative TMPK enzyme in immortal human BJ fibroblast cells (BJ).

Result and discussion

Isolated subcellular protein fractions from BJ were analyzed for catalytic enzyme activity and by western blot analysis using antibodies targeting human canonical TMPK. In the mitochondrial outer membrane (OM), we detected high catalytic activity by using the radiolabelled enzyme assay. However, the western blot analysis could not detect signals from the canonical TMPK, which suggested the existence of a novel TMPK mostly located in the OM fraction of mitochondrial preparations (Figure 12).

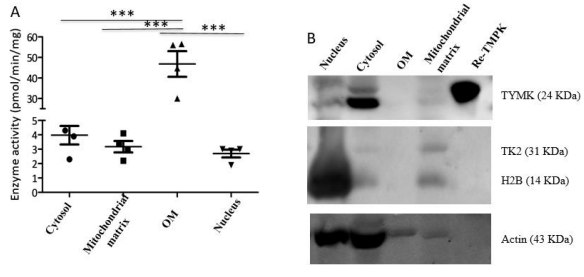


Figure 12. (A) TMPK activity in subcellular fractions assayed with radiolabeled dTMP as substrate. High activity was detected in the OM fraction. **(B) Subcellular localization of TMPK in immortal fibroblasts.** In the OM fraction, no signal was detected by using the human TMPK targeting antibody.

Next, we applied DEAE anion exchange chromatography to separate the OM protein fractions, and in parallel with cytosolic proteins. Enzyme activity to catalyze dTMP to dTDP was detected in fractions eluted with 50 mM KCl. The elution profile was similar to that of the TMPK enzyme found in the cytosolic compartment.

The DEAE fractions from OM protein with highest catalytic activity were pooled for steady-state kinetic analysis. This showed a sigmoidal substrate curve for the substrate dTMP and a hyperbolic curve for the substrate ATP.

In summary, our results suggest that the catalytic activity for dTMP to dTDP conversion detected in the OM fraction may originate from a novel dTMP kinase, distinct from the canonical TMPK.

2.3.3 Paper III: Structure and function analysis of human thymidylate kinase isoforms

Aim of the study

Human TMPK has six isoforms, encoding polypeptides ranging from 113 to 251 amino acids in length. Isoform 1 of human TMPK, is well-characterized regarding enzyme function and structure. This study was conducted to evaluate if any of these isoforms can function as the compensatory TMPK.

Result and discussion

Human TMPK isoforms 2-5 have partial deletions, whereas isoform 6 has an insertion of 39 amino acids, as compared to isoform 1. Human TMPK in complex with dTMP, ADP and Mg^{2+} (PDB code: 1E2F), from Protein Data Bank, was used as model for investigation. Isoform 2 lacks α5, β4 and one connecting loop region, which impairs ATP binding. Isoform 3 is missing α3, α4, β3 and two connecting loop regions, thus limiting the possibility for dTMP binding. Isoform 4 has a deletion of the entire active sites (α3, α4, α5, α6, α7, α8, β3, β4 and β5) and cannot be an active enzyme. Isoform 5 lacks two key helices (α3, α4) which bind dTMP. Based on the structural analysis, isoforms 2, 3, 4 and 5 can not be functional (Table 1).

Isoform 6, having an estimated molecular weight of 28 kDa, contains an insertion of 39 amino acids in the middle of α3, at position of P81. This residue functions as a kink to helix α3. Homology modelling with isoform using isoform 1 as template suggested that the 39 amino acid insertion may fold into a flexible loop. Thus, isoform 6 could be functional.

Recombinant isoform 6 was dominantly in oligomer, partly in dimer form. The activity of isoform 6 was one thousand times lower than that of isoform 1. Western blot using the human TMPK-targeting antibodies could detect signals in reduced protein samples from immortal fibroblasts. One of the dominant signals was from isoform 1, both as dimer and monomer. We could

also detect a band with a molecular weight close to that of isoform 6. No signal was detected for isoforms 2, 3, 4, and 5.

Table 1. Summary of structural analyses of human TMPK isoforms.

		Functional motifs			Structure stability	
	MW (kDa)	dTMP binding	DRX motif	ATP binding	β-sheet	Kink in helix α3
Isoform 1	23.8	+	+	+	+	+
Isoform 2	21.1	+	+	+	−	+
Isoform 3	19.0	−	−	+	−	−
Isoform 4	12.5	−	−	−	−	−
Isoform 5	21.9	−	−	+	−	−
Isoform 6	28.2	+	+	+	+	+

Our study reveals that human TMPK isoforms 2, 3, 4 and 5 miss important functional domains and cannot fold into active enzymes. These isoforms are not expressed in immortal fibroblasts. Isoform 6 has all structural elements but has very low catalytic activity. Hence, isoforms 2-6 cannot be the alternative TMPK.

2.3.4 Paper IV: Differential expression of enzymes in thymidylate biosynthesis in zebrafish at different developmental stages: implication for *dtymk* mutation-caused neurodegenerative disorders

Aim of the study

A recent clinical study with mutations in *DTYMK*, the gene coding for canonical TMPK, reveals that *DTYMK* mutants have a balanced dNTP pool in a cell culture model, but developed severe microcephaly and growth retardation in all affected infants. Zebrafish embryos with knockout *dtymk* develop microcephaly from 96 - 120 hours post fertilization (hpf), i.e., the phenotypic features are similar to the infants. However, the mechanism behind this is not well understood. Here we addressed this question by using the zebrafish model to study TMPK and thymidine kinases during earlier development to understand the role of nucleotide synthesizing enzyme´s role in neuropathology.

Result and discussion

The levels of the four dNTPs at different developmental stages in Zebrafish embryos were measured. The overall levels of dTTP and dCTP were clearly higher than those of dATP and dGTP from 0 to 144 hpf. dNTP levels were highest between 0 to 6 hpf and then declined gradually, which is inversely correlated to cell cycle length during early embryogenesis (Figure 13).

During early developmental stages, TMPK activity was maintained at a basal level and then increased. It reached the highest level after 96 hpf. Zebrafish embryos growing at 27°C hatch at approximately 72 hpf; and all zebrafishes become free swimming at 96 hpf. With respect to subcellular localization, TMPK activity was mostly found in the cytosolic compartment and was very low in the nuclear and mitochondrial fractions. Antibodies targeting zebrafish *dtymk*-encoded TMPK was used to detect the canonical TMPK levels. These studies revealed a fundamentally different pattern than the canonical TMPK activity. The TMPK protein increased gradually after fertilization and reached a maximum at 24 hpf (segmentation / neurulation period) and then declined gradually. At 120 and 144 hpf the canonical TMPK was non-detectable by Western blot analysis. The results from the enzyme activity and the protein determinations suggested that an alternative non-

canonical TMPK-like enzyme played a role after the hatching period. The canonical TMPK had high expression at 24 hpf, as shown in Figure 14.

Zebrafish proteins harvested at 24 hpf and 120 hpf were separated by anion exchange chromatography and eluted with stepwise increased KCl concentrations. The major TMPK activity was eluted in fraction 20 and 21 from the column, at which the KCl concentration was 200 mM. Both proteins harvested at 24 and 120 hpf shared similar elution profiles. The fractions with TMPK activity were further analyzed by size exclusion chromatography. Proteins harvested at 120 hpf showed a molecular weight of ~20-30 kDa.

Figure 13. dNTP level and cell cycle length are inversely correlated in early development. At earlier cell stages, the dNTP levels are high and the cell cycle progresses quickly. When the cells differentiate, dNTP level decreases and the cell cycle length increases.

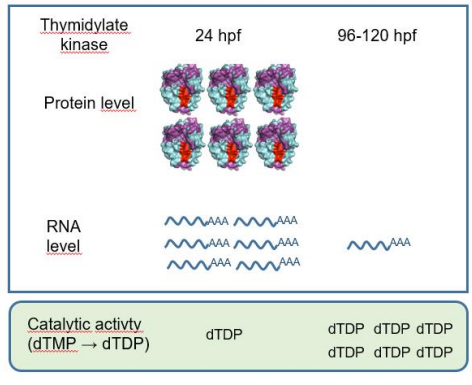


Figure 14. TMPK expression is in a dynamic pattern during the development of zebrafish embryos. TMPK protein levels, RNA levels and catalytic activities (dTMP to dTDP) were assessed and compared at two developmental stages, showing a huge difference at two time points. Protein and RNA levels of the TMPK was highest at 24 hpf; while the catalytic activity was highest at 96-120 hpf.

In contrast to the high TMPK activity during embryonic development, TK activity was very low. Based on DEAE analysis, the detected cytosolic TK activity in zebrafish was eluted with high concentration of KCl (100 mM) which corresponded to TK2. The activity measurements using dThd and dCyd as substrates further confirmed that this was the case.

TMPK activity in the head was ~50 times higher than in the body of zebrafish at 120 hpf. With zebrafish > 3 years, the brain had ~10 times higher activity than the rest of the body. From 10 to 24 hpf is the neurulation period, which is a critical period for organogenesis and neuromers development in zebrafish embryos. The canonical TMPK expression reached the highest level at 24 hpf. The loss of the canonical TMPK protein at the neurulation period impairs neuron development, which coincided with development failure of the brain, as observed in the zebrafish TMPK knockout model (Vanoevelen et al., 2022). The results demonstrate that the canonical TMPK is critical for neuronal development but can not be the only TMPK expressed in zebrafish.

2.4 Conclusions

TMPK in humans and zebrafish are the central theme of this thesis. Here is a summary of the main results of the investigations from the recent 4 years:

- *DTYMK* mutations found in human patients are loss of function mutations. Studies with patient-derived fibroblasts suggest the presence of additional enzyme that is compensatory to the canonical TMPK.

- A subcellular study of immortal fibroblasts reveals that a novel TMPK activity is present in the outer-membrane of mitochondria. Partially purified outer-membrane proteins suggests that the alternative TMPK has similar pI as the canonical TMPK.

- TMPK isoform 6 has all structural elements as compared with the canonical isoform 1. However, compared to isoform 1, the activity of isoform 6 is dramatically decreased. The alternative TMPK activity in immortal fibroblasts is most likely due to an unidentified enzyme encoded by a different gene, not by TMPK isoforms 2-6.

- In zebrafish embryos, the levels of dNTPs are inversely correlated to cell cycle length. Two main dTTP synthesis enzymes: TMPK and TK have prominent activity in the cytosolic fraction, but very low activity in the mitochondria of zebrafish embryos. With respect to tissue distribution of TMPK, brain has much higher activity than the rest of the body, in both zebrafish embryos and aged zebrafishes.

- TMPK activity reaches the highest level at 96 hpf, after hatching period. The canonical zebrafish TMPK antibody detects weakest signals at this time point. The TMPK protein level is highest at 24 hpf by western blot analysis, when organogenesis and neuronal development are maximal in zebrafish. These results indicate that the canonical TMPK protein and the alternative TMPK protein have compensatory expression patterns during zebrafish embryogenesis and that the canonical TMPK is essential for neurodevelopment.

2.5 Future Perspectives on TMPK Research

2.5.1 dTMP affinity column synthesis

We tried hard to isolate the alternative TMPK enzyme. The largest obstacle was the synthesis of a dTMP affinity column, which was critical to isolate the alternative TMPK-like enzyme. The first effort was to couple dTMP directly to CNBr-activated Sepharose 4 Fast Flow (GE healthcare). The activated Sepharose has a spacer arm, and we assumed that the free oxirane group of the spacer arm could directly interact with dTMP. The incorporation of dTMP was no success. We had cooperation with the chemistry department, Uppsala University. A modified dTMP derivative was synthesized. The dTMP derivative coupled to the matrix did unfortunately not aid in the isolation of the unknown TMPK-like enzyme. We are not clear whether the failure is dependent on the binding of protein. It is unfortunate that we did not get the dTMP affinity column work. Further efforts are thus required to purify the novel TMPK.

2.5.2 The canonical TMPK in different cell types

One of the unexpected result in this thesis is that loss of TMPK activity did not affect the dTTP level and did not impair the growth of human fibroblasts during extended time periods. In colon cancer cells, the dTTP level has been decreased using small hairpin RNA interference targeting the canonical TMPK (Hu CM & Chang ZF 2008). This result indicates that different cell types may vary in their dependence on the canonical TMPK. A variety of gene/protein expression profiles in different cell types caused by transcription regulation mechanisms may explain these findings. Further studies are needed to clarify such developmental control mechanisms in different cells and tissues.

2.5.3 Canonical TMPK mutation does not affect the mitochondrial complexes

Enzymatic activity of the mitochondrial respiratory complexes I-V showed no significant difference between $TMPK^{mut}$ and Cont in the zebrafish model. Mitochondria have independent dNTPs synthesis since mtDNA production is constitutive and differs from those for nuclear DNA synthesis. We

hypothesize that the alternative TMPK is essential for maintaining mtDNA and mitochondrial function, but may also have other roles in cellular nucleotide metabolism.

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Popular science summary

All life forms need storage and replication of genetic material, in the form of DNA. The four deoxyribonucleoside triphosphates (dNTPs): A, T, G and C, utilized in DNA should have three consecutive phosphorylations before they can be used for DNA synthesis. Our group´s research has focused on an enzyme which is responsible for catalysis of one intermediate step of T. The enzyme is called Thymidylate kinase (TMPK).

TMPK is strictly regulated in cells and has an elevated protein level in the S-phase of the cell cycle, since at that moment their reaction product is needed. dNTPs need to be in the right proportions for DNA polymerase to function in the most accurate way. Factors that inhibit the synthesis of one or more dNTPs can increase the mutation rate and lead to cell death.

In 2017, our partners in The Netherlands conducted a study on TMPK mutations in humans. Fibroblasts derived from a microcephaly patient´s biopsy with two TMPK mutations (one mutation in each allele) revealed that dNTP pools (A, T, G, and C) showed no significant differences between the patient and the control group (Cont). Two questions arose from these results: (1) Why did the fibroblasts with mutations have a normal dNTP pool even though the enzyme for biosynthesis of T was mutated and (2) how could TMPK mutation lead to severe microcephaly? Our studies followed this thread using human fibroblasts and zebrafishes as the investigation tools.

In our first study, we used fibroblasts derived from the patient ($TMPK^{mut}$) and a healthy human (Cont). Incubation of radiolabeled substrate in the fibroblasts showed that $TMPK^{mut}$ could convert substrate to product at the same rate as Cont. This suggested that an alternative enzyme may compensate for the mutated TMPK to perform the catalysis.

In the second study, the goal was to investigate a TMPK-like enzyme in immortal fibroblasts. We were able to detect a high catalytic activity from dTMP to dTDP in the outer-membrane of mitochondria; the antibody designed against the canonical TMPK could not recognize the new protein, which implied that a new enzyme existed in the outer-membrane fraction.

In the third study we investigated different isoforms of TMPK. TMPK has the canonical isoform 1, which has been studied in the recent decades. Isoforms 2, 3, 4 and 5 could not be folded into proteins or perform catalysis due to the absence of important elements identified in the structural analysis.

Isoforms 1 and 6 were produced in our lab. Isoform 6 showed thousand times lower activity than isoform 1. Cancer cells expressed isoform 1 and 6, but not 2, 3, 4 and 5, as judged by the different molecular weights. Therefore, isoforms 2-6 can not be the alternative TMPK.

In the fourth study, we studied TMPK in zebrafishes. We examined the protein level, the catalytic activity of the enzyme, and the RNA level over a period of 0-144 hpf. The result showed that the canonical TMPK had the highest protein level at 24 hpf (organogenesis and neurulation process quickly) and then decreased. However, the catalytic activity from dTMP to dTDP peaked at 96 hpf (when fish begins a free swimming life). TMPK shows a dynamically expressed pattern during early development of zebrafishes. Another TMPK-like enzyme may be present in high level from 96 hpf.

In summary, we investigated the TMPK in both human fibroblasts and zebrafish embryos. Our results suggested the existence of alternative TMPK in the human and zebrafish models. The alternative TMPK and canonical TMPK participate in a dynamic and compensatory expression pattern during embryonic development. The canonical TMPK mutation can lead to severe neuronal development disorders. Our studies were targeted to an increased understanding of TMPK and has contributed with important new results that will increase the knowledge of cell growth and the development of zebrafish embryos.

Populärvetenskaplig sammanfattning

Alla livsformer behöver lagring och replikation av det genetiska materialet i form av DNA. De fyra nukleosidtrifosfater (dNTPs), A, T, G och C, som används i DNA måste ha tre stegvis fosforyleringar innan de kan användas för DNA-syntes. Vår grupps forskning har fokuserat på ett enzym som ansvarar för katalysen av ett intermediärt steg av T. Enzymet heter Tymidylatkinas (TMPK).

TMPK är strikt reglerat i celler och har en förhöjd proteinnivå i S-fasen av cellcykeln, för att vid den tidpunkten behövs deras reaktionsprodukt (dTDP). dNTPs behöver vara i lagom proportioner för att DNA-polymeras ska kunna arbeta så korrekt som möjligt. Faktorer som hämmar produktionen av dNTPs kan leda till mutationer och celldöd.

År 2017 utförde våra samarbetspartners runt om i världen en studie om TMPK-mutation hos människor. Fibroblaster från en patients biopsi med två TMPK-mutationer (en mutation i varje allel) avslöjade att dNTP- poolerna från A, T, G och C-nukleotiderna inte visade signifikanta skillnader mellan patienten och kontrollgruppen (Kont). Detta väckte två frågor: Varför hade patienten en normal dNTP-pool i fibroblasterna trots att enzymet för biosyntes av T var muterat? Hur kan TMPK-mutation orsaka mikrocefali hos patienterna? Våra studier följde dessa trådar med fibroblaster och zebrafisk som undersökningsmodeller.

I vår första studie använde vi fibroblaster från patienten ($TMPK^{mut}$) och en frisk människa (Kont). Inkubation av radioaktivt märkta substrat med fibroblaster visade att $TMPK^{mut}$ kunde omvandla detta substrat i samma utsträckning som Kont. Detta tydde på att ett alternativt enzym fanns som kunde ersätta det muterade enzymet för att utföra katalysen.

I delarbete II var målet att undersöka om TMPK liknade enzymet i fibroblasterna. I yttermembran av mitokondrier kunde vi detektera en hög katalytisk aktivitet från dTMP till dTDP, men en antikropp mot det kanoniska TMPK kunde inte känna igen detta protein i en western blot-analys. Detta innebär att en ny typ av TMPK-enzym kan existera i yttermembranet.

I nästa delarbete (III) studerade vi olika isoformer (genfragment med snarlika sekvenser) av mänsklig TMPK i databasen. TMPK har den traditionella isoformen 1, vilket har studerats de senaste decennierna. Isoformer 2, 3, 4 och 5 kunde inte veckas till proteiner eller utföra katalysen på grund av att viktiga delar saknades från strukturanalysen. Men isoform 6 verkade ha möjlighet att fungera som ett enzym. Isoformer 1 och 6 producerades i vårt labb. Isoform 6 visade tusen gånger lägre aktivitet än isoform 1. Cancerceller uttryckte isoformer 1 och 6, men inte de övriga isoformerna. Bedömningen var baserad på deras molekylvikt. Därför kan isoformer 2-6 inte vara alternativa TMPK.

I det sista delarbetet (IV) använde vi zebrafiskar som modell. Vi undersökte TMPK-proteinet, enzyms katalytiska aktivitet och RNA-nivåer under ett tidsförlopp på 0-144 timmar efter befruktning (hpf). Det visade att det traditionella enzymet, TMPK, hade högst proteinnivå vid 24 hpf (organogenes och neurulation går då snabbt) och sjönk därefter. Men den katalytiska aktiviteten från dTMP till dTDP nådde en topp efter 96 hpf (när fiskar påbörjade ett fritt simmande liv). Detta avslöjade att TMPK uttrycks i ett dynamiskt mönster under zebrafiskens tidiga utveckling. Ett annat TMPK-liknande enzym bör finnas vid tidpunkten 120 hpf för att förklara resultaten.

Sammanfattningsvis undersökte vi TMPK hos fibroblaster från både människor och zebrafiskars embryon. Våra resultat antydde att det existerar ett alternativt TMPK i mänskliga celler och zebrafiskar. Det alternativa TMPK och det tidigare kända kanoniska TMPK har ett dynamiskt och kompensatoriskt uttrycksmönster under den embryonala utvecklingen. Den kanoniska TMPK-mutationen kan leda till allvarliga neuronala utvecklingsstörningar. Våra studier syftar till ökad förståelse av TMPK och har bidragit med viktiga nya resultat som kommer att öka kunskapen i hur celler växer och embryon utvecklas.

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Now I have reached the final stage of my PhD. Looking back on the recent 4 years, I would like to express my sincere gratitude to everyone, no matter named here or not, who has helped and encouraged me all the way. Your kindness is wonderful sunshine!

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Article

Biochemical Characterizations of Human TMPK Mutations Identified in Patients with Severe Microcephaly: Single Amino Acid Substitutions Impair Dimerization and Abolish Their Catalytic Activity

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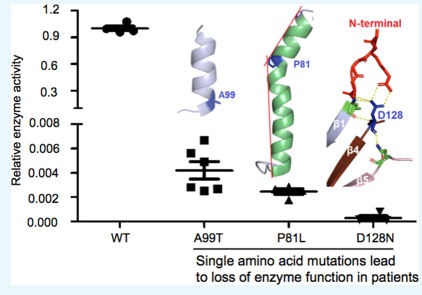
ABSTRACT: Deoxythymidylate kinase (TMPK) is a key enzyme in the synthesis of deoxythymidine triphosphate (dTTP). Four TMPK variants (P81L, A99T, D128N, and a frameshift) have been identified in human patients who suffered from severe neurodegenerative diseases. However, the impact of these mutations on TMPK function has not been clarified. Here we show that in fibroblasts derived from a patient, the P81L and D128N mutations led to a complete loss of TMPK activity in mitochondria and extremely low and unstable TMPK activity in cytosol. Despite the lack of TMPK activity, the patient-derived fibroblasts apparently grew normal. To investigate the impact of the mutations on the enzyme function, the mutant TMPKs were expressed, purified, and characterized. The wild-type TMPK mainly exists as a dimer with high substrate binding affinity, that is, low K_M value and high catalytic efficiency, that is, k_{cat}/K_M. In contrast, all mutants were present as monomers with dramatically reduced substrate binding affinity and catalytic efficiencies. Based on the human TMPK structure, none of the mutated amino acids interacted directly with the substrates. By structural analysis, we could explain why the respective amino acid substitutions could drastically alter the enzyme structure and catalytic function. In conclusion, TMPK mutations identified in patients represent loss of function mutations but surprisingly the proliferation rate of the patient-derived fibroblasts was normal, suggesting the existence of an alternative and hitherto unknown compensatory TMPK-like enzyme for dTTP synthesis. Further studies of the TMPK enzymes will help to elucidate the role of TMPK in neuropathology.



INTRODUCTION

Deoxynucleotide triphosphates (dNTPs) are essential building blocks for DNA synthesis. The synthesis of deoxythymidine triphosphate (dTTP) is accomplished by the de novo and salvage pathways. In the salvage pathway, thymidine kinase 1 (TK1, in cytosol) and thymidine kinase 2 (TK2, in mitochondria) phosphorylate thymidine (dT) to thymidine monophosphate (dTMP). In the de novo pathway, thymidylate synthase converts deoxyuridine monophosphate to dTMP in the presence of tetrahydrofolate. dTMP is then further phosphorylated to deoxythymidine diphosphate (dTDP) by deoxythymidylate kinase (TMPK) (EC 2.7.4.9). The final phosphorylation step from dTDP to dTTP is catalyzed by nonspecific nucleoside diphosphate kinases. Thus, TMPK is the bottleneck of dTTP synthesis since it is essential for both the de novo and salvage pathways of dTTP synthesis.[1]

In humans, *DTYMK* encodes TMPK and recently four *DTYMK* variants have been identified in human patients who suffered from severe congenital neurodegenerative diseases. In one study, compound heterozygous mutations (P81L and D128N) were found in one patient and a homozygous mutation (P81L) in another patient. Both patients suffered from severe neurodevelopment disorders with a vanishing brain syndrome and died at 18 and 32 months of age, respectively.[2] In another study, two siblings with compound heterozygous mutations (34 bp deletion causing frameshift and a missense A99T mutation) were reported, and the patients also suffered from neurodevelopmental disorders, severe microcephaly, hypotonia, and severe intellectual disability. Still, they were alive at 2 respective 7 years of age at the time of study. The authors suggested that the frameshift and A99T mutations may lead to loss of TMPK function and

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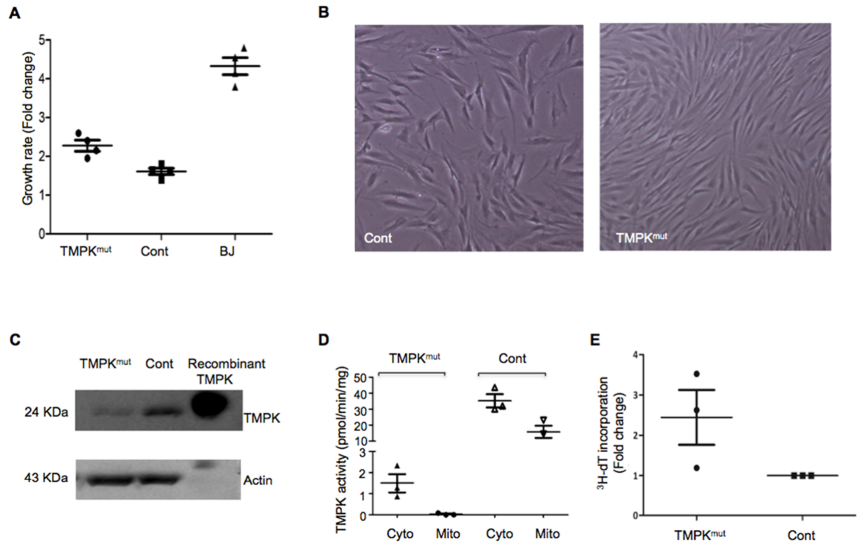

Figure 1. Characterization of TMPK^{mut} fibroblasts. (A) Growth rate of TMPK^{mut}, Cont, and immortal fibroblasts (BJ). The same number of cells were seeded and incubated for 3 days and then the cells were trypsinized and counted. The results represent fold changes in cell numbers during the 3-day incubation period; (B) comparison of the morphology of TMPK^{mut} and Cont cells. Approximately 1.5 million cells were seeded simultaneously; TMPK^{mut} reached 90% confluency faster than Cont. The TMPK^{mut} cells exhibited an elongated shaped compared with Cont. (C) Western blot analysis of the TMPK expression. Extracts from TMPK^{mut} and Cont cells were used; equal amounts of protein were loaded. Beta-actin was used a loading control; (D) cytosolic and mitochondrial TMPK activities; and (E) incorporation of ^{3}H-dT into DNA in TMPK^{mut} fibroblasts. DNA was extracted from TMPK^{mut} and control (Cont) fibroblasts after 10 h of incubation with ^{3}H-dT and the radioactivity was counted.

mitochondrial DNA depletion, albeit no experimental evidence was presented.[3]

To help to understand the role of TMPK in neurodegenerative disorders, we have here investigated the impact of the P81L and D128N mutations on the proliferation, dTTP synthesis capacity, and TMPK activity in patient-derived fibroblasts. We also expressed and characterized all three missense TMPK mutants identified in human patients in order to clarify the effects of these point mutations at the structural and functional levels. Finally, TMPK structure analysis helped to explain why these amino acid substitutions result in drastically reduced substrate binding affinity and catalytic activity and impaired dimerization.

RESULTS

P81L and D128N Compound Heterozygous Mutations Do Not Impair the Proliferation of the Fibroblasts Derived from a Patient. Considering the key role of TMPK in dTTP synthesis, we first asked whether the growth rate of primary fibroblasts derived from the patient might be impaired. Patient-derived fibroblasts (TMPK^{mut}), control fibroblasts (Cont), and a fibroblast cell line (BJ) were cultured under the same conditions and the number of cells was quantified. In the course of 1 month, the growth rate of TMPK^{mut} fibroblasts was clearly higher than that of Cont but lower than that of the BJ (Figure 1A). TMPK^{mut} cells attached to the culture flask displayed elongated shapes 6 h after seeding and reached 90% confluence faster than the Cont cells (Figure 1B). The proliferation capability of TMPK^{mut} diminished gradually after 2 months and stopped after 3 months, while the proliferation of Cont stopped in less than 2 months. Taken together, these data indicate that the mutations in the *DTYMK* gene do not impair the proliferation of the patient-derived fibroblasts.

TMPK Protein and Activity Levels and Subcellular Localization Are Affected in TMPK^{mut} Fibroblasts. The expression of the TMPK protein in cell lysates from both TMPK^{mut} and Cont was analyzed by Western blot using a human TMPK-specific antibody. As shown in Figure 1C, the level of TMPK protein in TMPK^{mut} fibroblasts was lower than that in the control cells. The TMPK activity in cytosolic and mitochondrial preparations was also measured using ^{3}H-dTMP as the substrate. In TMPK^{mut} cells, no TMPK activity could be detected in the mitochondria and low TMPK activity was detected in the cytosol, while in control cells, TMPK activity was detected both in the mitochondria and cytosol (Figure 1D). We also measured the thymidine kinase 1 (TK1) activity, a marker for cell proliferation, and found that TK1 activity in TMPK^{mut} fibroblasts was significantly higher than that in control cells (Figure S1), which was in-line with the higher proliferation rate observed for the TMPK^{mut} cells.

^{3}H-dT Uptake and Metabolism in TMPK^{mut} and Control Cells. The extremely low TMPK activity and fast growth of the TMPK^{mut} cells promoted us to investigate the dTTP synthesis using ^{3}H-dT (tritium-labeled thymidine). Stepwise phosphorylation of ^{3}H-dT into ^{3}H-dTTP and incorporation of ^{3}H-dT into DNA can be used as a measure for TMPK activity since the salvage pathway also requires TMPK for dTTP synthesis. As shown in Table 1, after 10 h of incubation with ^{3}H-dT in the culture medium, dTMP (68%) accounted for the highest percentage of ^{3}H-labeled nucleotides among the soluble nucleotides extracted from control cells. In TMPK^{mut} cells, dTDP and dTTP (52%) were the most

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Table 1. Distribution of ^{3}H-dT Nucleotides in Soluble Nucleotide Extracts and Media[a]

	extracts		media	
	Cont (%)	TMPK^{mut} (%)	Cont (%)	TMPK^{mut} (%)
dT	6	3	82	83
dTMP	68	39	6	5
dTDP + dTTP	24	52	4	5

[a]Data are shown as the percentage of total radioactivity recovered in extracts or media.

dominant nucleotides, indicating that the TMPK^{mut} cells have higher capacity to convert dTMP to dTDP. Furthermore, the percentage of ^{3}H-dT in control cells was also higher than that in TMPK^{mut} cells, and this could be explained by the higher TK1 activity in the TMPK^{mut} cells (Figure S1). In the culture media, after removal of the cells, >82% of the radioactivity remains as ^{3}H-dT for both types of cells. We could also detect labeled dTMP, dTDP, and dTTP in the media, which may be secreted by the cells or released from dead cells. In agreement with the intracellular ^{3}H-dT metabolism, in DNA extracted from TMPK^{mut} cells, the extent of radiolabeling was also higher than that of controls (Figure 1E). These results suggested that there is an alternative and previously unknown TMPK-like enzyme in TMPK^{mut} cells for dTTP synthesis and its high capability to synthesize dTTP may explain the observed higher growth rate.

Factors Affecting TMPK Activity in TMPK^{mut} Cell Extracts. The higher capacity of the TMPK^{mut} cells to synthesize dTTP raised the question as to whether there is a second TMPK-like enzyme that compensates for the lack of TMPK activity. Earlier studies have shown that TMPK is unstable in cell lysates and dTMP was needed to stabilize the TMPK activity.[4,5] Therefore, a pairwise comparison of the effect of dTMP on TMPK activity in cell lysates was conducted. As shown in Figure 2A, addition of dTMP to cell lysates resulted in higher TMPK activity in TMPK^{mut} cells. In contrast, the TMPK activity was relatively stable regardless of the presence of dTMP in control and BJ cell extracts (Figure 2A). The TMPK activity from TMPK^{mut} cells was also sensitive to the presence of salts such as NaCl in the lysis buffer. As shown in Figure 2B, there was a concentration-dependent reduction of TMPK activity in TMPK^{mut} cell lysates in the presence of NaCl. Since divalent metal ions are important cofactors for TMPK, we assayed TMPK^{mut} cell extracts in the presence of different divalent metal ions such as Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Ca^{2+} and found that only Mg^{2+} and Mn^{2+} could serve as cofactors for the enzyme. The highest TMPK activity in TMPK^{mut} cells was detected in the presence of Mn^{2+} (Figure 2C), while in control cells, the TMPK activity and recombinant human TMPK activity were higher in the presence of Mg^{2+} (data not shown), as was reported earlier.[5]

Molecular Characterization of the Wild-Type (WT) and Mutant TMPKs. To study the impact of the respective mutations on TMPK function, all three TMPK mutants and WT TMPK were expressed in *Escherichia coli* (*E. coli*), and the recombinant enzymes were affinity-purified to >95% purity as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 3A). We then compared the mutant and WT enzymes and found that the specific activity was substantially reduced in all three mutants ranging from 0.08 to 0.4% of that of the WT enzyme (Figure 3B).

Steady-State Kinetic Analysis. To provide further insight into the effects of the respective TMPK mutations on enzyme catalysis, a steady-state kinetic analysis was conducted. The WT enzyme showed high binding affinity for both dTMP and adenosine triphosphate (ATP) with K_M values of 1.75 and 1.11 μM, respectively. In contrast, the A99T and P81L mutant enzymes had remarkably low binding affinity for both substrates, with K_M values for dTMP 14- and 66-fold higher, respectively, than those of the WT enzyme. Moreover, the K_M value for ATP was ∼40-fold higher than that of the WT enzyme. Both the A99T and P81L mutants had also significantly lower catalytic efficiency (k_{cat}/K_M) compared with that of WT TMPK (Table 2).

The D128N mutant TMPK had too low activity to allow a reliable kinetic analysis. As shown in Figure S2, the D128N mutant activity, at variable dTMP and ATP concentrations, did not follow typical enzyme kinetics, and therefore, no K_M or k_{cat} was calculated from these data.

Effects of Mg^{2+}, Mn^{2+}, and Salt. Since the TMPK activity detected in the TMPK^{mut} cells was sensitive to both divalent metal ions and salt, we investigated the effects of these on the recombinant mutant enzymes. Of the three mutants, only the P81L mutant responded positively to the switch from Mg^{2+} to Mn^{2+} (Figure 3C). In contrast, for WT, A99T, and D128N mutant enzymes, replacement of Mg^{2+} with Mn^{2+} led to decreased activity, similar to what was reported earlier for the WT enzyme[5] (Figure 3C). Titration of Mn^{2+} and Mg^{2+} with P81L and WT enzymes revealed that at a 1:1 ratio (ATP/metal ion), the P81L mutant did not show any activity with

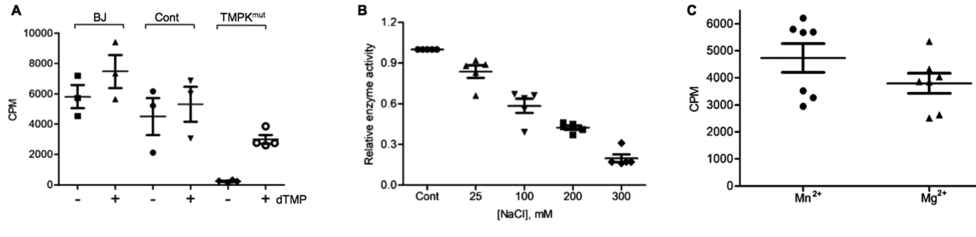

Figure 2. Factors affecting TMPK activity. (A) Effect of dTMP in the lysis buffer. Freshly harvested cells (0.2 million of each cell type) were homogenized in buffer ± 0.1 μM dTMP, and then equal amounts of protein were used for TMPK activity measurements (shown as CPM). (B) Effect of NaCl on the TMPK activity from TMPK^{mut} cells; TMPK activity at different NaCl concentrations relative to controls without NaCl (as 1.0). (C) Effect of Mn^{2+} and Mg^{2+}. Extracts containing equal amounts of proteins were used in the TMPK activity measurements in the presence of 4 mM Mg^{2+} or Mn^{2+} ions. The results are shown as CPM.

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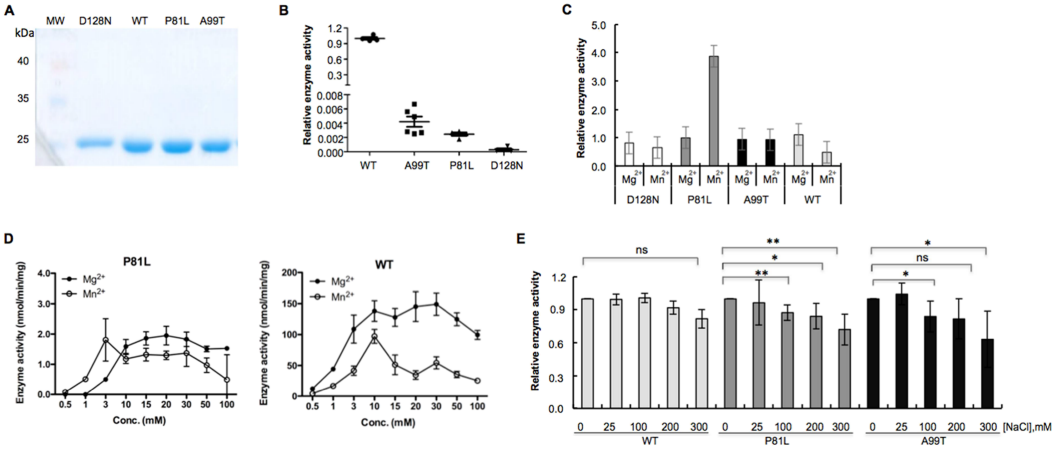

Figure 3. Expression and characterization of mutant TMPKs. (A) SDS-PAGE analysis of purified WT, P81L, A99T, and D128N mutant TMPK; (B) specific activity of mutant TMPK compared with that of the WT. Relative activity is shown (WT as 1.0); (C) effects of Mg^{2+} and Mn^{2+} on the TMPK activity. For each enzyme, the activity was normalized to that with Mg^{2+} (as 1.0); (D) titration of Mn^{2+} and Mg^{2+} with P81L mutant TMPK. ATP and dTMP concentrations were kept constant, 1 mM and 2 μM, respectively; and (E) effect of NaCl on WT and mutant TMPKs.

Table 2. Kinetic Parameters of Mutants and WT TMPK[a]

	dTMP			ATP		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
WT	1.75 ± 0.88	3.24 ± 0.23	1.85×10^6	1.11 ± 0.15	2.78 ± 0.03	2.51×10^6
A99T	24.6 ± 5.4	6.92 ± 0.42	0.28×10^6	41.3 ± 4.03	4.82 ± 0.07	0.11×10^6
P81L	115.9 ± 31.2	17.2 ± 2.13	0.14×10^6	43.1 ± 3.67	5.39 ± 0.07	0.12×10^6

[a]Activity determination was performed at 21 °C using a coupled spectrophotometric assay. Each measurement was repeated five times, and the data are given as mean ± SD. The data were fitted to the Michaelis−Menten equation using GraphPad Prism.

Mg^{2+}, but with Mn^{2+}, there was a detectable activity. At a 1:3 ratio (ATP/metal ion), the P81L enzyme showed maximal activity with Mn^{2+}, which was more than three times higher than that with Mg^{2+}. At greater than 10 times excess of metal ions, the P81L mutant showed higher activity with Mg^{2+}. For the WT enzyme, the activity was higher in the presence of Mg^{2+} at all concentrations (Figure 3D). This suggests that divalent metal ions may not only act as cofactors for enzyme catalysis but also interact with the protein and thus affect catalysis.

The influence of NaCl on the TMPK activity was also tested. Increasing NaCl concentrations had no effect on the WT TMPK activity but caused an approximately 30% reduction of the activity of the P81L and A99T mutant enzymes at the highest NaCl concentration used (Figure 3E). These results suggested that the salt-sensitive and Mn^{2+}-dependent TMPK activity detected in patient-derived fibroblasts ($TMPK^{mut}$) most likely originated from the P81L mutant enzyme.

Since both P81L and D128N are expressed in the patient-derived $TMPK^{mut}$ cells, we assessed whether the coexistence of these mutant enzymes affects the total TMPK activity. The recombinant P81L and D128N mutant enzymes were mixed at different ratios and their combined specific activities were determined. As shown in Table 3, when present alone, P81L had 0.25% of the WT enzymatic activity and D128N had 0.08% of the WT enzyme activity. When equal amounts of P81L and D128N were mixed, the specific activity was ∼0.2% of the WT enzymatic activity, which is approximately the level of TMPK activity detected in patient-derived fibroblasts compared with the controls. This suggests that both alleles are probably expressed equally.

Table 3. Specific Activities of Recombinant P81L and D128N Mutants Mixed at Different Ratios[a]

ratio	P81L:D128N (%)	D128N:P81L (%)
1:0	1033 ± 198 (0.25)	333 ± 33 (0.08)
1:1	831 ± 207 (0.20)	751 ± 175 (0.18)
1:2	607 ± 145 (0.15)	841 ± 139 (0.20)
1:5	440 ± 74 (0.11)	926 ± 156 (0.22)
1:10	360 ± 37 (0.09)	937 ± 178 (0.22)

[a]The activity determination was performed at 37 °C using ^{3}H-TMP as the substrate (1.2 μM). Each measurement was repeated five times and data are given as mean ± SD. Unit: pmol/min/mg. Numbers in parentheses are the percentage of WT TMPK.

Subunit Interactions. In the crystal structure, human TMPK is in a dimer form.[6] In order to understand the impact of mutations on TMPK catalysis, we studied the subunit interactions of these enzymes by using size-exclusion chromatography. As shown in Figure 4, WT TMPK was eluted predominantly in the dimer form (in fractions 15 and 16), as judged by activity measurements. In Western blot analysis, we could also observe a minor fraction of the WT enzyme in the monomer form (in fractions 18−20) with a very low specific activity. In contrast, all mutant TMPKs were

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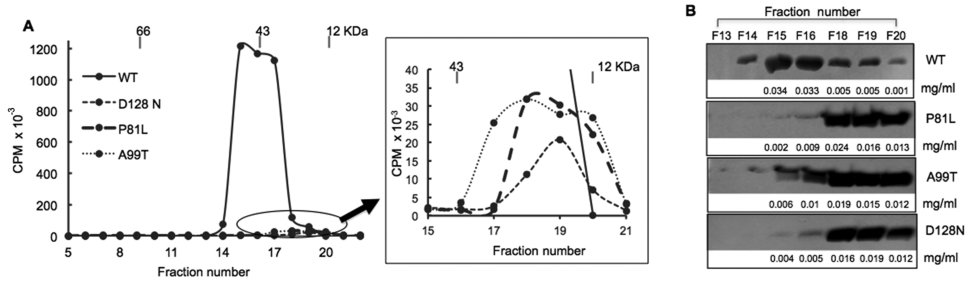

Figure 4. Subunit interaction of WT and mutant TMPKs. (A) Size-exclusion chromatography. Two hundred micrograms of each enzyme was analyzed. Fractions (0.4 mL) were collected and the TMPK activity in each fraction was measured (shown as CPM). Protein size markers were BSA (66 kDa), ovalbumin (43 kDa), and cytochrome C (12 kDa). (B) Western blot analysis. Ten microliters of protein from each fraction was used. The experiments were repeated at least three times for each protein; one representative image is shown. The protein concentrations in each fraction are indicated under the Western blot image.

```
                                  P-loop
Human      MAARRGALIVLEGVDRAGKSTQSRKLVEAL-SRGPPPELLRFPERSTEIGKLLSSYLQKK
Gorilla    MAARRGALIVLEGVDRAGKSTQSRKLVEALCAAGYRAELLRFPERSTEIGKLLSSYLQKK
G.pig      MAGRRGALIVLEGVDRAGKSTQSRKLVSALCAAGHRAELLRFPDRSTEIGKLLSSYLEKK
Mouse      MASRRGALIVLEGVDRAGKTTQGLKLVTALCASGHRAELLRFPERSTEIGKLLNSYLEKK
Z.fish     MSCRRGALIVLEGVDRAGKTTQCQKLVQAIQQSGRAAEIMRFPDRTTKIGQLISSYLEKK
Yeast      MMG-RGKLILIEGLDRTGKTTQCNILYKKLQPN---CKLLKFPERSTRIGGLINEYLTDD
           *   ** **::**:**:**:**   *   :       ::::**:*:*.** *:..** ..
                                                DRX motif
Human      S-DVEDHSVHLLFSANRWEQVPLIKEKLSQGVTLVVDRYAFSGVAFTGAK--ENFSLDWC
Gorilla    S-DVEDHSVHLLFSANRWEQVPLIKEKLSQGVTLVVDRYAFSGVAFTGAK--ENFSLDWC
G.pig      S-ELEDHSVHLLFSANRWEQVPLIKEKLYQGVTLVVDRYAFSGVAFTSAK--ENFSLDWC
Mouse      T-ELEDHSVHLLFSANRWEQVPLIKAKLNQGVTLVLDRYAFSGVAFTGAK--ENFSLDWC
Z.fish     S-NLEDHTVHLLFSANRWEMVPVMKQKLEEGINLVVDRYAFSGVAFTSAK--PGFSLEWC
Yeast      SFQLSDQAIHLLFSANRWEIVDKIKKDLLEGKNIVMDRYVYSGVAYSAAKGTNGMDLDWC
           : ::.*:::********** *  :* .* :* .:*:***.:****::.**   .:.*:**
                                                LID region
Human      KQPDVGLPKPDLVLFLQLQLAD-AAKRGAFGHERYENGAFQERALRCFHQLM----KDTT
Gorilla    KQPDVGLPKPDLVLFLQLQLAD-AAKRGAFGHERYENGAFQERALRCFHQLM----KDTT
G.pig      KQPDVGLPKPDLILFLQLQLTD-AATRGEFGHERYEEKTFQERALCGFQQLM----RDST
Mouse      KQPDVGLPKPDLILFLQLQLLD-AAARGEFGLERYETGTFQKQVLLCFQQLM----EEKN
Z.fish     MNPDVGLPKPDLVMFLQLNPNV-AANRGEYGNERYETSAFQRTVQQRFEELM----QDTS
Yeast      LQPDVGLLKPDLTLFLSTQDVDNNAEKSGFGDERYETVKFQEKVKQTFMKLLDKEIRKGD
           :***** **** :**. :     * :. :* ****   **. .   * :*:    ..

Human      LNWKMVDAS-KRLEAVHEELRVLSEDAIRTATEKPLGELWK
Gorilla    LNWKMVDAS-KSIEAVHEDIRVLSEDAIRTATEKPLGELWK
G.pig      LNWKVVDAS-RRIEEVHEEICTLSKAAILSAAQKPLGKLWQ
Mouse      LNWKVVDAS-KSIEEVHKEIRAHSEDAIRNAAQRPLGELWK
Z.fish     INWKVIDAA-RTIEEVHKDIKDLSENIISLAEEQPVGELWR
Yeast      ESITIVDVTNKDIQEVEALIWQIVEPVLSTHIDHDKFSFF-
            . .::*.: : :: *.  :    :  :    ::   .::
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Figure 5. TMPK sequence analysis. Amino acid sequence alignment of thymidylate kinases from different species. Human: CAA38528.1, Gorilla: XP_004033529.1, Guinea pig (G. pig): XP_003474598.3, Mouse: NP_001099137.1, Zebrafish (Z.fish): NP_001032187.1, and Yeast: AAA35158.1. The alignment was performed at https://mafft.cbrc.jp/alignment/server/. Residues P81, A99, and D128 are marked in red. Important functional motifs, for example, P-loop, DRX motif, and LID region are marked in blue. Green-labeled residues have important functions in protein folding and ligand binding.

eluted mainly as monomers (in fractions 18−20), which was also confirmed by Western blot analysis (Figure 4).

TMPK Sequence and Structure Analysis. At the amino acid level, TMPK is highly conserved across different species, particularly for the functional domains, for example, the p-loop, the DRX motif, and the LID region (Figure 5). These point mutations identified in human patients are conserved in all vertebrates, but in yeast, the residues corresponding to human P81 and A99 were replaced by D and V, respectively (Figure 5). To elucidate the possible mechanisms of the observed drastic changes in substrate binding, catalysis, and altered subunit interactions of the TMPK mutants, we performed a structural analysis of human TMPK using PyMol software and the known structure of human TMPK in complex with dTMP, an ATP analogue, and a Mg^{2+} ion (www.rcsb.org, PDB code: 1e2f).

In the WT human TMPK structure, the binding pocket for dTMP is buried while the ATP binding site is exposed, with the Mg^{2+} ion lying between them (Figure 6A). As shown in Figure 6B−D, two α helices, that is, α4 (in light blue) and α3 (in lime), are needed to position the thymine base; this is due to the hydrophobic residues Y105, A104, V103, G102, F100, A99, and Y98 on α4 forming a hydrophobic environment for the thymine base (Figure 6B). The side chain of F72 on α3 contributes with $\pi-\pi$ stacking to the thymine base within 3.5 Å (the cutoff of $\pi-\pi$ stacking is 4 Å) (Figure 6D). The side

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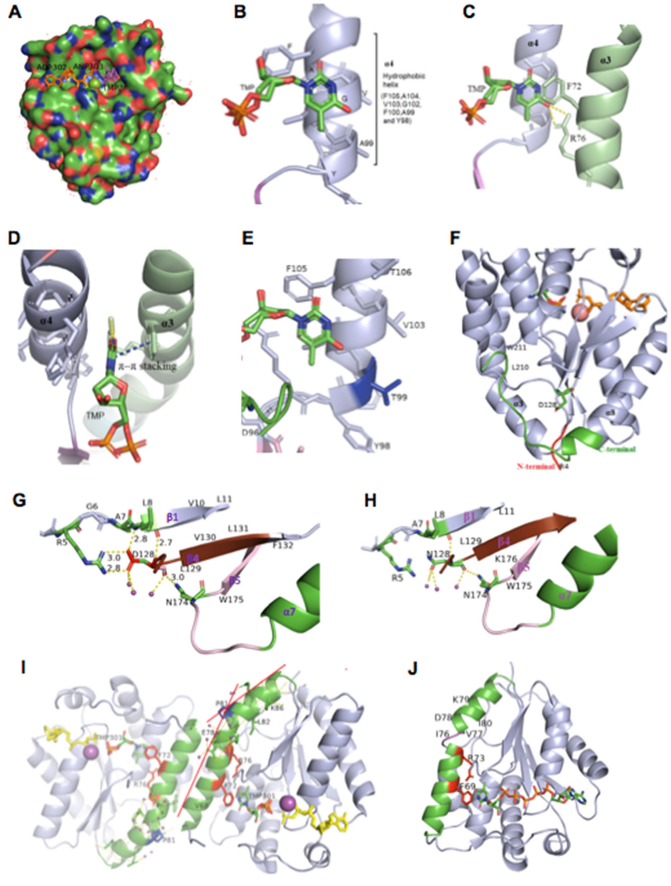

Figure 6. Structure analysis. The human TMPK structure (PDB code: 1e2f) was extracted from the Protein Data Bank (www.rcsb.org) and PyMol 2.3.4 was used in the analysis. (A). Overall human TMPK structure with bound substrates; dTMP (in magenta), ATP analogue ADPANP (in orange), and Mg^{2+} ion (in green); (B) α4 (in light blue) functions as a hydrophobic spine to the thymine base of dTMP (colors of the atoms are according to the element. C: green, H: gray, N: blue, O: red, and S: orange); (C) two helices position the thymine base in the middle by hydrophobic interactions. The benzyl ring of F72 on α4 (in light blue) and α3 (in lime) is in parallel with the thymine base. Two salt bridges formed between residue R72 and the thymine base further stabilize it; (D) top view of α3, dTMP, and α4. α3 and α4 are in parallel position and the thymine base is held in between; (E) A99 is replaced with T99. This changes the hydrophobic environment; (F) D128 in the 3D structure. The *N*-terminal is in red; *C*-terminal is in green; residue D128 is marked; the ATP analogue is in orange, and dTMP is colored according to the elements; (G) Interaction of D128 with neighboring residues; (H) D128 is replaced with N and changes in interaction with neighboring residues; (I). The human TMPK dimer. P81 (blue) locates at α3 (green). P81 introduces a kink resulting in a 30° bend of the helix, which directs R76 and F72 to the ligand TMP; and (J) yeast TMPK monomer structure (PDB code: 3TMK). No Pro is present in the yeast sequence. Therefore, the corresponding helix in human TMPK (e.g., helix α3 (green)) in yeast consists of two shorter helices connected by a loop structure (red color), which forms the right angle. Thus, residues R73 and F68 could interact with the thymine base in the same manner as in human TMPK.

chain of R76 forms two polar contacts with the oxygen atom of the carbonyl group on c4 of the thymine base at ideal distances of 2.7 and 3.3 Å (Figure 6C). Thus, the residues on α3 and α4 play an important role in dTMP binding (Figure 6C,D).

A systematic study by Pace and Scholtz[7] suggested that the propensity of amino acids to form helices is determined by their conformational entropy, that is, the energy required for proper folding. Ala has a helix propensity value of 0 kcal/mol, and the helix propensity value of Thr is 0.66 kcal/mol. Therefore, to form a helix, Thr needs higher energy than Ala, in particular, when the nearby residues have already relatively high helix propensity (F100, 0.54 kcal/mol and Y98, 0.53 kcal/mol). An A99 to T99 mutation, that is, a change from a nonpolar (Ala) to a polar (Thr) residue, may introduce new polar interactions such as H-bonds, resulting in local structural alterations that disrupt substrate binding and subunit interactions, leading to a lower binding affinity and catalytic efficiency (Figure 6B,E).

D128 is located at the bottom of the β-sheet, adjacent to the *N*-terminal (in red) and *C*-terminal (in green) (Figure 6F). A stable β-sheet is critical for the TMPK active site structure. D128 stabilizes β1 and β4 through interactions with the

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backbone of A7 and L8 and electrostatic interactions, that is, two pairs of salt bridges with the side chain of R5 at 2.8 and 3.0 Å distances, respectively (Figure 6G). The backbone of D128 interacts with the side chain of N174, which further stabilizes β5 and α7 since N174 locates between β5 and α7 (Figure 6G). Therefore, D128 plays a key role in stabilizing the internal dynamic structure of the enzyme (Figure 6G). D128 to N128 mutation (with an uncharged side chain) leads to the loss of electrostatic attraction to R5 and disruption of the salt bridges to A7 (Figure 6H). Thus, the D128N mutation could destabilize the β-sheet, which in turn destabilizes the active site and thus reduces the binding affinity and catalytic efficiency.

P81 is located on helix α3 that forms the monomer−monomer interface of the TMPK dimer (Figure 6I). The helix α3 contains 25 residues and is the longest helix of the protein. Proline has a rigid ring structure on its side chain, and rotation is not possible. Therefore, when proline is present in the middle of a helix, it usually destabilizes the structure element or causes a kink. The latter is relevant for P81 in human TMPK, which causes a 30° bend of the helix α3 (Figure 6I).[8] This kink makes the interaction of F72 and R76 with dTMP possible, which contributes partly to the specificity of the enzyme. The kink also makes dimer formation possible (Figure 6I). In yeast, Pro is replaced by Asp at this position and thus the kink is not present. However, residues 75−77 form a loop structure that results in two helices instead, which provides a right angle so that R73 and F69 can interact with the thymine base in the same manner as in human TMPK (Figure 6J). Replacing P81 with L81 causes the helix to lose the kink and thereby the helix may become straight, and the residues F72 and R76 will be diverged away from the dTMP binding site and also preclude dimer formation, which results in decreased substrate binding affinity and catalytic efficiency.

DISCUSSION

Deoxynucleoside triphosphates including dTTP are the fundamental building blocks for DNA and are synthesized through a highly regulated process catalyzed by specific enzymes, with TMPK being a key enzyme in dTTP synthesis. Using primary fibroblasts derived from a patient with compound heterozygous mutations in TMPK (P81L and D128N), we here studied the impact of these mutations on cell proliferation and dTTP synthesis. Strikingly, we found that these mutations did not affect the proliferation rate or the morphology of the fibroblasts although the TMPK activity and protein levels were extremely low. In fact, despite the low TMPK activity, the TMPK^{mut} cells have a higher dTTP synthesis capacity compared with the controls, suggesting that there is an alternative and hitherto unknown TMPK-like compensatory enzyme present, similar to the TMPK-like activity detected recently in BJ cells.[9]

Here, we expressed and characterized three mutant TMPK enzymes (P81L, D128N, and A99T) in order to clarify the impact of the respective mutations on the enzyme function. All three mutants showed very poor substrate binding affinity and drastically reduced catalytic activity, in particular the D128N mutant, compared with the WT enzyme. Using size-exclusion chromatography, we demonstrated that all three mutants are in the monomer form. This is in contrast to the WT TMPK, which is predominantly in the dimer form with a high enzymatic activity. Notably, the monomer form of the WT enzyme had very low specific activity, suggesting that dimerization is critical for efficient catalysis. In the human TMPK structure, the P81, A99, and D128 residues have no direct interaction with the substrates. However, by structural analysis we were able to explain why the single amino acid substitutions (P81L, A99T, and D128N) result in not only drastically reduced catalytic activity and decreased substrate binding affinity but also impaired dimerization.

We also show that the residual TMPK activity in the TMPK^{mut} cells and of the recombinant P81L mutant enzyme prefers Mn^{2+} to Mg^{2+} as a cofactor for catalysis, which deviates from the WT enzyme. In a kinase-catalyzed reaction, divalent metal ions such as Mg^{2+} or Mn^{2+} coordinate with the phosphoryl groups of ATP and facilitate nucleophilic attack. These metal ions may also interact with the protein and induce conformational changes.[11] In the human TMPK, one Mg^{2+} interacts with ATP in each subunit. However, there is also a Mg^{2+} ion present in the monomer−monomer interface (PDB code: 1e2f).[6] Although the role of this Mg^{2+} ion has not been studied, it is likely that it may interact with the protein through electrostatic interactions and thus might play a role in dimer formation. P81 is located in the middle of helix α3, which forms the monomer−monomer interface. The change in α3 caused by the P81L mutation, as described above, may alter the interaction between the two monomers with metal ions, and Mn^{2+} (with a larger radius compared with Mg^{2+}) might fit better in this position and may thus facilitate dimerization, resulting in a higher activity.

The four reported human cases with TMPK mutations showed different degrees of disease severity. The patient with compound heterozygous P81L and D128 mutations died at 18 months of age, whereas the patient with the P81L mutation died at 32 months of age.[2] The two siblings with 34 bp deletion and an A99T mutation also showed different degrees of severity and were alive at 2 respective 7 years of age at the time of study.[3] Notably, the A99T variant TMPK has the highest activity, whereas the D128N TMPK has the lowest activity among the three mutants, which may indicate that there is a correlation between the residual TMPK activity and the severity of the diseases.

In mitochondria, dTTP needed for mtDNA synthesis can be synthesized in situ by the salvage pathway, and thus, a mitochondrial TMPK is required.[10] In the study describing the two siblings with the A99T mutation and 34 bp deletion, the authors suggested that the mutations likely caused loss of TMPK activity, resulting in mtDNA depletion.[3] Indeed, the lack of mitochondrial TMPK activity observed in the TMPK^{mut} cells may indicate impaired mitochondrial dTTP synthesis. However, to prove the effect of TMPK mutations on mtDNA copy number in neuronal cells, it would be necessary to evaluate patients' tissue material or to conduct studies in transgenic animals.

At present, the mechanism behind why a deficiency in TMPK activity causes neurodegenerative disorders is not known. Neurons, unlike other cells, have two sets of extensions outward in opposite directions, that is, dendrites and axons, which are essential for neuronal communication. Mitochondria are the most abundant organelles in neurons and are located throughout axons and dendrites. Except for energy production, mitochondria in neurons play an essential role in calcium homeostasis, which is vital for synaptic function and brain cell growth.[12,13] The loss of TMPK activity caused by genetic alterations, that is, missense mutations and deletion, in human patients may eventually lead to mtDNA depletion and

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mitochondrial dysfunction, which in turn can lead to dysfunctional neurons and apoptosis of developing neurons.[14,15]

Defects in enzymes involved in the pyrimidine nucleotide metabolism have profound impacts on human neuropathology.[16,17] Except for TMPK deficiency causing neurodevelopmental disorders as described by us and others,[2,3] deficiency in dihydropyrimidine dehydrogenase causes seizure, intellectual disability, and microcephaly;[18] deficiency in dihydroorotate dehydrogenase leads to Miller syndrome, possibly through dysfunctional mitochondria;[19] thymidine phosphorylase deficiency causes mitochondrial neurogastrointestinal encephalopathy;[20] and thymidine kinase 2 (TK2) deficiency causes devastating mitochondrial DNA depletion and/or deletion diseases with neuromuscular involvement.[21,22] However, the mechanism behind the tissue specificity of these diseases is still not well understood although tissue-specific expression of these enzymes may play an important role.[22] Therefore, future investigations regarding the expression and distribution of these enzymes during different developmental stages and their effects on mitochondrial function are critical to answer the question why neurons are excessively vulnerable to impaired pyrimidine nucleotide metabolism.

CONCLUSIONS

The TMPK mutations identified in human patients represent loss of function mutations but, surprisingly, the proliferation rate of the patient-derived fibroblasts carrying such mutations was normal, suggesting the existence of an alternative and hitherto unknown compensatory TMPK-like enzyme for dTTP synthesis. The present study may contribute to the understanding of basic biochemical pathways in dTTP synthesis as well as to understanding the role of TMPK in neurodegenerative diseases. Furthermore, the present study may aid in future attempts to design therapeutic interventions for diseases caused by defects in enzymes involved in the pyrimidine nucleotide metabolism.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) and sodium pyruvate were obtained from Sigma-Aldrich; heat-inactivated fetal bovine serum (FBS) and penicillin−streptomycin were obtained from Thermo Fisher; trypsin−EDTA was obtained from the Swedish National Veterinary Institute; Bradford protein determination solution was obtained from AppliChem; DEAE filter paper (DEAE filtermat) and liquid scintillation fluid (Optiphase HiSafe 3) were purchased from PerkinElmer; and a PEI cellulose F plate was obtained from Merck group. The polyclonal antibody against human TMPK was produced by using the C-terminal peptide sequence as antigen (GenScript Inc).[9] The antibody against beta-actin was from Santa Cruz Biotechnology. Secondary fluorescence antibodies were from LI-COR Biosciences.

Cell Culture. The TMPK^{mut} fibroblasts were derived from the patient with compound heterozygous mutations (P81L and D128N) and Cont were derived from the patient's mother as described earlier.[2] BJ cells immortalized with hTERT[23] was kindly provided by Prof. Staffan Johansson (Uppsala University, Sweden). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in complete DMEM media containing 10% heat-inactivated FBS, 1 mM sodium pyruvate, and 1% penicillin−streptomycin. The medium was changed when the cells reached 80−90% confluence.

Subcellular Fractionation. Freshly harvested cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then used to isolate subcellular fractions by differential centrifugation, essentially as described.[24] The resulting subcellular fractions were stored in aliquots at −80 °C until further analysis. The protein concentrations were determined using the Bradford method and bovine serum albumin (BSA) as the standard.

Expression and Purification of the WT and Mutant TMPKs. All mutant (P81L, A99T, and D128N) TMPKs were cloned into the pET-14b vector with an *N*-terminal fusion 6xHis tag and expressed in *E. coli* strain BL21 (DE3) pLysS. The recombinant enzymes were expressed and purified as previously described.[25] WT TMPK was run in parallel for comparison. The purity of the proteins was >95% as judged by the SDS-PAGE analysis.

Western Blot Analysis. Appropriate amounts of cell extracts or recombinant TMPKs were resolved on 12% SDS-PAGE gels. After gel electrophoresis, the protein bands were transferred to a PVDF membrane. After blocking, the membranes were probed with a primary antibody against human TMPK, and the TMPK protein bands were visualized using a fluorescence secondary antibody and the Odyssey system (LI-COR Biosciences).

Radiochemical Enzyme Assays. TK and TMPK activities were determined as described previously.[25,26] Briefly, the TK reaction mixture contained 10 mM Tris/HCl pH 7.6, 5 mM $MgCl_2$, 0.5 mg/mL BSA, 5 mM dithiothreitol (DTT), 1 mM ATP, 2 μM ^{3}H-dT, 28 μM dT, and an appropriate amount of proteins. For TMPK activity determination, ^{3}H-dTMP was used as the substrate.[26] The reaction mixtures were incubated at 37 °C for a total of 30 min. At different time points, aliquots of the reaction mixture were spotted onto the DEAE filter paper. After drying, the unreacted substrate was washed away with ammonium formate (1 mM for the TK assay and 50 mM for the TMPK assay). The products were eluted with 0.5 mL of HCl (0.1 M) and KCl (0.2 M). After addition of a scintillation fluid, the radioactivity was counted (Tri-Carb, PerkinElmer).

Coupled Spectrophotometric Assay. Steady-state kinetic studies of the WT and TMPK mutants were carried out using a coupled spectrophotometric method essentially as previously described.[25,27] The reaction mixture contained 10 mM Tris/HCl pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.5 mM phosphoenolpyruvate, 0.1 mM NADH, 4 units/mL pyruvate kinase, 4 units/mL lactate dehydrogenase, ATP (1 mM or at variable concentrations), and dTMP (100 μM or at variable concentrations). The reactions were started by the addition of the TMPK enzymes, and the rate of NADH oxidation was monitored at 340 nm using a spectrophotometer for 2 min at room temperature (21 °C). All assays were repeated at least three times, and the results are given as mean ± SD. The kinetic parameters were calculated by fitting the initial velocity data to the Michaelis−Menten equation, $V_0 = V_{max}[S]/(K_M + [S])$.

^{3}H-dT Uptake and Metabolism. A total of 300,000 cells were seeded in T25 cell culture flasks for 24 h, and then 0.5 μM ^{3}H-dT was added to the cell culture. The cells were then incubated for 10 h. The cells were harvested and the media was collected. The cells were washed three times with ice-cold PBS, and the nucleotides were extracted with 10% PCA on ice. One microliter of the soluble nucleotide extracts or the cell culture

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media was spotted on a thin-layer chromatography (TLC) plate and then developed in 0.2 M sodium dihydrogen phosphate buffer.[28] After developing, the TLC plate was dried and cut into 1 cm in length, transferred into vials, and eluted with 0.5 mL elution buffer (0.2 M KCl and 0.1 M HCl) for 25 min on a shaker at room temperature. The radioactivity was counted after the addition of the scintillation fluid. ^{3}H-dT, ^{3}H-dTMP, ^{3}H-dTDP, and ^{3}H-dTTP were used as the standards.

DNA Extraction from Fibroblasts. Freshly harvested cells were washed three times with ice-cold PBS, and then the cell pellet was resuspended in 500 μL buffer (100 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.25 mg/mL proteinase K, 0.1% SDS, and 200 mM NaCl) and incubated at 50 °C overnight. The mixture was then centrifuged at 16,000 ×g for 20 min at 4 °C and the supernatants were transferred into new tubes. Next, 300 μL of ice-cold isopropanol was added and incubated for 1 h on ice and then centrifuged for 20 min at 16,000 ×g at 4 °C. The pellet was washed with ice-cold 70% ethanol and then air-dried. Water (100 μL) was added to the pellet to dissolve the DNA. Finally, 10 μL of DNA was mixed with the scintillation fluid and the radioactivity was counted.

Size-Exclusion Chromatography. A Superdex G200 column (GE Healthcare) was pre-equilibrated with a buffer containing 10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, and 5 mM DTT. BSA (66 kDa), ovalbumin (43 kDa), and cytochrome C (12 kDa) were used as protein size markers. WT and mutant TMPK proteins (200 μg) were injected into the column and eluted with the same buffer. The flow rate was 0.4 mL/min. Fractions of 0.4 mL were collected. Each fraction was subjected to Western blot and enzyme activity analyses. The protein concentrations were determined using the Bradford method with BSA as the standard.

Structure Analysis. The human TMPK structure with bound ligands TMP, ATP analogue (ADPANP), and Mg^{2+} ion was extracted from the Protein Data Bank (www.rcsb.org, PDB code: 1e2f). The structure was analyzed using PyMol 2.3.4 software. The rotamer of an amino acid with fewest steric clashes was chosen in each mutagenesis. Due to the limitation of PyMol, energy minimization of the protein structure is not taken into consideration in the mutagenesis.

Statistical Analysis. All data were analyzed using Microsoft Office Excel 2010 and GraphPad Prism software. Statistical comparisons were performed using a two-tailed t-test. All data were derived from at least three independent experiments and are presented as the mean ± S.D.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05288.

Thymidine kinase activity and kinetic study of the D128N mutant (PDF)

Accession Codes

Accession ID for human TMPK: UniProt: P23919 (KTHY_HUMAN). NCBI: CAA38528.1.

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Notes

The authors declare no competing financial interest.

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II

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Identification of a novel thymidylate kinase activity

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ABSTRACT

Thymidylate kinase (TMPK, EC2.7.4.9) is the enzyme that converts deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP) in the synthesis of dTTP, an essential building block of DNA. To date, there is only one gene (*TYMK*) known to encode TMPK in mammalian cells. In this study, we investigated the distribution of TMPK activity and protein in subcellular fractions by using activity measurements and by using a specific antibody against *TYMK*-encoded TMPK (canonical TMPK). TMPK activity was detected in all subcellular fractions, of which the mitochondrial outer membrane contained the highest activity. High levels of canonical TMPK protein were detected in the cytosolic fraction, whereas low levels were found in the nuclear and mitochondrial matrix fractions. Strikingly, despite the detection of high TMPK activity in the mitochondrial outer membrane, canonical TMPK protein was not detected in this fraction. These results suggest that the TMPK activity detected in the outer membrane fraction may originate from a novel dTMP kinase, distinct from the canonical TYMK.

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dTTP synthesis; thymidylate kinase (TMPK); subcellular fractions; mitochondrial outer membrane

1. Introduction

Deoxythymidine triphosphate (dTTP), an essential building block of DNA, is synthesized by the salvage and *de novo* pathways. In the salvage pathway, the formation of dTTP starts from thymidine, followed by three successive phosphorylation steps. Thymidine kinase 1 (TK1) in the cytosol or thymidine kinase 2 (TK2) in mitochondria catalyzes the first phosphorylation step, i.e., conversion of thymidine to thymidine monophosphate (dTMP). In the *de novo* pathway, thymidylate synthase (TS) converts deoxyuridylate (dUMP) to dTMP. The formation of dTDP from dTMP is an essential step for dTTP synthesis because it converges the salvage and the *de novo* pathways. This reaction is catalyzed by thymidylate kinase (TMPK, EC 2.7.4.9). Human TMPK was isolated and characterized during the 1970s; until today

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there is only one known gene coding for TMPK in all organisms and humans it is encoded by the *TYMK* gene.[1,2]

Considering the essential role of TMPK in dTTP synthesis, loss of TMPK function would lead to a depleted dTTP pool, which in turn leads to failure in DNA synthesis and lethality. However, challenging this view, knock-down of *TYMK* in human colon cancer cells does not affect the viability of the cells.[3] Furthermore, children with deletions and missense mutations, respectively, in the *TYMK* gene were reported recently; these genetic alterations were predicted to cause loss of TMPK function, however, despite these mutations, the patients survive, albeit their manifestation with microcephaly and hypotonia.[4] Together, these studies point to the possibility that *TYMK* may not be the only gene coding for proteins with TMPK activity. To address this, we investigate the distribution of TMPK activity and levels of canonical TMPK protein in different subcellular fractions of human fibroblasts. Intriguingly, we found high levels of TMPK activity in cellular compartments devoid of canonical TMPK protein, suggesting the existence of a novel enzyme with TMPK activity.

2. Materials and methods

2.1. Materials

Antibody against human *TYMK*-encoded TMPK was produced by using C-terminal peptide sequence of human TMPK, i.e., IRTATEKPLGELWK, as antigen to immunize rabbits and affinity purified (Genscript Inc).

2.2. Cell culture

Human BJ fibroblasts, immortalized with hTERT, were kindly provided by Prof. Staffan Johansson (Uppsala University).[5] The cells were incubated at 37 °C incubator supplied with 5% CO_2 in complete DMEM medium (SigmaAldrich) containing 1 mM sodium pyruvate, 6% FBS and 1% PEST. The cells were harvested when reaching 90% confluence.

2.3. Subcellular fractionation

Subcellular fractions were isolated by using differential centrifugation as previously described[6] with minor adjustments. Approximately 30 million cells were homogenized in a glass-glass conical grinder on ice. The nuclear fraction was collected after centrifugation at 800 × g for 10 min; the supernatant was centrifuged at 12,000 × g for 30 min at 4 °C to pellet the mitochondrial fraction and the remaining supernatant was regarded as the cytosolic fraction. The mitochondrial fraction was gently mixed with 0.01%

digitonin to remove the outer membrane from the mitochondria, and the mitoplasts were removed by centrifugation; the resulting supernatant corresponded to the mitochondrial outer membrane fraction. Proteins extracted from mitoplasts were regarded as the mitochondrial matrix fraction. Protein concentration was determined by using the Bradford method.

2.4. Western blot analysis

Twenty microliters of each fraction were separated on a 12% SDS gel and transferred to PVDF membrane at 200 mA for 75 min. The membrane was probed by using antibodies against human TYMK, human TK2,[7] H2B (Abcam) and β-actin (Santa Cruz Biotechnology) and detected by infrared labeled secondary antibodies using the Odyssey system (LI-COR). Purified human recombinant TMPK (with His tag, 8 ng) was used as the positive control.

2.5. Enzyme activity

TMPK activity was measured by using [^{3}H]-dTMP as substrate essentially as described.[8] The reaction mixture was incubated at 37 °C for 30 min and then 12 μl of the reaction mixture was spotted on DEAE filters (DEAE filtermat, PerkinElmer) and dried. The unreacted dTMP was washed away with 50 mM ammonium formate. The product ([^{3}H]-dTDP) was eluted in 0.5 ml of 0.2 M KCl and 0.1 M HCl. After addition of 2 ml scintillation liquid (Optiphase Hisafe 3, PerkinElmer), the radioactivity was counted (Tri-Carb, PerkinElmer).

2.6. Anionic exchange column chromatography

Cytosolic and outer membrane proteins were loaded on an anionic exchange column (DEAE sepharose, Fast Flow, GE Healthcare) equilibrated with buffer A (10 mM Tris/HCl pH 7.6, 20 mM KCl and 0.1 M sucrose). The bound proteins were eluted with stepwise increased KCl concentrations in buffer A. Fractions were collected and the protein concentration was determined by the absorbance at 280 nm. Each fraction was assayed for its ability to form [^{3}H]-dTDP from [^{3}H]-dTMP. Fractions with high activity were pooled together for further analysis.

2.7. Steady-state kinetic analysis of partially purified mitochondrial outer membrane TMPK-like enzyme

Pooled DEAE fractions were used to determine kinetic parameters with [^{3}H]-dTMP as the labeled substrate as described above. K_M and V_{max}

values were determined by using variable dTMP concentrations (0.5–500 μM) and fixed ATP concentration (2 mM), or fixed dTMP concentration (3 μM) and variable ATP concentrations (2–200 μM). Each measurement was repeated at least 3 times and data were fitted into Hill equation, and given as mean ± SD. Hill coefficient, $n < 1$, indicating negative cooperativity and $n = 1$, indicating Michaelis-Menten kinetics.

2.8. Statistical analysis

All original data were analyzed by using the Microsoft Excel 2010 and GraphPad Prism. Statistical analyses were performed by using a two-tailed t-test with the assumption of unequal variance. All data were derived from two to five independent experiments and are presented as the mean ± S.D. $P > 0.05$ considered as not statistically significant, and $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ as statistically significant.

3. Results

To investigate the distribution of TMPK in subcellular compartments, different subcellular fractions were isolated from cultured human BJ fibroblasts. To access the quality of the subcellular fractions, TK2 (a mitochondrial matrix protein) and histone 2B (H2B, a nuclear protein) were used as markers for mitochondria and the nucleus, respectively, in western blot analyses. As shown in Figure 1(A), TK2 was present mainly in the mitochondrial matrix fraction. H2B was mainly found in the nucleus, whereas only low amounts were found in mitochondria and the cytosolic fraction. Conceivably, the faint H2B band in the cytosolic fraction could represent newly produced protein from ribosomes, rather than contamination from the nuclear compartment.

The TMPK activity in each subcellular fraction was measured by using [^{3}H]-dTMP as substrate. As shown in Figure 1(B), the mitochondrial outer membrane (mt-OM) fraction contained the highest level of enzyme activity converting dTMP to dTDP, while the cytosolic, nuclear and mitochondrial matrix (mt-matrix) fractions had considerably lower enzyme activities, approximately 2–5 pmol/min/mg.

An antibody against the C-terminus of human canonical TMPK was used to identify the location of the TMPK protein. As seen in Figure 1(A), the *TYMK*-encoded (canonical) TMPK protein was detected in cytosol, nucleus and mitochondrial matrix, with the highest levels seen in the cytosolic fraction. However, the canonical TMPK specific antibody did not provide a positive signal when applied to the outer mitochondrial membrane fraction (mt-OM) (Figure 1(A)), despite that the highest TMPK activity

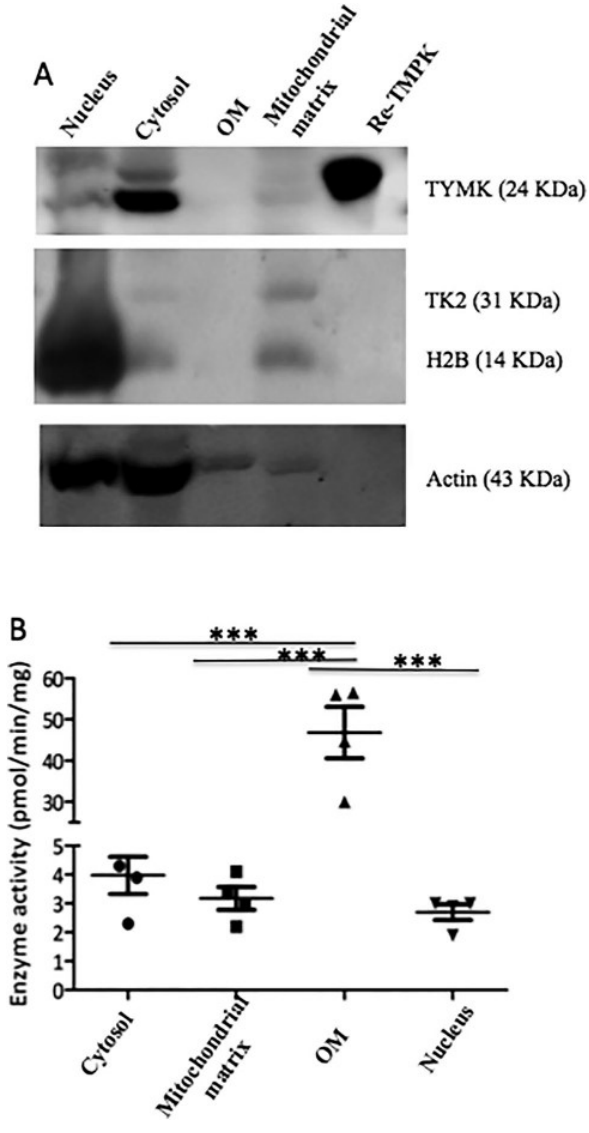

Figure 1. (A) Western blot analyses of subcellular proteins. Antibodies against TK2, H2B and actin were used as markers for subcellular fractions. The TYMK antibody was raised in rabbits using the C-terminal peptide sequence of human *TYMK*-encoded TMPK as antigen; (B) TMPK-like enzymatic activity in subcellular compartments of BJ fibroblasts. [^{3}H]-dTMP was used as a substrate and formation of [^{3}H]-dTDP was measured as described in the experimental procedures.

was found in this location (Figure 1(B)). These results suggest the presence of an alternative enzyme having TMPK activity in the outer mitochondrial membrane, i.e., with the ability to convert dTMP to dTDP.

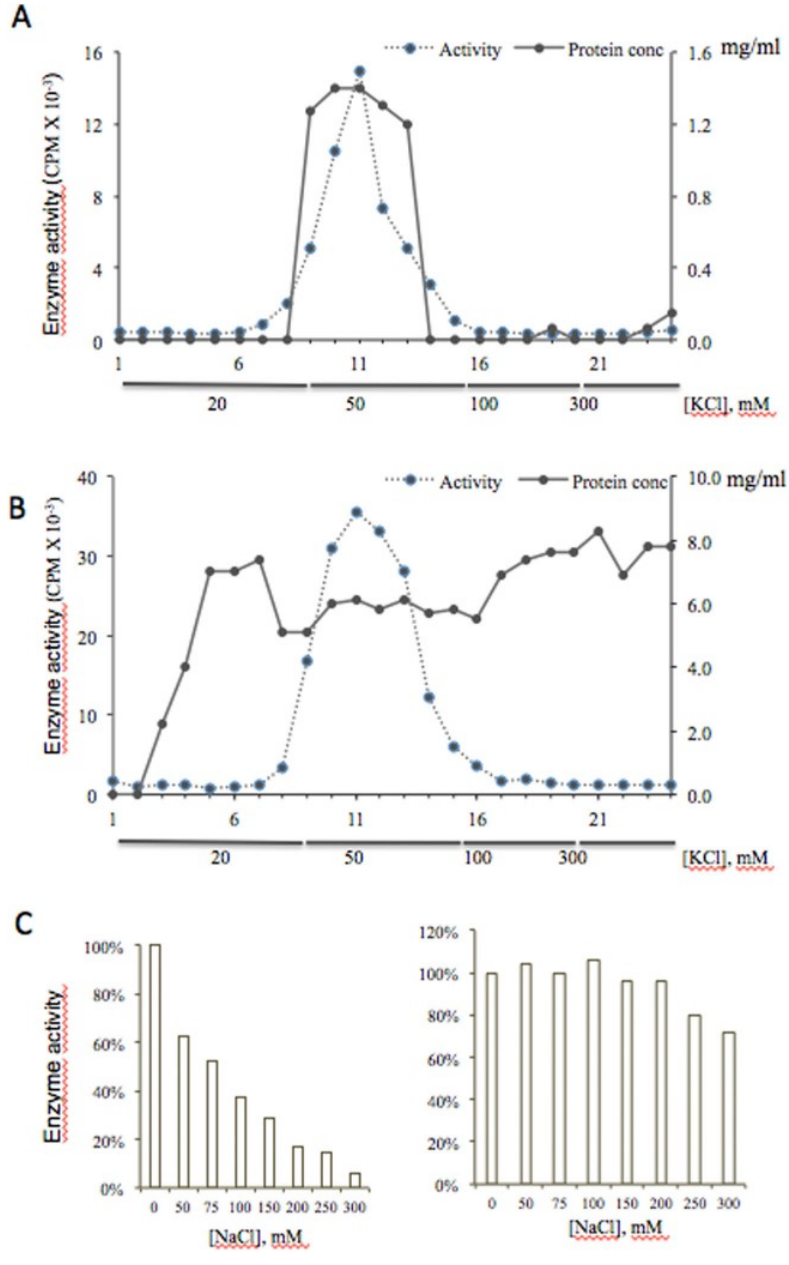

Figure 2. Characterization of mitochondrial outer membrane TMPK-like enzyme. DEAE chromatograms of the mitochondrial outer membrane (A) and cytosolic (B) proteins. Fractions were collected and the enzyme activity that converts [^{3}H]-dTMP to [^{3}H]-dTDP was measured. Protein concentration was determined by absorbance at 280 nm. Fractions with the highest activity (from mitochondrial outer membrane) were pooled and assayed in the presence of an increasing concentration of NaCl (C, left panel); recombinant human TMPK was also assayed in the presence of an increasing concentration of NaCl (C, right panel), and data are presented as percentage of the activity in the absence of NaCl.

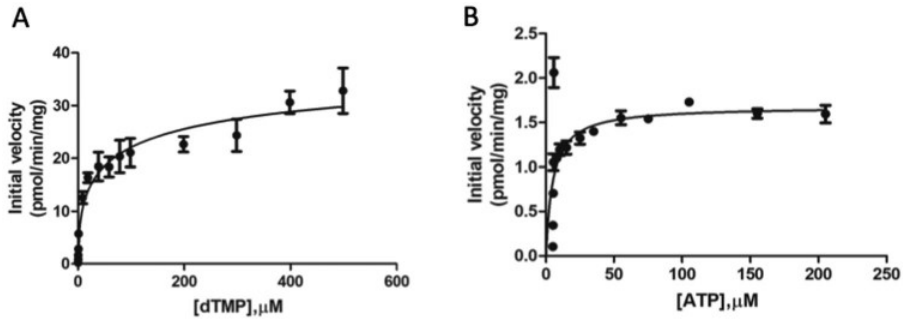

Figure 3. Steady-state kinetic analysis of mitochondrial outer membrane TMPK-like enzyme. Substrate saturation curves (V versus [S]) with dTMP (A) and ATP (B) as the variable substrate. Data with was fitted into Hill equation and given as mean ± SD.

To identify this putative novel enzyme, the outer mitochondrial membrane protein fractions were separated on an anionic exchange (DEAE) column. As depicted in Figure 2(A), enzyme activity converting dTMP to dTDP was found in fractions eluted at approximately 50 mM KCl (Figure 2(A)). This elution position is thereby similar to that of the TMPK enzyme found in the cytosolic compartment (Figure 2(B)). To further characterize the TMPK-like novel enzyme activity from the outer mitochondrial membrane, the fractions with the highest enzyme activities (after DEAE column chromatography) were pooled and studied further. These analyses showed that the outer mitochondrial membrane enzyme was unstable and lost activity rapidly; it also lost activity in the presence of a high concentration of NaCl (Figure 2(C), left panel). In contrast, the canonical TMPK is stable and its activity is not affected by the presence of NaCl (Figure 2(C), right panel).

Steady-state kinetic analysis of the partially purified mitochondrial outer membrane showed a sigmoidal substrate saturation curve for dTMP (Figure 3(A)), indicating negative cooperativity for dTMP phosphorylation with a $K_{1/2}$ value of 8.6 ± 2.0 μM, a V_{max} value of 42.4 ± 9.9 pmol/min/mg, and Hill coefficient of 0.5. However, a hyperbolic substrate saturation curve was observed when ATP was the variable substrate, suggesting Michaelis-Menten kinetics (Figure 3(B)). The K_M and V_{max} values for ATP were 5.1 ± 1.2 μM and 1.7 ± 0. 1 pmol/min/mg, respectively.

These results thus reveal that the TMPK-like enzyme present in the outer mitochondrial membrane has properties that are distinct from those of the canonical, *TYMK*-encoded TMPK, supporting the existence of a novel TMPK-like enzyme.

4. Discussion

Nucleotides are essential for cellular metabolic processes including DNA replication and RNA synthesis. As discussed earlier, the conversion of

Table 1. TMPK subcellular localization in human and yeast cells.

	Nucleus	Cytoplasma	Mitochondria	Mitochondrial outer membrane	Reference
Yeast (cdc8)	+	+	–	ND	[5]
Normal cells (*TYMK*)	+	+	+	ND	[6]
Cancer cells (*TYMK*)	+	+	+	–	This work

ND, not determined.

dTMP to dTDP is an important step that connects the salvage and the *de novo* pathways of dTTP synthesis. If *TYMK*-encoded TMPK would be the only source of dTDP production, the loss of function mutations or deletions of the *TYMK* gene would be lethal. However, knock-down of *TYMK* in cultured cells and genetic alterations observed in human patients have demonstrated that this is not the case.[3,4] In 2008, Chen et al claimed that an open reading frame found in the GenBank™ with sequence tag Loc 129607 encoded a TMPK (denoted as TMPK2), which was localized in mitochondria.[9] However, the same protein was later confirmed as a UMP-CMP Kinase (denoted as UMP-CMPK2) but with no detectable TMPK activity.[10] Thus, so far there is only one gene – the *TYMK* gene, known to code for TMPK.

We show here that TMPK activity is present in all subcellular fractions and that the highest activity is present in the mitochondrial outer membrane. However, this outer membrane enzyme did not react with antibodies against the canonical, *TYMK*-encoded TMPK. Furthermore, the elution profile of this mitochondrial outer membrane enzyme on anionic exchange column was similar to that of cytosolic TMPK but, in contrast to the canonical *TYMK*-encoded TMPK, the enzyme was unstable and sensitive to NaCl. Steady-state kinetic analysis revealed negative cooperativity for the enzyme with dTMP as substrate, and with ATP as the substrate, it followed Michaelis-Menten kinetics. However, the canonical *TYMK*-encoded TMPK showed Michaelis-Menten kinetics for both dTMP and ATP in a previous study.[11] Hence, the mitochondrial outer membrane enzyme of BJ fibroblasts had unique properties that differ from the canonical TMPK.

To further reinforce the concept of a putative novel enzyme with TMPK-like activity, we examined published data on TMPK from other cell types and species (Table 1). In yeast, *cdc8* encodes a TMPK protein, which is present in the nucleus and cytoplasm but not in mitochondria; however, there is no study where the existence of TMPK in the mitochondrial outer membrane has been examined.[12] In normal human cells, TMPK is detected in nucleus, cytosol and within mitochondria, whereas its possible existence in the mitochondrial outer membrane has not been studied.[13] Hence, it is possible that the existence of a TMPK-like enzyme in the mitochondrial outer membrane may have escaped the detection in earlier investigations. Altogether, based on the results presented in this investigation

and data from the literature, we propose that the TMPK-like activity detected in the mitochondrial outer membrane of BJ fibroblasts may originate from a hitherto unknown protein. Identification of this novel enzyme is underway.

5. Conclusion

An alternative enzyme with similar function as *TYMK*-encoded TMPK exists in the outer membrane of mitochondria in human cells. The identification of this new enzyme will have a profound impact on our understanding of the metabolism of nucleotides.

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III

NUCLEOSIDES, NUCLEOTIDES AND NUCLEIC ACIDS
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Structural and functional analysis of human thymidylate kinase isoforms

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ABSTRACT

Thymidylate kinase (TMPK) phosphorylates deoxythymidine monophosphate (dTMP) and plays an important role in genome stability. Deficiency in TMPK activity due to genetic alterations of *DTYMK*, i.e., the gene coding for TMPK, causes severe microcephaly in humans. However, no defects were observed in other tissues, suggesting the existence of a compensatory enzyme for dTTP synthesis. In search for this compensatory enzyme we analyzed 6 isoforms of TMPK mRNA deposited in the GenBank. Of these, only isoform 1 has been characterized and represents the known human TMPK. Our results reveal that isoform 2, 3, 4 and 5 lack essential structural elements for substrate binding and, thus, they are considered as nonfunctional isoforms. Isoform 6, however, has intact catalytic centers, i.e., dTMP-binding, DRX motif, ATP-binding p-loop and lid region, which are the key structural elements of an active TMPK, suggesting that isoform 6 may function as TMPK. When isoform 6 was expressed and purified, it showed only minimal activity (<0.1%) as compared with isoform 1. A putative isoform 6 was detected in a cancer cell line, in addition to the dominant isoform 1. However, because of its low activity, isoform 6 is unlikely be able to compensate for the loss of TMPK activity caused by deletions and/or point mutations of the *DTYMK* gene. Thereby, future studies to identify and characterize the compensatory TMPK enzyme found in patients with *DTYMK* mutations may contribute to the understanding of dTTP synthesis and of the pathophysiological role of *DTYMK* mutations in neurodegenerative disorders.

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1. Introduction

Thymidylate kinase (TMPK) is a key enzyme in the synthesis of DNA. In the presence of magnesium ions and ATP, it catalyzes the transfer of the γ-phosphate group from ATP to the 5′-phosphate group of deoxythymidine monophosphate (dTMP), and the product, i.e., deoxythymidine

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5′-diphosphate (dTDP), will then be further phosphorylated to deoxythymidine triphosphate (dTTP) by nucleoside diphosphate kinases. This is a highly regulated process and the level of dTTP is critical for nuclear genome stability and mitochondrial DNA copy number maintenance.[1]

The expression of human TMPK mRNA and the levels of TMPK protein are highest during the S-phase the cell cycle in order to cope with rapid DNA synthesis, and once the cells enter mitosis the levels of TMPK protein decline rapidly from mitosis to early G1 phase. This cell cycle regulated proteolysis of TMPK protein is mediated by the anaphase promoting complex/cyclosome (APC/C)-complex since human TMPK contains several sequence motifs that are recognized specifically by the APC/C complex, which targeted TMPK for degradation.[2,3] Defects in enzymes involved in dTTP synthesis cause neurodegenerative disorders in humans.[4,5] For instance, genetic alterations of *DTYMK*, i.e., the gene coding for human TMPK, cause severe microcephaly in humans.[5,6] Notably, TMPK is up-regulated in cancer cells and tissues, implicating TMPK as a potential target for cancer treatment.[7] In line with this notion, it has been demonstrated that inhibition of TMPK activity sensitizes tumor cells to doxorubicin treatment, both *in vitro* and *in vivo*.[8]

As noted above, point mutations or deletions in *DTYMK* in humans lead to loss of function of TMPK and cause neurodegenerative disorders. However, two of the four reported patients were still alive at 2 respective 7 years old and there were no abnormalities observed in non-nervous tissues.[5,6] This suggests that there is a compensatory mechanism for dTTP production in tissues other than nervous tissue. Indeed, an earlier study performed on cancer cell line has identified a novel TMPK activity that is not recognized by an antibody against the *DTYMK*-encoded TMPK protein.[9] However, the identity of this alternative TMPK is still unknown.

To approach this issue, we here searched for TMKP isoforms in databases and assessed whether any of these could have TMPK enzymatic activity. In GenBank there are six isoforms of human TMPK mRNA deposited, and the encoded polypeptide ranges from 113 to 251 amino acids in length. Isoform 1, consisting of 212 amino acids, is well-characterized regarding enzyme function and 3D-structure and is considered as the human TMPK.[10–12] However, the structures and functions of isoforms 2 to 6 are still not known. To evaluate if any of these isoforms can function as the compensatory TMPK enzyme, we conducted structural and functional studies. Structural analysis revealed that isoform 2-5 lacked essential structure elements required for TMPK activity, whereas isoform 6 contains all necessary functional sequence motifs and may fold into an active enzyme. Therefore, isoform 6 was cloned and expressed and the recombinant protein was characterized. In addition,

the presence of the various TMPK isoforms in cell extracts was also studied.

2. Materials and methods

2.1. Sequence and structural analysis

Protein sequences of all 6 isoforms were extracted from GenBank and aligned by using the Clustal Omega algorithm at www.ebi.ac.uk. The 3D structure of human TMPK (isoform 1, PDB code: 1E2F) was extracted from the Protein Data Bank and analyzed by using PyMOL. Structure modeling of isoform 2 to 6 was performed by using the Swiss model (https://swissmodel.expasy.org/), with human TMPK structures in complex with dTMP, ADP and Mg^{2+} (PDB code: 1E2F) and in complex with AZTMP and ADP (PDB code: 1E98) as templates. The global model quality estimate (GMQE) is 0.83 and sequence identity is 99.06%. Local quality estimate of the newly built model is between 0.8 to 1.0 (1.0 is the highest grade), except for the loop region (residue 81–120 in isoform 6).

2.2. Protein expression, purification and enzyme assay

Human TMPK isoforms 1 (NP_036277.2) and 6 (NP_001307834.1) were cloned into the pET-14b vector with an N-terminal fusion 6x His tag and expressed in *E. coli* strain BL21 (DE3) pLysS. Recombinant proteins were expressed and purified as previously described.[13] The purity of the recombinant proteins were >95% as judged by SDS-PAGE. The recombinant proteins were used in TMPK activity measurement using [^{3}H]-dTMP as substrate, essentially as previously described.[9]

2.3. Size-exclusion chromatography

The Äkta Prime system was connected to a Superdex 200 10/300 GL column (GE healthcare). The column was washed extensively and equilibrated with buffer containing 10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$ and 5 mM DTT. Prior to loading, protein samples were filtered through a 0.22 µM filter (Merck). The flow rate was 0.2 ml/min. Fractions (0.4 ml) were collected and used for TMPK activity measurement and western blot analysis using a human TMPK specific antibody.[9] Blue dextran 2000 was used to determine the void volume and bovine serum albumin (66 kDa), ovalbumin (43 kDa) and cytoschrome c (12 kDa) were used as standards.

2.4. Cell culture and western blot analysis

Human fibroblasts, immortalized with hTERT,[14] provided by Prof Staffan Johansson, Uppsala University, were maintained in a 37 °C incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma Aldrich) containing 6% heat inactivated Fetal Bovine Serum (Thermofisher), 1% Penicillin-Streptomycin (Thermofisher) and 1 mM sodium pyruvate (Sigma Aldrich). Fibroblast cell cultures were harvested after reaching ~80 – 90% confluency. Total protein was extracted and used in western blot analyses using a polyclonal antibody against human TMPK, targeting all TMPK isoforms except isoform 4,[9] following standard protocols.

3. Results

3.1. Sequence and structural analysis

The amino acid sequences of the 6 human TMPK isoforms vary considerably in length, and sequence alignment shows that isoforms 2–5 have partial deletions, whereas isoform 6 has an insertion of 39 amino acids, as compared with isoform 1 (Figure 1). In all known TMPK structures there are three important sequence motifs: the p-loop, the DRX (X = Tyr or Phe) motif and the Lid region (Figure 1). The p-loop is involved in the binding and proper positioning of the phosphoryl group of ATP. The Asp residue of the DRX motif assists in binding and positioning the Mg^{2+} ion. The Arg residue of the DRX motif may aid in catalysis by bringing the phosphate donor (ATP) and acceptor (dTMP) into proximity. The Lid-region covers the ATP molecule and undergoes conformational changes during catalysis.[12]

To investigate if the corresponding deletions in isoforms 2-5 and the insertion in isoform 6 have any impact on the structure and function of the enzymes, the structure of human TMPK (isoform 1) in complex with ligands (dTMP, ADP and Mg^{2+}) was analyzed by using PyMol. As shown in Figure 2A, human TMPK (isoform 1) has a globular structure in which the ATP-binding domain is present on the surface, whereas the dTMP-binding domain is located in the interior of the enzyme. ATP binding is mediated by two flexible loops, i.e., the Lid region extending from helices α6 and α7, and the p-loop located between β1 and α1 (Figures 1 and 2). The binding of dTMP is mediated by a series of intricate interactions, including H-bonds with residues R76 in α3 and D96 in β3 and three pairs of hydrophobic π-π stacking mediated by F72 at α3, F105 at α4, and Y151 at the Lid region (Figure 2B, these residues are labeled in blue). Notably, this highlights that hydrophobic interactions are a driving force in protein and ligand interactions in human TMPK. The five β-strands make up the

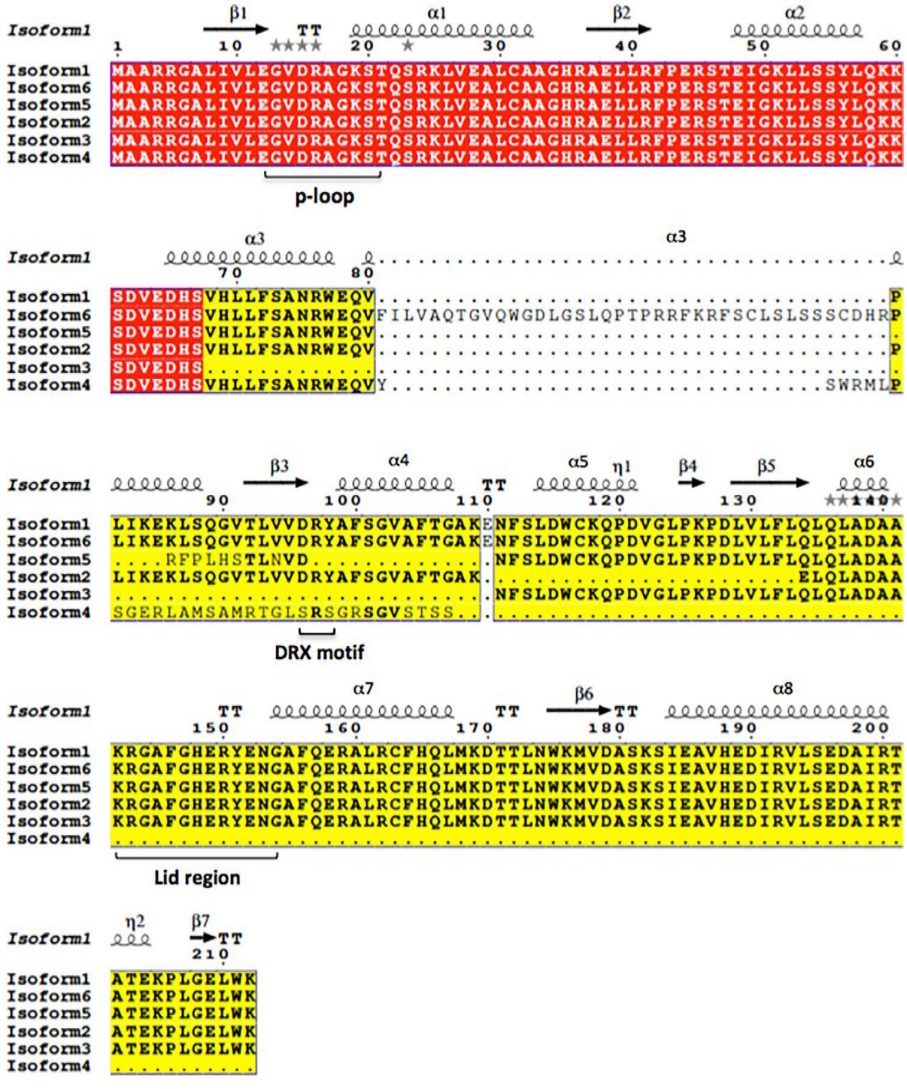

Figure 1. Sequence alignment of human TMPK isoforms. Accession numbers: Isoform 1 (Np_036277.2), isoform 2 (Np_001158503.1), isoform 3 (Np_001307831.1), isoform 4 (Np_001307832.1), isoform 5 (Np_001307833.1), isoform 6 (Np_001307834.1). Important functional motifs are marked. The structure-based sequence alignment was performed by using the ESPript 3.0 program.[15]

core structure of the enzyme. Each β-strand forms three to five H-bonds with adjacent β-strands, which is essential for folding and stability of the active site structure (Figure 2C).

Based on the key structural elements outlined above, the structure of the various human TMPK isoforms i.e., isoforms 2-6, was analyzed with the aim to identify variants that could possess catalytic activity.

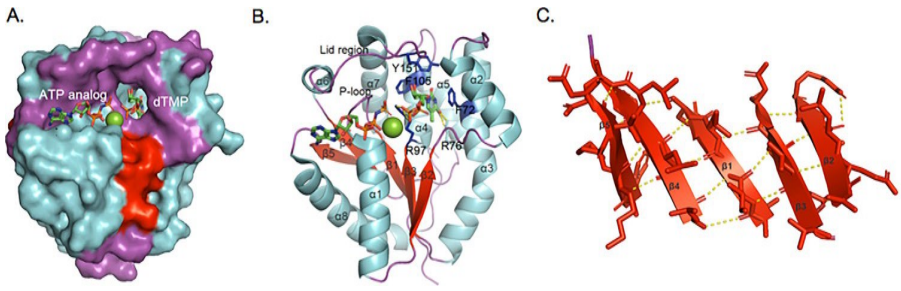

Figure 2. Human TMPK structure (isoform 1). (A) The human TMPK structure shown as surface model. The helices are labeled with cyan, the β-sheet with red and loops are labeled with magenta. In the ligand-binding pocket: ATP is located partly on the surface and dTMP binds in the interior of the protein. (B) Human TMPK structure shown as a cartoon. Residues interacting with dTMP: hydrophobic interaction with F72, F105 and Y151, labeled in blue, the side chain of R76 binds to the thymine ring and the side chain of R97 interacts directly with the phosphate group of dTMP via a H-bond. (C) The β-sheet structure of human TMPK. The five β-strands are placed in the same direction and form the backbone of the protein structure. The human TMPK structure used in the analysis was from https://www.rcsb.org/ (pdb code: 1E2F).

As shown in Figure 1, isoform 2 lacks α5, β4 and one connecting loop region. The lack of β4 leads to instability of the entire β-sheet. Since β4 makes direct contact with the Lid region (α6 and α7), this may pose constraints on the conformation of the Lid region. Thus, isoform 2 is most likely not capable of binding ATP, resulting in an inactive enzyme. Isoform 3 lacks α3, α4, β3 and two connecting loop regions. Both α3 and α4 are located in the dTMP-binding pocket, donating two pairs of π-π stacking (F72 and F105) and a H-bond (R76) to dTMP. With all these essential elements for dTMP binding missing, it is unlikely that isoform 3 can bind dTMP. Furthermore, β3 is important for the entire β-sheet stability and the loop that connects to β3 contains the DRX motif, which is crucial for substrate binding and catalysis. Therefore, isoform 3 is most likely catalytically inactive. Isoform 4 is the shortest of all isoforms and the deletions present in this isoform encompasses the entire active site, including α3, α4, α5, α6, α7, α8, β3, β4 and β5. Thus, isoform 4 cannot be an active enzyme. In isoform 5, two key helices (α3 and α4) are missing. Both of these are essential for dTMP binding, and thus isoform 5 is most likely not catalytically active.

Isoform 6 has an insertion of 39 amino acids, and has a calculated molecular weight of 28.2 kDa. The insertion is placed in the middle of α3. To clarify the effect of this insertion, it is important to understand the role of α3 in the TMPK structure and function. Helix α3 has three unique and essential roles in TMPK function: (1) active human TMPK is a homodimer (Figure 3A). Hydrophobic residues (L70, L71, A74 and W77) in α3 form the interface between the two monomers by hydrophobic

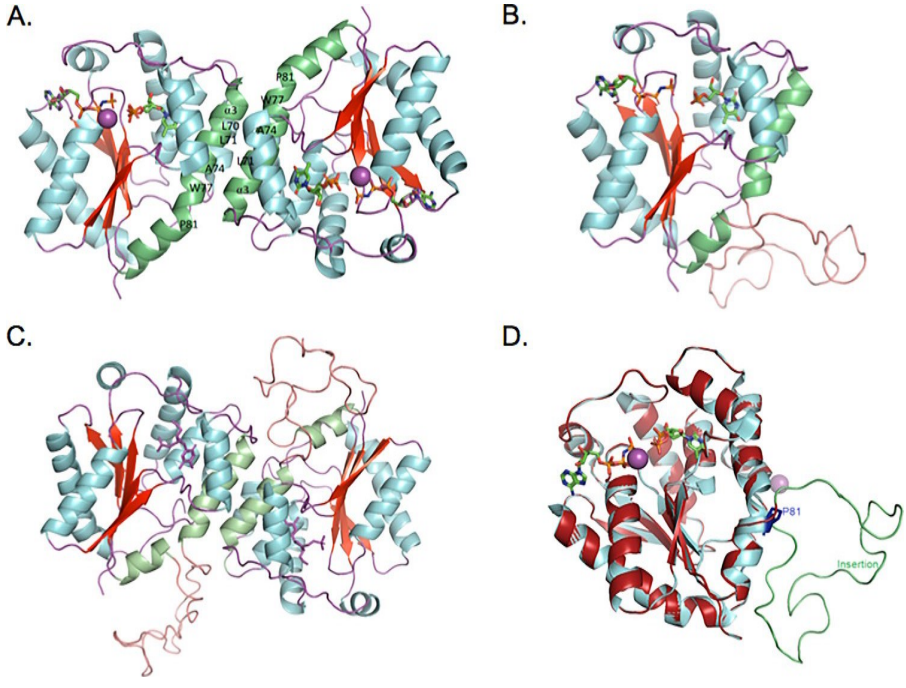

Figure 3. Structure model of TMPK isoform 6. (A) Human TMPK (isoform 1) homodimer. The α3 makes interface between the two monomers, through hydrophobic interactions (in green); (B) Homology model of the TMPK isoform 6 monomer. The 39-amino acids insertion is shown as a loop (salmon). (C) Structural model of the TMPK isoform 6 dimer. (D) Superimposed structures of isoform 1 and 6. Isoform 1 is in cyans and isoform 6 is in firebrick color. The 39 amino acid insertion is in green. Structural modeling was performed at https://swissmodel.expasy.org/ with human TMPK structure as template (PDB code: 1E2F).

interactions to the corresponding helix in the other monomer (Figure 3A); (2) residue F72 forms a hydrophobic interaction and R73 forms a H-bond with dTMP; (3) Residue P81 makes a kink of α3, which enables the interaction of F72 and R73 with dTMP (Figure 3A). The P81 residue is conserved in almost all TMPKs, including TMPK from viruses, bacteria and eukaryotes, although with one exception: *Mycobacterium tuberculosis* (*M. tuberculosis)* TMPK has a glycine instead of proline at this position (G76 in *M. Tuberculosis*, PDB code: 1N5I). Glycine has a similar function as proline, i.e., it can partly break a helix and make a kink. Mutation of P81 to P81L in human TMPK resulted in loss of TMPK function and severe microcephaly in human patients,[5,16] further demonstrated the essential role of this "kink" in TMPK function. How could this be achieved in isoform 6 with the 39 amino acid insertion prior to the conserved proline? As shown in Figure 3B, in the structural model of isoform 6 the 39 amino acids insertion folds into a flexible loop that may form the "kink" between α3 and α4 (labeled as "insertion" in the figure). This loop

Table 1. Summary of structural analyses of human TMPK isoforms.

		Functional motifs			Structure stability	
	MW (kDa)	dTMP binding	DRX motif	ATP binding	β-sheet	Kink in helix α3
Isoform 1	23.8	+	+	+	+	+
Isoform 2	21.1	+	+	+	–	+
Isoform 3	19.0	–	–	+	–	–
Isoform 4	12.5	–	–	–	–	–
Isoform 5	21.9	–	–	+	–	–
Isoform 6	28.2	+	+	+	+	+

+, present
–, absent

structure may provide the structure flexibility needed for dimerization (Figure 3C). Nonetheless, isoform 6 is predicted to have a preserved 3D-structure with all functional important motifs intact as shown in superimposed structures of isoform 1 and 6, and could therefore be catalytically active (Figure 3D). A summary of the predicted structural and functional features of the various human TMPK isoforms is presented in Table 1.

3.2. Expression, purification and characterization of isoform 6

Our molecular modeling approach suggested that isoform 6 might represent a catalytically active enzyme, although it cannot be excluded that the extra insertion may affect enzyme function. To address this, we cloned and expressed TMPK isoform 6 in *E. coli* and the recombinant protein was affinity purified to > 95% purity as judged by SDS-PAGE. The human TMPK (isoform 1) was also expressed and purified, and used as control (Figure 4A). Upon size-exclusion chromatography analysis isoform 1 is in dimer form,[16] however, isoform 6 eluted in various forms, from monomer, dimer to oligomer and the oligomer form was the dominant form (Figure 4B). The activity of the purified isoforms 1 and 6 were determined by using [^{3}H]-dTMP as substrate. This analysis revealed that the specific activity of isoform 6 is only 0.20 nmol/min/mg, which is < 0.1% of the activity of the human TMPK (isoform 1) (Figure 4C). Hence, the 39-amino acid insertion in isoform 6 has a major negative impact on the catalytic activity of the enzyme.

3.3. Expression of isoform 6 in cells

We next examined if isoform 6 is expressed in cells by using a human TMPK-specific antibody raised against the C-terminal sequence. This antibody will recognize all TMPK isoforms (except for isoform 4) if they are

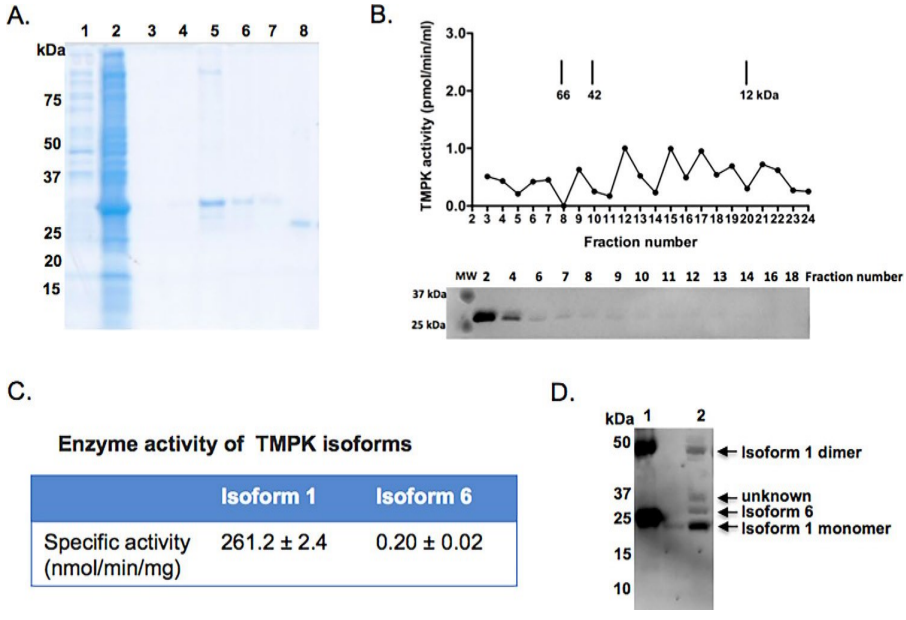

Figure 4. Functional characterization of TMPK isoform 6. (A) Purified TMPK isoform 6 and isoform 1. Lane 1, *E. coli* extracts of uninduced culture; lane 2: *E. coli* extracts of induced culture for isoform 6; lane 3 to 7, correspond to fractions 1-5 of the purified isoform 6; lane 8, purified isoform 1. (B) Size-exclusion chromatography of purified isoform 6. Fractions were collected and assayed for TMPK activity using [^{3}H]-dTMP as substrate and shown as pmol/min/ml. Selected fractions were analyzed by SDS-PAGE and western blot using a human TMPK specific antibody. The elution positions of molecular weight standards are indicated. (C) TMPK activity. Recombinant isoform 1 and 6 were assayed for enzymatic activity using [^{3}H]-dTMP as substrate; data are from three independent measurements and given as mean ± SD; (D) Western blot analysis using a human TMPK-specific antibody. Lane 1, recombinant human TMPK (isoform 1), Lane 2, extracts from fibroblasts.

expressed. Total protein from a cultured human fibroblast cell line was extracted and used in western blot analysis. As shown in Figure 4D, four protein bands were detected by the antibody. Of these, the 24 kDa and ~ 50 kDa bands correspond to the monomer and dimer forms of human TMPK (isoform 1), respectively, which represent the dominant form of TMPK in this cell line. In addition, a ~28 kDa TMPK band was observed, most likely representing isoform 6. Bands corresponding to isoforms 2, 3 and 5 were not detected. An additional band at ~37 kDa was also detected by the antibody. However, the identity of the latter band is uncertain.

4. Discussion

Alternative splicing is a mechanism of RNA maturation and can generate either degradable nonsense mRNAs or mature mRNAs of different splicing

variants, which may encode proteins with altered functions. For example, an alternative spliced dominant negative isoform of PPARγ (peroxisome-proliferator-activated receptor γ), which lacks the entire ligand binding domain, could impair PPARγ function.[17] In search for compensatory TMPK enzymes we here sought to characterize the isoforms of human TMPK mRNA that are deposited in GenBank, and to evaluate if any of these isoforms could exert TMPK enzymatic activity. Our sequence and structural analysis revealed that isoform 2, 3, 4 and 5 all lacked the structural elements that are essential for substrate binding and for formation of a globular structure. Thus, they are considered as nonfunctional isoforms. In contrast, isoform 6 has intact catalytic centers i.e., dTMP binding, DRX motif, ATP binding p-loop and Lid region, which are the key structural elements of an active TMPK. Therefore, isoform 6 may have TMPK activity. However, when isoform 6 was expressed and purified, it showed only minimal activity as compared with isoform 1. Furthermore, a putative isoform 6 was detected in a fibroblast cell line in addition to isoform 1, the latter being the dominant TMPK present in these cells. Since isoform 6 has minimal TMPK activity, it is unlikely that isoform 6 contributes significantly to dTTP synthesis *in vivo* but it cannot be excluded that it may have regulatory functions. For example, Isoform 6 may bind to both dTMP and ATP, and thus compete with isoform 1 for substrates, thereby interfering with dTTP synthesis in cells expressing isoform 6. It is also possible that isoform 1 and 6 form heterodimers, resulting in altered TMPK activity. At present, we do not have any experimental evidence for the presence of isoforms 2 to 5 in cells, however, if these inactive isoforms are expressed, they might interact with the active TMPK (isoform 1) and interfere with its function. To clarify this, further investigations are needed.

Studies of human patients with TMPK deficiency suggest that there is a compensatory TMPK in tissues other than nervous tissue.[5,16] In a fibroblast cell line, a high level of TMPK activity was detected in the mitochondrial outer membrane fraction and this TMPK activity had different biochemical properties as compared with the *DTYMK*-encoded TMPK.[9] Our results presented here suggest that the alternative TMPK identified in the fibroblast cell line, or present in TMPK-deficient patients, does not represent any of the TMPK isoforms characterized here, and is most likely encoded by a different gene. Since all other known nucleoside monophosphate kinases, i.e., AMPKs (adenylate kinases), CMP-UMP kinase, GMP kinase have narrow substrate specificity and none of them are able to phosphorylate dTMP.[18] In future investigations, identification and characterization of this alternative TMPK enzyme may contribute to the understanding of dTTP synthesis in different tissues, and may also provide further insight into the pathophysiological role of TMPK in neurodegenerative disorders.

5. Conclusion

For human TMPK, there are 6 mRNA isoforms present. Isoform 1 is the TMPK enzyme that is well-characterized. Our study revealed that, except for isoform 1, only isoform 6 might fold into an active enzyme. However, functional studies revealed that isoform 6 has minimal TMPK activity. Although isoform 6 may be expressed in cells, it is unlikely that it contributes significantly to dTTP synthesis, but may instead have regulatory functions. Therefore, the alternative TMPK activity that has been identified in human patients and cancer cell line is most likely not due to the TMPK isoform 6, but rather due to a hitherto unidentified enzyme encoded by a different gene.

Disclosure statement

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RESEARCH ARTICLE

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Differential expression of enzymes in thymidylate biosynthesis in zebrafish at different developmental stages: implications for *dtymk* mutation-caused neurodegenerative disorders

Junmei Hu Frisk[1], Stefan Örn[2], Gunnar Pejler[1,3], Staffan Eriksson[1] and Liya Wang[1*]

Abstract

Background: Deoxythymidine triphosphate (dTTP) is an essential building block of DNA, and defects in enzymes involved in dTTP synthesis cause neurodegenerative disorders. For instance, mutations in *DTYMK*, the gene coding for thymidylate kinase (TMPK), cause severe microcephaly in human. However, the mechanism behind this is not well-understood. Here we used the zebrafish model and studied (i) TMPK, an enzyme required for both the de novo and the salvage pathways of dTTP synthesis, and (ii) thymidine kinases (TK) of the salvage pathway in order to understand their role in neuropathology.

Results: Our findings reveal that maternal-stored dNTPs are only sufficient for 6 cell division cycles, and the levels of dNTPs are inversely correlated to cell cycle length during early embryogenesis. TMPK and TK activities are prominent in the cytosol of embryos, larvae and adult fish and brain contains the highest TMPK activity. During early development, TMPK activity increased gradually from 6 hpf and a profound increase was observed at 72 hpf, and TMPK activity reached its maximal level at 96 hpf, and remained at high level until 144 hpf. The expression of *dtymk* encoded Dtymk protein correlated to its mRNA expression and neuronal development but not to the TMPK activity detected. However, despite the high TMPK activity detected at later stages of development, the Dtymk protein was undetectable. Furthermore, the TMPK enzyme detected at later stages showed similar biochemical properties as the Dtymk enzyme but was not recognized by the Dtymk specific antibody.

Conclusions: Our results suggest that active dNTP synthesis in early embryogenesis is vital and that Dtymk is essential for neurodevelopment, which is supported by a recent study of *dtymk* knockout zebrafish with neurological disorder and lethal outcomes. Furthermore, there is a novel TMPK-like enzyme expressed at later stages of development.

Keywords: Thymidylate kinase, *Dtymk*, Thymidine kinase, *Tk*, dTTP synthesis, dNTPs, Zebrafish development, Neuronal development

Background

Zebrafish (*Danio rerio*) is a useful vertebrate model organism because of the external fertilization and fast embryonic development with a high level of transparency, which provides a unique opportunity for

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biomedical and genetic studies. The rapid generation of genetically modified zebrafish lines by Crispr/Cas9 or morpholino approaches allows detailed analyses of gene/protein functions in a vertebrate context. The zebrafish genome-sequencing project reveals that approximately 70% of the human genes have zebrafish orthologues [1]. Therefore, the zebrafish model has contributed profoundly to our knowledge of vertebrate genetics and biology [2–4].

Nucleotides are essential components of cellular metabolic processes and are synthesized by two distinct pathways - the de novo and salvage pathways. Deoxythymidine triphosphate (dTTP) is an essential building block of DNA and can be synthesized by the de novo pathway where deoxyuridine monophosphate is converted to thymidine monophosphate (dTMP) by thymidylate synthase in the presence of tetrahydrofolate, or by the salvage pathway where deoxythymidine (dThd) is phosphorylated to dTMP by either cytosolic thymidine kinase 1 (TK1) or mitochondrial thymidine kinase 2 (TK2). dTMP, i.e., produced either by the de novo or salvage pathway, is then further phosphorylated to dTTP in two consecutive steps catalyzed by thymidylate kinase (TMPK) and the non-specific nucleoside diphosphate kinases. TMPK is thus an enzyme required for both the salvage and de novo pathways of dTTP synthesis [5–7].

A balanced deoxynucleoside triphosphates (dNTPs) pool is essential for DNA synthesis, repair and cell proliferation. Therefore, enzymes involved in nucleotide synthesis play important roles in cell proliferation and survival. Loss of function mutations or dysregulation of key enzymes in nucleotide metabolism can lead to severe pathophysiological conditions, including neurological and myopathic disorders, such as neonatal liver failure, nystagmus, hypotonia, and growth retardation [8]. In humans, deficiency in TK2 activity due to genetic alterations causes devastating mitochondrial DNA depletion and/or deletion diseases affecting multiple tissues [6, 9]. Genetic alterations of the *DTYMK* gene, coding for TMPK, cause severe neurodegenerative disorders [10, 11], suggesting that TMPK is essential for neuronal development.

In the recent two decades, using different genetic approaches, a large number of genes coding for enzymes in nucleotide metabolism have been studied in zebrafish [2, 12–15]. For example, mutations in *gart* and *paics* in the de novo purine biosynthesis cause pigmentation and ocular developmental disorders [14]; mutations in *cad*, encoding carbamoyl-phosphate synthetase 2 - aspartate transcarbamoylase - dihydroorotase in pyrimidine de novo synthesis, lead to severe dismorphogenesis of the jaw and fin, and to a small eye phenotype; and mutations in ribonucleotide reductase, an essential enzyme for the de novo dNTP synthesis, result in severe deficits and early embryonic lethality [13].

To understand the essential role of enzymes in dTTP synthesis in development, we used zebrafish as a model to study the expression and activity of TMPK and TK enzymes at different developmental stages. Our findings suggested the existence of two TMPK enzymes, which apparently were differentially expressed. Further, we demonstrated that the expression and activity of salvage pathway enzymes, i.e., TK1 and TK2, were low and probably insignificant for dTTP synthesis during early embryogenesis. Our results also showed that dNTP levels were inversely correlated to cell cycle length during early embryogenesis, thus representing limiting factors for cell division in zebrafish.

Results

dNTP levels are inversely correlated to cell cycle length during early embryogenesis

Zebrafish embryos undergo rapid cell division during early stages of development. By the time zygotic genome activation starts, approximately 3.5 hpf, the embryos have gone through 12 cycles of cell division with approximately 15 to 30 min per cycle [16]. This rapid cell division requires adequate supply of dNTPs for nuclear DNA synthesis. Here we determined the levels of dNTPs at different developmental stages. As shown in Fig. 1, the overall levels of dCTP and dTTP were higher than those of dATP and dGTP during the entire measurement period, and the highest dNTP levels were maintained from 0 to 6 hpf and then started to decline gradually to a low level at 144 hpf. These results suggest that there is an active dNTP synthesis during the first 6 h after fertilization, probably by maternal supplied essential enzymes and mRNA transcripts encoding dNTP synthesis enzymes before zygotic genome activation. The zebrafish genome contains 1.41×10^9 base pairs with an AT content of 61.4%. Using the dNTP concentrations determined at 0 hpf and method described by Song et al. [17], we could conclude that the embryo contained at least 70 nuclear equivalents of dATP, which was sufficient for 6 rounds of nuclear division, and the levels of dTTP, dGTP and dCTP could sustain even higher numbers of cell divisions (Fig. 1). Until the 10th cell division (3 hpf) each cell cycle needs only 15–20 min, and the high levels of dNTPs measured here from 0 to 6 hpf ensured that there are sufficient dNTPs to sustain rapid cell growth. From 6 hpf and onward, the dNTP levels decreased gradually to their lowest levels at 144 hpf. Similarly, from cell division cycle 12 and onwards, the time required for each cell

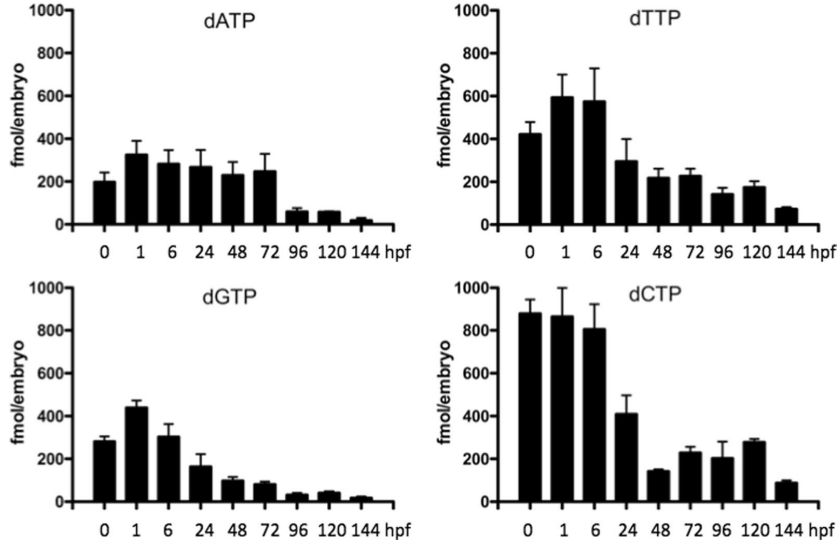

Fig. 1 The levels of dNTPs at different developmental stages. A total of 50–75 embryos/larvae were collected at each time point and used in dNTP measurement (see Materials and Methods for details). Data were from 9 to 12 independent measurements and given as fmol dNTP per embryo (mean ± SD)

division gets progressively longer, with 240 min needed for the 16th cell cycle [16]. These results indicated that the levels of dNTPs are limiting factors during cell division and that levels of dNTPs inversely correlate with the time required for each cell cycle.

High TMPK activity in developing zebrafish embryos

TMPK is a key enzyme in dTTP synthesis both for the de novo and the salvage pathways. Therefore, we studied zebrafish TMPK activity in embryos and larvae harvested at different developmental stages. The embryos/larvae were homogenized and fractionized directly into cytosolic, nuclear, and mitochondrial fractions. Total protein from these fractions was extracted and used to measure TMPK activity using tritium labeled dTMP as substrate. TMPK activity was detected mostly in the cytosolic compartment (Fig. 2A), while in mitochondrial and nuclear fractions TMPK activities were very low, only approximately 1–5% of the TMPK activity detected in the cytosol (Fig. 2B and C). As shown in Fig. 2A, directly after fertilization the cytosolic TMPK activity was at basal level and slightly decreased in comparison with the zero time point, but then started to increase, reaching a maximum at 72 hpf, after which the TMPK activity was profoundly increased; this coincided with the hatching point. The highest TMPK activity was reached at 96 hpf and TMPK activity was maintained at this high level throughout the studied time frame (up to 144 hpf) (Fig. 2A).

Two TMPK proteins are differentially expressed at different developmental stages

Using a specific antibody raised against the active site region of the *dtymk*-encoded TMPK (hereafter referred to as the "canonical Dtymk"), the canonical Dtymk protein levels were determined. Notably, the western blot analysis revealed a fundamentally different pattern of the canonical Dtymk protein expression in comparison with the TMPK activity profile shown in Fig. 2A; low levels of the canonical Dtymk protein were detected at 0 and 1 hpf, thereafter, it started to increase from 6 hpf and reached its maximum at 24 hpf, after which it decreased gradually down to being barely detectable at 96 hpf. From 120 hpf and onwards the canonical Dtymk protein was undetectable (Fig. 2D). These findings suggest that the high TMPK activity detected from 96 to 144 hpf (see Fig. 2A) is most likely not attributed by the canonical Dtymk protein, hence introducing the notion that an alternative non-canonical TMPK like enzyme is expressed at high levels at later stages of development. This non-canonical TMPK is most likely encoded by an unknown gene, since in the zebrafish genome there is only one known TMPK-encoding gene: *dtymk*.

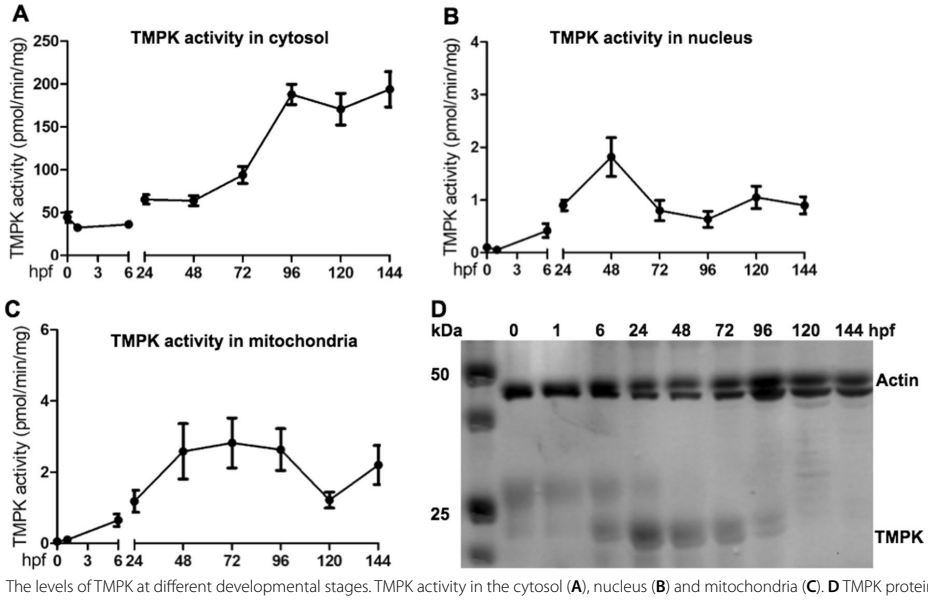

Fig. 2 The levels of TMPK at different developmental stages. TMPK activity in the cytosol (**A**), nucleus (**B**) and mitochondria (**C**). **D** TMPK protein levels in cytosolic fractions. Cytosolic, mitochondrial and nuclear fractions were isolated from zebrafish at 0, 6, 24, 48, 72, 96, 120 and 144 h post fertilization (hpf). Total protein was extracted from these fractions and used to measure TMPK activity by using [^{3} H]-dTMP as substrate and the data was plotted as mean ± SD. TMPK protein levels was assessed by western blot analysis using a specific antibody designed against zebrafish canonical TMPK and only the relevant part of the full image is shown

Biochemical properties of the zebrafish TMPKs

Next, we analyzed the cytosolic TMPK proteins by using anion exchange chromatography. For this, we used TMPK protein isolated from embryos harvested at 24 hpf, a stage at which the canonical Dtymk protein was expressed at its highest level, and from larvae at 120 hpf at which the canonical Dtymk protein was undetectable. Total cytosolic proteins were loaded onto DEAE columns, followed by elution with stepwise increased salt concentrations. Fractions were collected and TMPK activity determined. As shown in Fig. 3A, the major TMPK activity that bound to the column was eluted with 200 mM KCl, with the highest TMPK activity observed in fraction 20 and 21 for proteins isolated from embryos/larvae harvested both at 24 and 120 hpf. The protein concentration in each fraction was also determined (Fig. 3B). Fractions with TMPK activity (fraction number 16, 18, 20, 21, 25 and 28) were then analyzed by western blot using the antibody against the canonical Dtymk protein. As shown in Fig. 3C, the canonical Dtymk protein was detected in fractions with TMPK activity isolated from embryos harvested at 24 hpf, with the highest TMPK protein levels seen in fraction 20 and 21, which coincided with the TMPK activity as shown in Fig. 3A. In addition to the ~ 25 kDa band representing monomeric Dtymk protein, we also noted a band of ~ 75 kDa. Most likely, this band corresponds to oligomerized Dtymk. However, for proteins isolated from larvae harvested at 120 hpf, the canonical Dtymk protein was below the level of detection in all of the fractions containing TMPK activity (Fig. 3C). Hence, these findings are consistent with the results shown in Fig. 2D, providing support for the existence of two different TMPK enzymes, expressed at 24 and 120 hpf embryos/larvae, respectively.

We further analyzed the partially purified TMPKs by size exclusion chromatography to determine their molecular weight (MW). TMPK from embryos harvested at 24 hpf eluted with a MW of ~ 20–30 kDa, and the TMPK from larvae harvested at 120 hpf eluted at a similar MW (Fig. 3D). Thus, the TMPK proteins presented in the cytosol isolated from 24 to 120 hpf embryos/larvae apparently have similar biochemical properties and molecular weight but clearly different immuno-reactivity towards antibody against the canonical Dtymk.

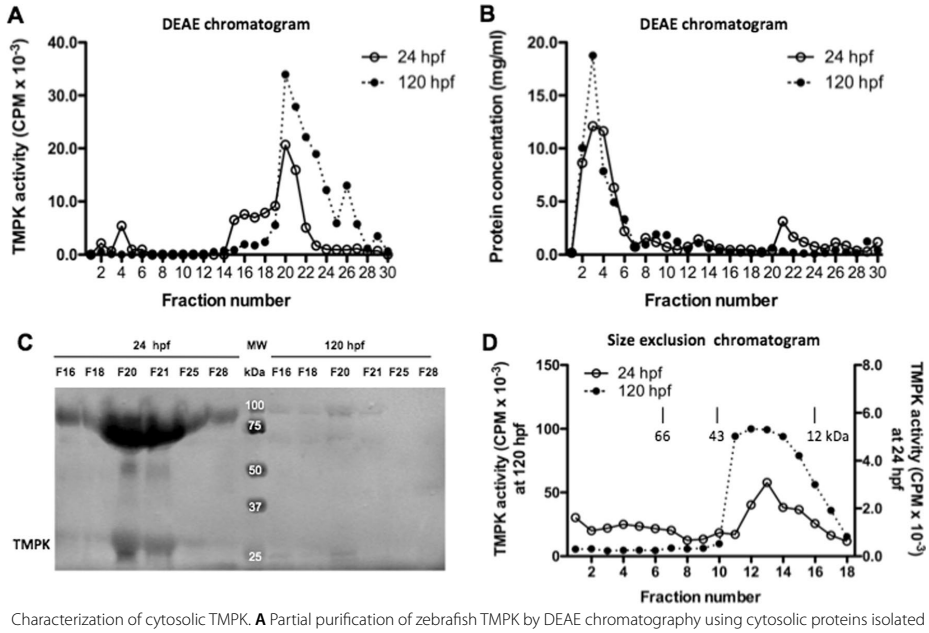

Fig. 3 Characterization of cytosolic TMPK. **A** Partial purification of zebrafish TMPK by DEAE chromatography using cytosolic proteins isolated from zebrafish embryos harvested at 24 hpf and 120 hpf. The bound proteins were eluted with stepwise increase of KCl concentration (from 50 to 250 mM) in DEAE buffer. Fractions were collected and assayed for TMPK activity; **B** Protein concentration of the corresponding DEAE fractions; **C** Identification of the canonical TMPK proteins in the TMPK activity peak fractions by western blot analysis using a zebrafish Dtymk-specific antibody. Fractions F16, F18, F20, F21, F25, and F28 from DEAE chromatography were used and only part of the image is shown; **D** Size exclusion chromatography of partially purified zebrafish TMPK. Fractions F20 and F21 from the DEAE chromatography were used. Fractions were collected and assayed for TMPK activity. The Y-axis on the left side corresponded the TMPK activities from 120 hpf embryos and the Y-axis on the right side corresponded the TMPK activities from 24 hpf embryos. The elution positions of molecular weight markers are indicated

Tk2 is the dominant cytosolic Tk expressed in zebrafish

The contribution of the salvage pathway synthesis of dTTP was studied by measuring Tk activity in embryos and larvae harvested at different developmental stages. In contrast to the high cytosolic TMPK activity during embryonic development, Tk activity in the cytosolic compartment was very low at early time points, whereafter it increased gradually with time and reached the highest levels at 144 hpf (Fig. 4A). The Tk activity in the nuclear fraction was even lower, approximately half of that in the cytosol (Fig. 4B), whereas no Tk activity was detected in the mitochondrial fractions (data not shown). Notably, this is in contrast to TKs from other species, where high TK activity is present in the cytosol, and lower TK activity is present in mitochondrial and nuclear fractions from rapidly dividing cells [5, 18].

We next analyzed the cytosolic proteins by DEAE column chromatography. As shown in Fig. 4C, two major Tk activity peaks were identified, with the first peak eluting at low salt (fraction 3–4) and the second peak eluting in the high salt fractions (fraction 14–16). Western blot analysis of the Tk activity peak fractions using a Tk1-specific antibody showed the presence of Tk1 protein in the fractions corresponding to the Tk activity eluting with low salt during DEAE chromatography, whereas no Tk1 protein was detected in fractions eluting with high salt (Fig. 4D). When fractions 14–16 (corresponding to the high salt peak of Tk activity) were assayed with ^{3}H-dThd in the presence of excess of deoxycytidine (dCyd), Tk activity was reduced by 65% (Fig. 4E). This indicates that the Tk activity recovered in the high salt peak corresponds to Tk2, since Tk2 uses both dThd and dCyd as substrates whereas Tk1 phosphorylates only dThd [5].

Organ distribution of TMPKs and TK in zebrafish

TMPK and TK1 are known to be cell cycle regulated with the highest expression levels in proliferating cells and tissues [19, 20]. Neurons are usually regarded as postmitotic without the ability to re-enter the cell cycle [21], and therefore, it is conceivable to assume that TMPK

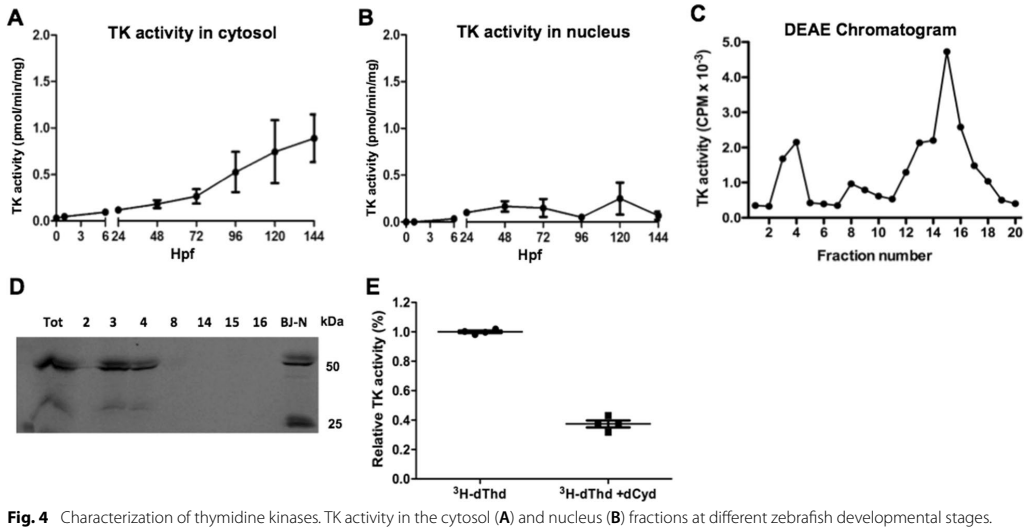

Fig. 4 Characterization of thymidine kinases. TK activity in the cytosol (**A**) and nucleus (**B**) fractions at different zebrafish developmental stages. **C** DEAE chromatography of cytosolic protein isolated from zebrafish at 120 hpf. Fractions were collected and TK activity was determined by using [^{3} H]-dThd as substrate. **D** Western blot analysis of the TK peak fractions (2, 3, 4, 8, 14,15 and 16) from DEAE chromatography using a TK1 specific antibody and only part of the image is shown. Cell extracts from a fibroblast (BJ-N) cell line was used as control. **E** Fractions from DEAE chromatography (fraction 15 and 16) were assayed in the absence and presence of excess dCyd using [^{3} H]-dThd as the substrate. Data was plotted as mean ± SD

and TK1 activity would be low. Here, we examined tissue distribution of TMPK and TK activity in zebrafish larvae at 120 hpf and adult fish (> 3 years old). The cytosolic, mitochondrial and nuclear TMPK activity in the zebrafish head were compared with the remainder of the body (decapitated zebrafish) from zebrafish larvae at 120 hpf and adult fish (> 3 years old), as well as in different organs from adult fish. Similar to the results shown in Fig. 2, the highest TMPK activity was detected in the cytosolic fractions of all samples and the levels of TMPK activity in the nuclear and mitochondrial fractions were very low (Fig. 5A–C). In zebrafish larvae from 120 hpf, TMPK activity was much higher in the head than the remainder of the body, and a similar pattern was also observed in adult fish (Fig. 5A, B). Compared with adult fish, brain TMPK activity from zebrafish larvae at 120 hpf was 3 times higher, and the remaining body of zebrafish larvae at 120 hpf contained 50 times higher TMPK activity (Fig. 5A and B). In other organs from adult fish, the specific TMPK activity was more than 4 times lower as compared with the brain. Furthermore, heart, spleen and gill had higher TMPK activity levels than liver and unfertilized egg (Fig. 5C).

Next, we analyzed the levels of the canonical Dtymk protein in different organs from adult fish by using western blot analysis, and found that the canonical Dtymk protein was not detectable in any of the organs from adult fish, while in the 24 hpf embryos and unfertilized egg the canonical Dtymk protein could be detected (Fig. 5D). These results indicate that the canonical Dtymk is mainly expressed during early embryogenesis and that the alternative non-canonical TMPK enzyme is expressed in adult fish.

TK activity in different organs was also measured. In agreement with the data displayed in Fig. 4, TK activities were higher in the cytosolic fractions recovered from different organs, in comparison with TK activities in mitochondrial and nuclear fractions. However, the overall TK activity was very low in all tested organs (Fig. 5E).

The mRNA expression profile of enzymes in thymidine nucleotide biosynthesis

We next assessed the mRNA expression profiles of enzymes involved in thymidine nucleotide biosynthesis: *dtymk* (coding for the canonical Dtymk), *tyms* (coding for Tyms), *tk1* (coding for Tk1) and *tk2* (coding for Tk2) during different developmental stages, using data available at the Expression Atlas database (http://www.ebi.ac.uk/gxa/experiments/E-ERAD-475) [22]. As shown in Fig. 6, the expression of *tyms* mRNA is the highest of all four genes included in our analysis, and *dtymk* mRNA levels is lower than that of *tyms* mRNA but higher than the levels

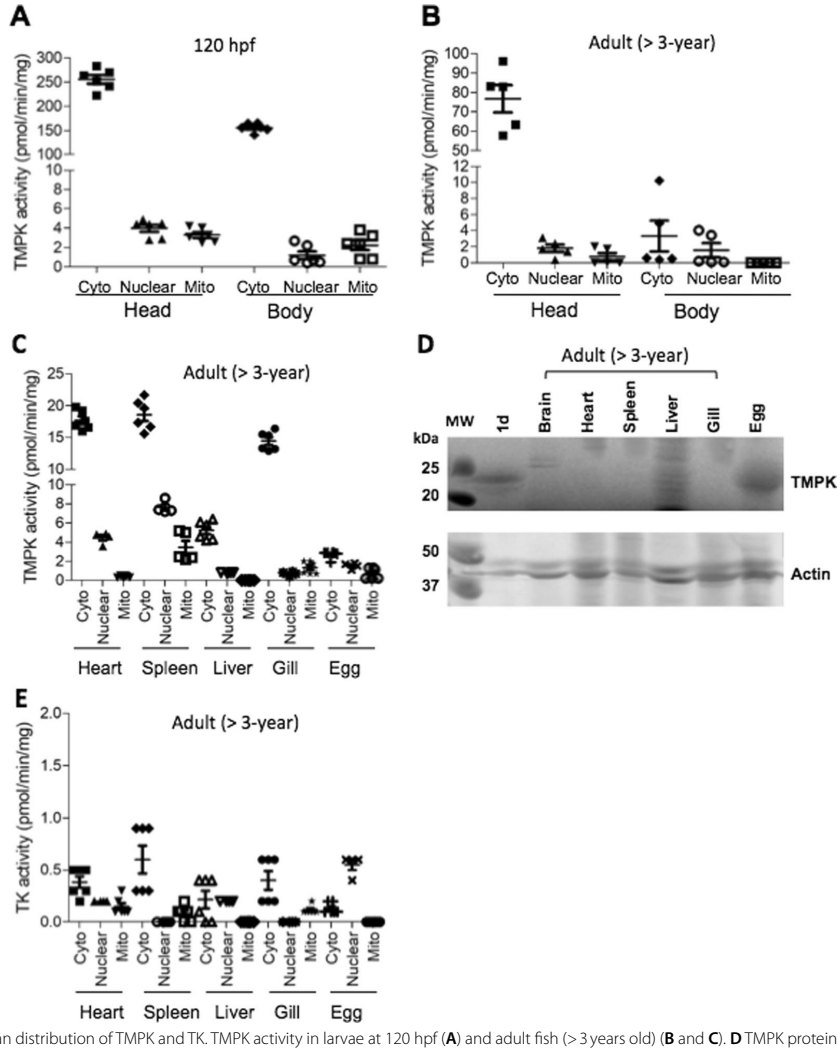

Fig. 5 Organ distribution of TMPK and TK. TMPK activity in larvae at 120 hpf (**A**) and adult fish (> 3 years old) (**B** and **C**). **D** TMPK protein levels in different organs. **E** TK activity in different organs. The head and truncated body (without head) as well as organs from adult fish were used to isolate cytosolic, mitochondrial and nuclear fractions. Total proteins were extracted and used to measure TMPK and TK activity by using [^{3} H]-labeled substrates and the data was plotted as mean ± SD. Western blot analysis was conducted by using the zebrafish Dtymk-specific antibody and only parts of the images are shown

of *tk1* and *tk2* mRNA. Prior to zygotic genome activation (pre-ZGA), the levels of *tyms, dtymk, tk1 and tk2* mRNA are relatively high and then decline to baseline levels at 6 hpf. After zygotic genome activation, the *dtymk* mRNA levels increase again during blastula/gastrula stages and reach its peak levels at approximately 10 hpf and then decline to baseline levels at 48 hpf, remaining at low levels onwards (Fig. 6B). Similar to *dtymk*, the level of *tyms* mRNA also increased after zygotic genome activation and reaches a peak level during somitogenesis (24 hpf), and then declines to baseline at 72 hpf and remains at the same level during the study time frame (Fig. 6C). The

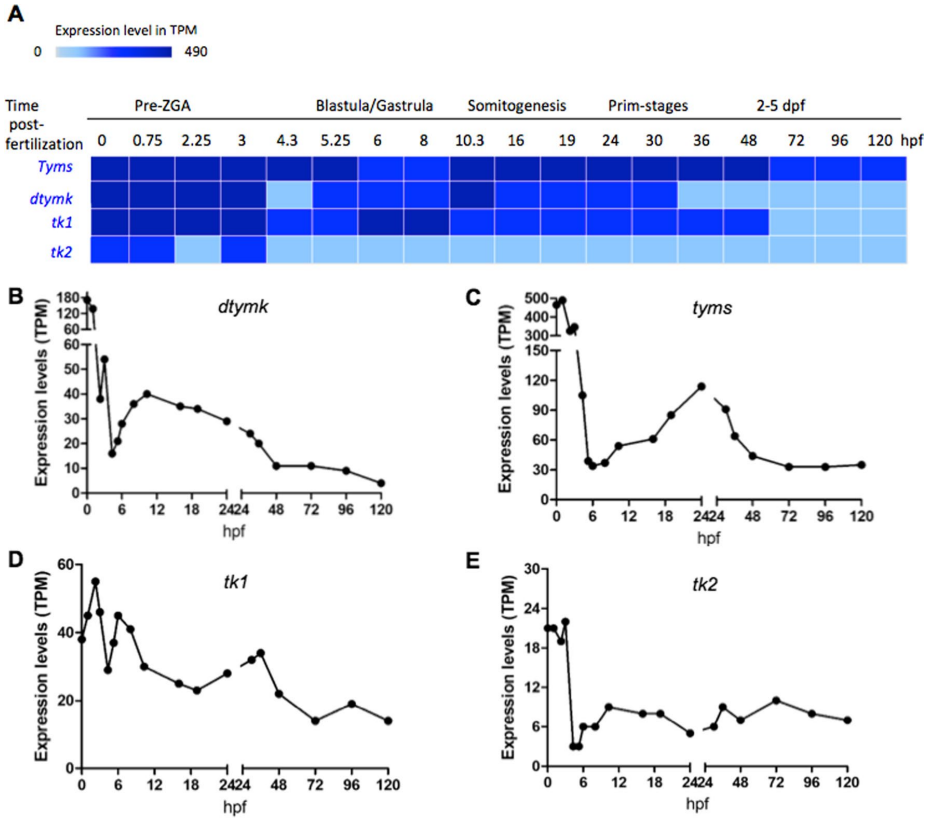

Fig. 6 mRNA expression profiles of selected enzymes involved in dTTP synthesis at different zebrafish developmental stages. **A** mRNA expression heatmap. *dtymk*, thymidylate kinase; *tyms*, thymidylate synthesis; *tk1*, thymidine kinase 1; *tk2*, thymidine kinase 2. Plots of mRNA levels of *dtymk* (**B**), *tyms* (**C**), *tk1* (**D**) and *tk2* (**E**). TPM, transcript per million. Data are shown as average expression (mean TPM) and retrieved from Expression atlas – baseline expression of transcriptional profiling of zebrafish developmental stages (https://www.ebi.ac.uk/gxa/experiments/E-ERAD-475/Result)

levels of *tk1* and *tk2* mRNA are low as compared with those of *dtymk* and *tyms*, and fluctuate during the different developmental stages (Fig. 6D, E).

Discussion

An adequate supply of dNTPs is essential for DNA replication and repair, and thus, cell proliferation. Early studies have shown that maternal-stored dNTPs is only sufficient for a limited number of cell divisions during early embryogenesis, for example, 11 rounds of cell division in *Xenopus* and 12 division cycles in *Drosophila* [17, 23]. Therefore, embryos must produce their own dNTPs in early stages in order to cope with rapid cell division during embryogenesis. Our data show that, in zebrafish embryo, the maternal-stored dNTP is only sufficient for 6 rounds of cell division and that the dNTP synthesis capacity is high in zebrafish embryos to sustain repaid cell division. Furthermore, the levels of dNTP detected at different developmental stages inversely correlate with cell cycle length [16], demonstrating that dNTP levels are limiting factors for cell proliferation.

There are four nucleoside monophosphate kinases, i.e. adenylate kinases, guanylate kinase, cytidylate-uridylate kinases, and TMPK, which catalyze the phosphorylation of nucleoside monophosphates to their corresponding nucleoside diphosphates. These enzymes have very narrow substrate specificity, and TMPK is the only nucleoside monophosphate kinase that is known to be able to phosphorylate dTMP [24]. Therefore, TMPK is essential for dTTP biosynthesis and thereby plays a critical role

in cell growth. Our study demonstrated that in zebrafish TMPK activity is mainly present in the cytosol with very low activity in mitochondria and nucleus, irrespective of developmental stages or organ/tissue origin. This cytosolic distribution of zebrafish TMPK is similar to what has been observed in other species [24–26]. We also showed that, during early embryonic development, TMPK activity was at a relatively low but stable level (from 0 to 6 hpf), which is sufficient to sustain an adequate dTTP pool for rapid DNA synthesis and cell division. After activation of the zygotic genome, from 6 to 24 hpf, TMPK activity increased gradually and a drastic increase in TMPK activity was seen after 72 hpf, i.e. at a stage where zebrafish switch from the embryonic to larval stage, from which cell growth escalates [27]. Hence, this marked increase in TMPK activity is consistent with the high demand for thymidine nucleotides at this phase of development.

We also compared TMPK activity in different organs and found that brain contained the highest TMPK activity, both at the larval stage (120 hpf) and in adult fish (> 3 years). Furthermore, the overall TMPK activity was much higher at the larval stage than in adult fish, and in different organs from adult fish the TMPK activity level were in the order of brain > heart > spleen > gill > liver.

An intriguing finding in this study was the presence of high TMPK activity that may not be attributed by the canonical Dtymk protein (encoded by *dtymk*). This non-canonical TMPK activity appeared to be expressed from ~ 96 hpf and onwards into adulthood. Notably, at 120–144 hpf and also in adult fish, the canonical Dtymk protein was undetectable by Western blot analysis using a Dtymk specific antibody despite the high TMPK activity detected. Notably, the Dtymk antibody was raised against a 13-amino acid peptide sequence that is localized within the Lid region of human TMPK sequence, which is an essential structural element for enzyme catalysis [28]. Hence, an alternatively spliced isoform lacking this sequence would not fold into an active enzyme, and the failure of the antibody to detect any alternative variants of Dtymk suggests that enzymatically active, alternatively spliced isoforms of Dtymk are not expressed in the zebrafish. Together, these results provide support for the existence of an alternative non-canonical TMPK gene in zebrafish. Along the same line, we noted that the canonical *dtymk* mRNA expression was high at earlier stages of embryonic development but dropped to low levels at later stages (from 48 hpf and onwards), which is not correlated with the TMPK activity levels. These results also support the presence of an alternative TMPK gene.

In fibroblasts derived from a human patient with loss of function mutations in the *DTYMK* gene, the cells grew normally and level of dTTP showed no significant difference from that of the controls, and the loss of function mutations affected only the brain but not other organs [11, 29], suggesting the presence of a compensatory TMPK like enzyme expressed in other organs. Furthermore, in a human fibroblast cell line, the existence of an alternative TMPK enzyme has recently been described [30]. Our results show that the non-canonical TMPK activity detected at late stages had similar biochemical properties as the canonical Dtymk protein regarding isoelectric point and molecular weight, whereas it differs in not being recognized by antibody raised against the canonical Dtymk. However, the identity of this potential non-canonical TMPK, either in zebrafish or in humans is presently not known, but represents an important task for further investigations.

Zebrafish embryos start their morphogenesis at 4.5 hpf, and the first neurons become post-mitotic in the neural plate shortly after gastrulation (5.5 hpf). The time between 10 and 24 hpf is a critical time period when the embryo's primary organs start to develop, the nervous system is further expanded, and the brain is further developed at the end of the first day (24 hpf). During the next two days (48 and 72 hpf), the completion of the primary organ system is reached [31–33]. The expression pattern of the canonical Dtymk protein showed a close correlation with organogenesis and neuronal development; i.e. the levels of the canonical Dtymk protein increased significantly at 6 hpf and reached its maximum at 24 hpf, and then declined gradually until 72 hpf, a time point before the expression of the non-canonical TMPK activity starts to increase. These results suggest that the canonical Dtymk protein may play an important role in neurodevelopment, and that deficiency in TMPK activity caused by mutations in the *dtymk* gene would preferentially affect nervous system development. As shown in a recent study where *dtymk* knockout in zebrafish resulted in microcephaly, neuronal cell death and early lethality [11].

In the zebrafish genome database, one *tk1* transcript (GenBank: AAO64437.1) and two *tk2* transcripts (GenBank: NP_001002743.2 and AAH76441.1) were reported. The two *tk2* transcripts differ mainly in their N-terminal sequences: one (NP_001002743.2) with a putative mitochondrial targeting signal sequence, which is lacking in the second transcript (AAH76441.1), and thus, the second transcript is most likely encoding a cytosolic protein. Thus, the first TK activity peak seen after DEAE chromatography corresponds to Tk1, which was also confirmed by western blot analysis using a Tk1 specific antibody, and the second TK activity peak corresponded to the cytosolic form of Tk2, which was also confirmed by TK activity assay in the presence of excess of dCyd. Altogether, these results suggest that Tk2 is

the prominent TK present in the cytosol of zebrafish cells.

Thymidylate synthase (Tyms) is also a key enzyme in the de novo dTTP synthesis pathway and is expressed throughout all of the developmental stages, with the highest levels of Tyms protein detected at 1–4 cell (0.2 to 1 hpf) stage [34], and the levels of Tyms protein correlate with its mRNA expression profile [22]. Knockdown of *tyms* by siRNA technique or insertion mutagenesis resulted in a serious delay in tail and head development [13, 34]. However, thymidine supplement could reverse the abnormality observed in *tyms*-knockdown zebrafish [34]. These results suggest that dTTP is a limiting factor in zebrafish development and that the salvage pathway of dTTP synthesis through TK-catalyzed reactions can compensate for the defect in the de novo dTTP synthesis pathway caused by *tyms*-knockdown if sufficient deoxynucleosides are available, although the levels of Tk1 and Tk2 activity and mRNA are low.

In cultured cells the expression of the canonical TMPK is cell cycle-regulated with the highest level during the S-phase, and TMPK activity levels correlated with cell growth rates. In non-proliferating cells/tissues, the canonical TMPK activity is very low [19]. In yeast, defects in dTTP synthesis due to mutations in either *cdc8* (coding for TMPK) or *cdc21* (coding for Tyms) leads to telomere shortening and growth arrest [35]. In humans, mutations of the canonical TMPK coding gene (*DTYMK*) causes severe neurodegenerative disorders, but have no clear effects on other organs, nor on overall metabolism in the affected patients [10, 11]. Together, these results suggest that *DTYMK* is required for neuronal development during early embryogenesis, whereas the putative non-canonical TMPK is expressed at a later stage of development. Future studies of the canonical TMPK and identification of the putative non-canonical TMPK may help to elucidate the mechanism by which TMPK deficiency leads to neurodegenerative disorders.

Conclusions

Our results suggest that active dNTP synthesis in early embryogenesis is vital. TMPK and TK activities are prominent in the cytosol of embryos, larvae and adult fish and brain contains the highest TMPK activity. The expression of the *dtymk*-encoded Dtymk protein is correlated to neurodevelopment but not to the TMPK activity levels detected at late stages of development. These results indicate that Dtymk is essential for neurodevelopment, which is supported by a recent study of *dtymk* knockout zebrafish with neurological disorders and fatal outcomes [11]. The TMPK-like enzyme expressed at later stage of development showed similar biochemical properties as the Dtymk protein but could not be recognized by the Dtymk specific antibody. This study expands our knowledge of nucleotide biosynthesis during zebrafish development, and also provided evidence for a compensatory TMPK-like enzyme expressed at later stages of development.

Materials and methods

Zebrafish embryo maintenance

Adult zebrafish of a laboratory bred population were maintained under climate controlled conditions in the Aquatic Laboratory at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden. The fish were kept at a photoperiod of 12 h light/12 h dark cycle and a water temperature of 27 °C. The fish were fed twice daily with commercial flake food Sera Vipan Nature® (Djurhobby, Uppsala, Sweden). Groups of 10 individual adult zebrafish were placed in stainless steel mesh spawning cages in 10-L tanks the day before experiments started. Spawning was initiated at onset of light the day after at 9:00 a.m. Approximately 2000 eggs were collected and assessed for fertilization under a stereo-microscope. The selected fertilized eggs were placed in Petri dishes with filtered oxygenated water (0.22 µM filter). The developing embryos were sampled at 0 (n = 60), 6 (n = 60), 24 (n = 60), 48 (n = 45), 72 (n = 30), 96 (n = 30), 120 (n = 30) and 144 (n = 30) hours post fertilization (hpf). Embryos were not fed during development. Adult zebrafish (n = 200) euthanized due to old age (3 to 4 years old of mixed gender) were also sampled. At sampling, adult fish were euthanized by decapitation after immersion in sodium bicarbonate buffered tricaine methanesulfonate solution (MS222: 500 mg/L). Dissection of body parts and organs were performed under a stereo-microscope. All experiments with zebrafish were conducted according to the ARRIVE guidelines [36].

Protein isolation and subcellular fractionation

The nuclei, mitochondria and cytosol fractions were prepared essentially as described [30, 37]. Briefly, zebrafish embryos were washed with ice cold water at least five times before addition of protein extraction buffer (10 mM Tris-HCl pH 7.6, 25 mM KCl, 1 mM dithiothreitol (DTT), 25 mM sucrose and 1x protease inhibitor). After homogenization, the homogenates were centrifuged at 800 x g for 15 min at 4 °C to extract nuclei; the supernatant was then centrifugation at 12 000 x g for 30 min at 4 °C to isolate mitochondria. The remaining supernatant was centrifuged again two more times and the final supernatant was saved as the cytosolic fraction. The nuclei and mitochondria preparations were washed three times

with the extraction buffer to avoid cytosolic protein contamination. Finally, 0.1% NP-40 was added to the nuclei and mitochondria preparations and incubated on ice for 20 min to extract the total protein. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as standard.

Enzyme assays

TK and TMPK activities were measured by using radiolabeled substrates essentially as described previously [30, 38] but with the reaction temperature set to 27.5 °C and the duration of the assays to 30 or 60 min.

dNTP pool measurement

A pool of 50–75 embryos/larvae was collected at each developmental stage and homogenized immediately. Soluble nucleotides were extracted by using 60% methanol and total dNTP pools were determined essentially as described [39]. Briefly, appropriate amounts of soluble nucleotide extracts or dNTP standards were added to a reaction mixture containing 40 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.25 µM specific primed oligonucleotide, 0.75 µM ^{3} H-dTTP or ^{3} H-dATP, and 0.30 unit Taq DNA polymerase in a total volume of 20 µl. The reaction mixtures were incubated at 48 °C for 60 min, and then 15 µl of the reaction mixture were spotted onto DEAE filter paper (DEAE filtermat, PerkinElmer), and dried. The filter papers were then washed three times with 5% NaH_2PO_4, once with water and once with 95% ethanol. The filters were dried and the products were quantified by liquid scintillation counting (Tri-carb, PerkinElmer) after the addition of scintillation fluid (Optiphase Hisafe, PerkinElmer). The results are given as mean ± SD from 9 to 12 independent measurements.

Anionic exchange column chromatography

Zebrafish proteins from 24 hpf embryos or 120 hfp larvae were isolated and stored at −80 °C until further analysis. Each batch provided ~ 2000–4000 fertilized embryos and three to four batches were pooled together for protein separation. A diethylaminoethanol (DEAE)-Sepharose Fast Flow (GE Healthcare) column was equilibrated with DEAE buffer (10 mM Tris/HCl pH 7.6, 25 mM KCl and 0.1 M sucrose) before the proteins were applied to the column. The flow through fraction was collected and the bound proteins were eluted with stepwise increasing KCl concentrations (50 to 250 mM) in the DEAE buffer. Fractions were collected and used to determine protein concentrations by using Bio-Rad protein assay (Bio-Rad) with BSA as standard, TK and TMPK activities determinations by using tritium labeled substrates and western blot analysis using specific antibodies.

Western blot analysis

Polyclonal antibody against zebrafish Dtymk was designed and produced by Genscript Inc. The 13-amino acid peptide sequence chosen for immunization represented amino acid number 139–152. The corresponding sequence in human TMPK encompasses the Lid region in the human TMPK structure, which is exposed on the enzyme surface [28]. Antibody specificity was validated by ELISA (by the manufacturer) and in-house by peptide blocking experiments for western blot analysis using the 13-amino acid peptide used for immunization (data not shown). Monoclonal anti-TK1 antibody was a gift from Dr. Kiran Kumar Jagarlamudi [40]. Anti-actin antibody was purchased from Abcam. Infrared-labeled secondary antibody was from Li-COR. All western blot analyses followed standard protocol. The images shown in figures were cropped in order to improve clarity and conciseness and all original, full-length and uncropped images are provided as Additional file 1.

Size exclusion chromatography

The Äkta Prime system was connected to a Superdex 200 10/300 GL column (GE healthcare). The column was washed extensively and equilibrated with buffer containing 10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$ and 5 mM DTT. Prior to loading, protein samples were filtered through a 0.22 µM filter (Merck). The flow rate was 0.3 ml/min. Fractions (0.4 ml) were collected and saved for further analysis.

Statistical analysis

All experiments were repeated at least three times and data were analyzed with GraphPad Prism. Statistical analyses were performed by T-test, two-tailed. $P > 0.05$ was considered as not statistically significant, and $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as significant. All data are given as mean ± standard deviation and for western blot analyses representative blots are shown.

Abbreviations

TMPK: Thymidylate kinase; TK: Thymidine kinase; dNTP: Deoxynucleoside triphosphate; dThd: Thymidine; dCyd: Deoxycytidine; dTMP: Thymidine 5′-minophosphate; hpf: Hours post fertilization; MW: Molecular weight.

Supplementary Information

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Additional file 1. Additional figures.

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Not applicable.

Authors' contributions
JMHF and LW performed the experimental work, analyzed the data and made the figures. LW, GP and SE supervised the project. SÖ supervised the practical work with zebrafish. LW and JMHF wrote the first draft of the manuscript and all authors participated in manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated in this study are included in the manuscript.

Declarations

Ethics and approval and consent to participate
The use of adult animals and procedures described in this study were approved by the regional ethical committee for animal experimentation (Uppsala Djurförsöksetiska Nämnd, permission number Dnr.5.8.18-008/2017). Zebrafish embryos at developmental stages earlier than free-feeding are not considered research animals according to Swedish research animal regulations (SJVFS 2019:9) [41] why no ethical permit is needed for embryo studies. At sampling, adult fish were euthanized by decapitation after immersion in sodium bicarbonate buffered tricaine methanesulfonate solution (MS222: 500 mg/L).

Consent to publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Doctoral Thesis No. 2022:26

Thymidylate kinase (TMPK) is an essential enzyme in dTTP synthesis. In this thesis, TMPK was investigated using human fibroblasts and zebrafish embryos. The results suggest that a novel form of TMPK enzyme exists and *DTYMK* encoded TMPK is important during embryonic development. The studies expand our understanding of the TMPK enzyme, and provides a correlation between TMPK and neuropathology during early development.

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