國立臺灣大學醫學院生物化學暨分子生物學研究所

碩士論文

Graduate Institute of Biochemistry and Molecular Biology,
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Master Thesis

人類去氧尿嘧啶三磷酸核苷酸水解酶對細胞生長及 Methotrexate 抗藥性影響之探討

Functional analysis of human deoxyuridine triphosphatase in cell growth and methotrexate sensitivity

研究生: 黃婷筠 (Tyng-Yun Huang)

指導教授： 張智芬 博士 (Zee-Fen Chang, Ph.D.)

指導教授： 張富雄 博士(Fu-Hsiung Chang, Ph.D.)

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國立台灣大學碩士學位論文
口試委員會審定書

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本論文係黃婷筠君(R97442024)在國立台灣大學醫學院生物化學暨分子生物學研究所完成之碩士學位論文，於民國九十九年六月二十三日承下列考試委員審查通過及口試及格，特此證明

口試委員：

謝誌

生命就像是一盒巧克力，即使不知道會拿到什麼口味，還是得鼓起勇氣去嘗試, 才能親自體會到箇中滋味。兩年的碩士班生涯, 嚐到許多未曾試過的新口味, 新奇的, 深刻的, 獨一無二的!

感謝指導教授張智芬博士，提供這麼好的環境讓我學習, 並且感受研究的樂趣! 老師總能適時的提醒我指點我, 讓我更進步。謝謝張富雄老師，陳美如老師，李財坤老師，提供許多寶貴的意見讓我從更多方面檢視, 使論文更臻完整!

からすてんぐ,像是繽紛圖畫裡的潔白, 不是最顯眼最醒目, 卻是最不可或缺的! 謝謝你實質上的幫助, 精神上的鼓勵!魚雁往返的字字珠璣, 跨越時空修改的點點滴滴, 轉換成最銘心的感激!謝謝你!

謝謝我的師父，春美學姊，是你一步一步教我，讓我有工具並帶我踏入研究的領域。謝謝實驗室的大家，你們是不同的音符, 交織出兩年來喜怒哀樂的樂章; 是不同調味料, 調配出豐富的酸甜苦辣!

最重要的，我親愛的爸爸、媽媽和哥哥，你們是我最安心的避風港，謝謝你們包容我，並給我一個無憂無慮的環境，讓我成長茁壯，讓我即使身處異地，還是保有繼續下去的勇氣!

生命無法停止追逐，峰迴路轉走不出白雲深處!我會繼續努力，盼能以不悔的步伐，踏出精采的人生!

中文摘要

去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)負責催化去氧尿苷三磷酸(dUTP)水解為去氧尿苷單磷酸(dUMP)和焦磷酸鹽(PPi)。有許多證據顯示去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)的表現量和腫瘤形成以及化療抗藥性有關連。本篇研究的目的在於了解去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)在細胞週期進行時的調控及其所扮演的必要角色。

在本篇研究中發現去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)的表現量隨著細胞週期改變，在 S phase 時大量表現。在 293T 細胞中抑制去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)的表現，會稍微減緩細胞生長速度。利用 aphidicolin 進行同步化(synchronization)後，進一步發現抑制去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)表現不但會影響細胞週期的進行，在細胞週期經過 S phase 時還會有去氧核糖核酸(DNA)損傷的訊號產生，並引發細胞凋亡(apoptosis)，顯示去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)對複製壓力(replication stress)反應的重要性。

胸苷酸(dTMP)代謝一直是癌症化療應用的重要作用點。像是 5-氟尿嘧啶(5-FU)或葉酸拮抗劑(antifolate)。抑制胸苷三磷酸新生成(*de novo* dTTP synthesis)的途徑會降低細胞內胸苷三磷酸(dTTP)含量，並導致去氧尿苷單磷酸(dUMP)累積，進一步磷酸化為去氧尿苷三磷酸(dUTP)。dUTP/dTTP 比例的增加會導致去氧尿苷三磷酸(dUTP)誤嵌入 DNA，引發 DNA 損傷反應。因此提出去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)的表現量是否會影響細胞對滅殺除癌錠(methotrexate)的感受性。結果顯示在 293T 細胞中降低去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)的表現量會增加細胞對滅殺除癌錠(methotrexate)的敏感度，並且增強 DNA 損傷反應。另一方面，表現去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)則可減少因滅殺除癌錠(methotrexate)所引起的損傷訊號。

總結本論文，說明了去氧尿嘧啶三磷酸核苷酸水解酶(dUTPase)在細胞從複製壓力(replication stress)復原時的重要性，以及在利用抑制胸苷三磷酸新生成(*de novo* dTTP synthesis)途徑化療藥物治療產生抗藥性所扮演的角色。

關鍵詞: 去氧尿嘧啶三磷酸核苷酸水解酶、滅殺除癌錠、複製壓力

Abstract

Deoxyuridine triphosphatase (dUTPase) catalyses the hydrolysis reaction of dUTP to form dUMP and pyrophosphate. Accumulating evidence has shown the association of dUTPase expression with tumor and chemotherapy resistance. The aim of my study is to understand the regulation and the essential role of dUTPase in the cell cycle progression.

I found that the expression of dUTPase is regulated in a cell cycle-dependent manner, being maximal in the S phase. Depletion of dUTPase in 293T cells reduced the cell growth rate slightly. By aphidicolin synchronization, I further found that depletion of dUTPase not only affected cell cycle progression, but also induced DNA damage and apoptosis when progression through S phase, indicating the importance of dUTPase in response to replication stress.

Thymidylate metabolism is an important target for chemotherapeutic agents such as fluoropyrimidines, and antifolates. Inhibition of *de novo* dTTP synthesis results in dTTP pool depletion and a subsequent accumulation of dUMP, which may then be phosphorylated to form dUTP. The increase in the dUTP/dTTP ratio induces dUTP misincorporation, resulting in DNA damage responses. Therefore, I addressed the question whether the expression level of dUTPase affects methotrexate sensitivity of cells. The results showed that knockdown of dUTPase sensitized 293T cells to methotrexate, accompanied by an enhancement of DNA damage response. In addition, enforced expression of dUTPase diminished methotrexate-induced damage signals.

Accordingly, these results elucidated the crucial role of dUTPase in cells resumed from replication stress and in resistance to anti-cancer treatment by blocking *de novo* dTTP synthesis.

Key words: deoxyuridine triphosphatase (dUTPase)、methotrexate、replication stress

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Introduction

Part I: Overview of dUTPase

Deoxyuridine triphosphatase (dUTPase, EC 3.6.1.23), also named dUTP pyrophosphatase or dUTP nucleotidohydrolase, is a ubiquitous enzyme that is responsible for the hydrolysis of deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP) and inorganic pyrophosphate (PPi), using Mg^{2+} as a cofactor (Bertani et al., 1961; Greenberg and Somerville, 1962; Shlomai and Kornberg, 1978; Williams and Cheng, 1979)(figure I-1). It has the exquisite specificity for dUTP because of the steric exclusion and hydrogen bonding pattern specific only for uracil (Vertessy and Toth, 2009).

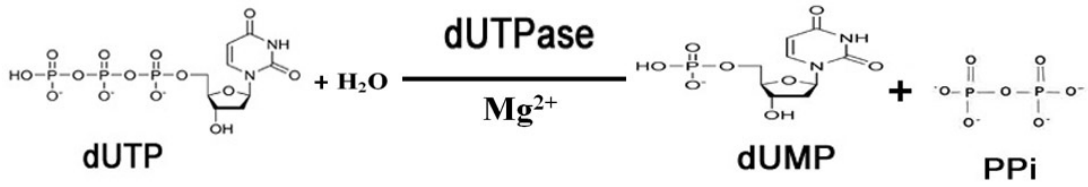


Figure I-1. dUTPase reaction mechanism

dUTPase was first purified from *E. coli,* and the structures of the enzyme from various sources such as virus, yeasts, plants and human have been well studied.

Amino acid sequence alignments have been identified with five conserved motifs(Harris et al., 1999) (figure I-2). Motif 3 is responsible for substrate binding and motifs 1, 2, and 4 coordinate the metal ion and the phosphate chain of the nucleotide (Harris et al., 1999). Because dUTPase is essential for viability in both *E coli* (el-Hajj et al., 1988)and *Saccharomyces cerevisiae* (Gadsden et al., 1993), its conservation in evolution revealed an important role of dUTPase.

In humans, nuclear and mitochondrial isoforms of dUTPase have been identified to have the same kinetic affinities for dUTP (Ladner et al., 1996). They share the same gene and are generated by alternative splicing (Ladner and Caradonna, 1997)(Figure I-3). The molecular weight of the nuclear dUTPase is 18 kD and the mitochondrial form with an additional mitochondria targeting presequence is 27kD (Ladner and Caradonna, 1997). Mitochondrial dUTPase is constitutively expressed, whereas the expression of nuclear dUTPase is under cell cycle control (Ladner et al., 2000).

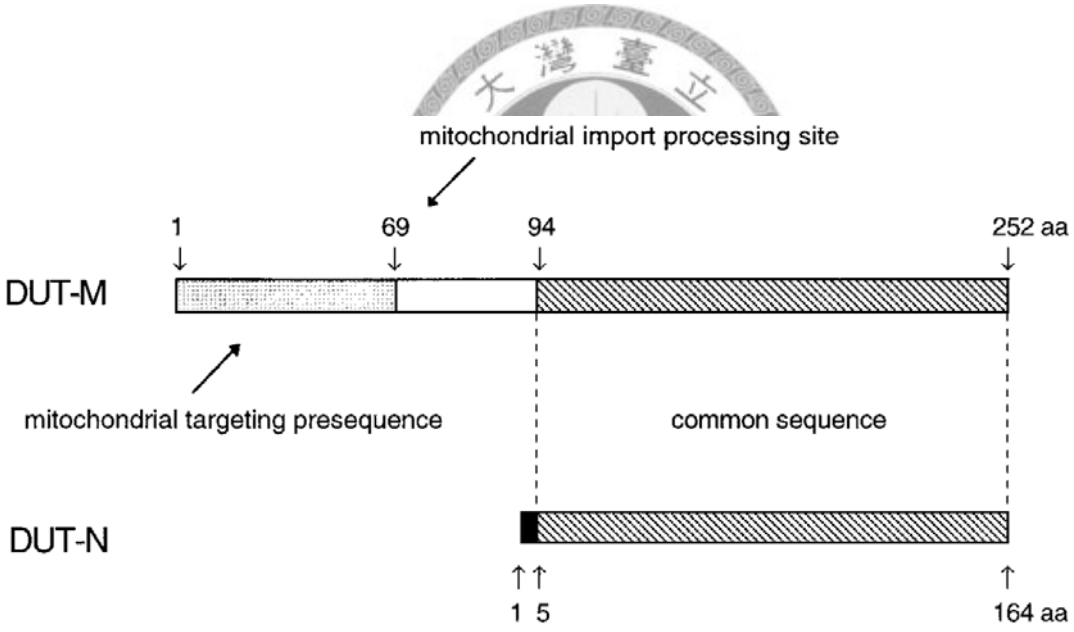


Figure I-3. Comparison of the amino acid sequences of DUT-N and DUT-M.

(Ladner and Caradonna, 1997)

Part II: Role of dUTPase in thymidylate metabolism and genome integrity

1. Thymidylate metabolism

In cells, dUTPase is a member of nucleotide metabolism and is involved in dTTP synthesis. Deoxyribonucleotide triphosphates (dNTPs) play important roles in all cells, and maintenance of the adequate and balanced dNTP pools is required for DNA replication and repair (Reichard, 1988). Among the four dNTPs, thymidine triphosphate (dTTP) is the most stringently regulated. There're two pathways responsible for dTTP synthesis (figure II-1), one is the *de novo* pathway, and the other is the *salvage* pathway (Arner and Eriksson, 1995).

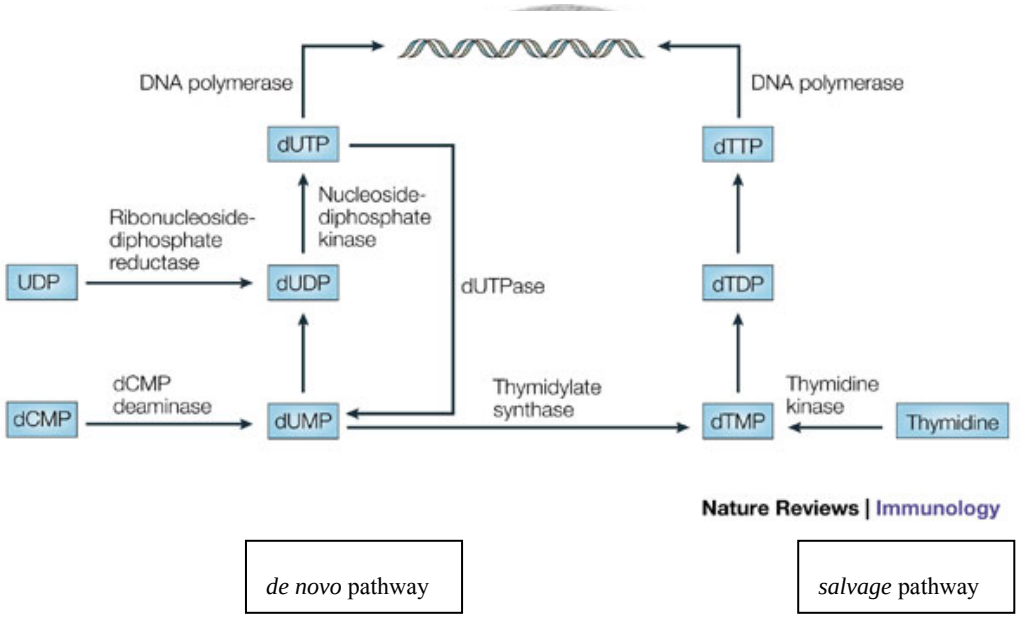


Figure II-1. Overview of dTTP synthesis pathway.

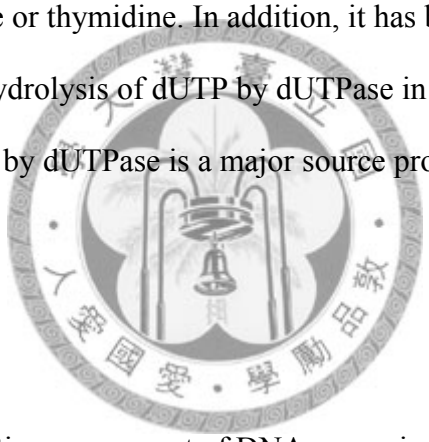
(Neuberger et al., 2005)

In the *de novo* pathway, dUMP, which was produced from dCMP deamination by dCMP deaminase or dUTP hydrolysis by dUTPase, is the substrate for thymidylate synthase (TS) (Gadsden et al., 1993; Zhang et al., 2007). For converting dUMP to dTMP, thymidylate

synthase transferred a one-carbon unit from N^5,N^{10}-methylenetetrahydrofolate to dUMP, and reduced to a methyl group coupled with the oxidation of tetrahydrofolate to dihydrofolate (Ladner, 2001; McIntosh and Haynes, 1997; Reichard, 1988).

In the *salvage* pathway, thymidine kinase (TK) transferred γ -phosphate from ATP to the 5' hydroxyl group of thymidine to form dTMP (Arner and Eriksson, 1995). dTMP from both pathways can be further converted to dTDP by thymidylate monophosphate kinase (TMPK), and followed by subsequent phosphorylation to dTTP by a ubiquitious nucleoside diphosphate kinase (NDPK) for DNA synthesis (Reichard, 1988).

In many cells, *de novo* pathway is the main source for dTTP synthesis, especially in the absence of exogenous thymine or thymidine. In addition, it has been reported that 60% of dUMP pool comes from the hydrolysis of dUTP by dUTPase in *S. cerevisiae* (Gadsden et al., 1993). Therefore, the reaction by dUTPase is a major source providing dUMP as substrate for thymidylate synthase.



2. Genome integrity

Uracil, which is not a native component of DNA, can arise either by the spontaneous deamination of cytosine residues (Pearl and Savva, 1996) or through dUTP utilization by DNA polymerase during replication and repair (Bertani et al., 1963; Lindahl, 1993). There're two enzymes responsible for keeping uracil out of DNA, one is uracil-DNA glycosylae (UDG), and the other is dUTPase(Caradonna and Muller-Weeks, 2001; Vertessy and Toth, 2009).

Because most DNA polymerases can't distinguish dTTP from dUTP , deregulation of dUTP/dTTP ratio will result in dUTP incorporation into DNA(Bessman et al., 1958; Brynolf et al., 1978; Dube et al., 1979; Yoshida and Masaki, 1979). Thus, to keep dUTP out of DNA, dUTP level should be highly regulated. When the incorporation of dUTP into DNA occurs,

this misincorporation can be removed through base excision repair. At first, the bond between uracil and deoxyribose is cleaved by uracil DNA N-glycosylase (Lindahl, 1993). After the removal of uracil, the gap will be filled by DNA polymerase (Caradonna and Muller-Weeks, 2001). Nevertheless, the DNA polymerase can still incorporate dUTP instead of dTTP again if the high ratio of dUTP/dTTP is present. Therefore, the cell engages in repeated cycles of uracil misincorporation and UDG-mediated repair. This iterative process results in increased recombination, DNA strand breaks, and ultimately cell death, which is called futile cycle(el-Hajj et al., 1988; Goulian et al., 1986). (Figure II-2)

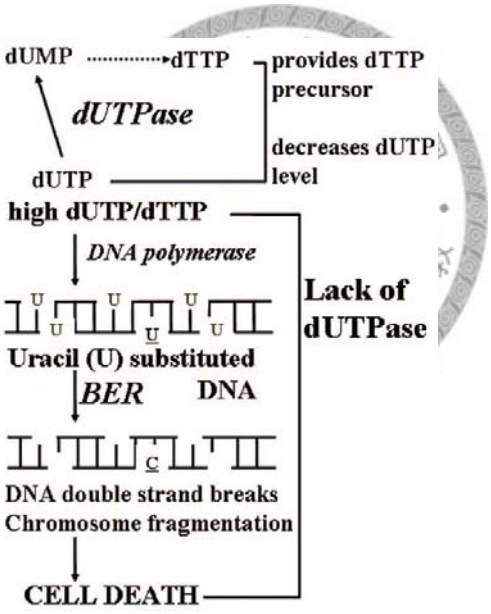


Figure II-2. Futile cycle of uracil incorporation

(Vertessy and Toth, 2009)

Therefore, dUTPase cooperates with TS function to limit the expansion of dUTP pool, avoiding the misincorporation of uracil into DNA as the guardian of DNA integrity.

Part III: Targets in Chemotherapy

In many kinds of cancer therapy, dTTP metabolism has been an important target for a long time because of the demand for dNTP supply for DNA synthesis in cancer cells (Heidelberger et al., 1957; Longley et al., 2003; Moertel, 1994). The blockage of the *de novo* pathway in dTTP synthesis resulting in the depletion of dTTP pool(Cohen, 1971; Goulian et al., 1986), and the accumulation of dUMP, which will further be phosphorylated to dUTP. As a result, the misincorporation of dUTP into DNA inhibits cancer cell growth and initiation of apoptosis. These effects make tumor cells, which have higher proliferation rate than normal cells, to be killed (Ladner et al., 2000; Wilson et al., 2008).

Antifolate compounds are used as anti-cancer agents. In clinical application, methotrexate (MTX) is not only used in rheumatoid arthritis treatment at low dosage, but also used as a thymidylate synthase inhibitor indirectly (Goulian et al., 1980a, b). The structure of polyglutamate metabolite of methotrexate is similar to folic acid. It can inhibit dihydrofolate reductase (DHFR) by the competition of the binding site (Jacobs et al., 1975; Whitehead, 1977). There're two main biological function of dihydrofolate reductase. One converts dietary folic acid to the coenzymatic form, tetrahydrofolate, via dihydrofolate. The other catalyses the regeneration of tetrahydrofolate following the biosynthesis of thymidylate from deoxyuridylate when tetrahydrofolate is oxidized to dihydrofolate, then catalyses the regeneration of tetrahydrofolate. Tetrahydrofolate then is transferred to 5,10-Methylene tetrahydrofolate(MTHF) by serine hydroxymethyl transferase. MTHF is a methyl donor in the reaction that dUMP is catalyzed to dTMP by thymidylate synthase. (Schnell et al., 2004)(Figure III-1)

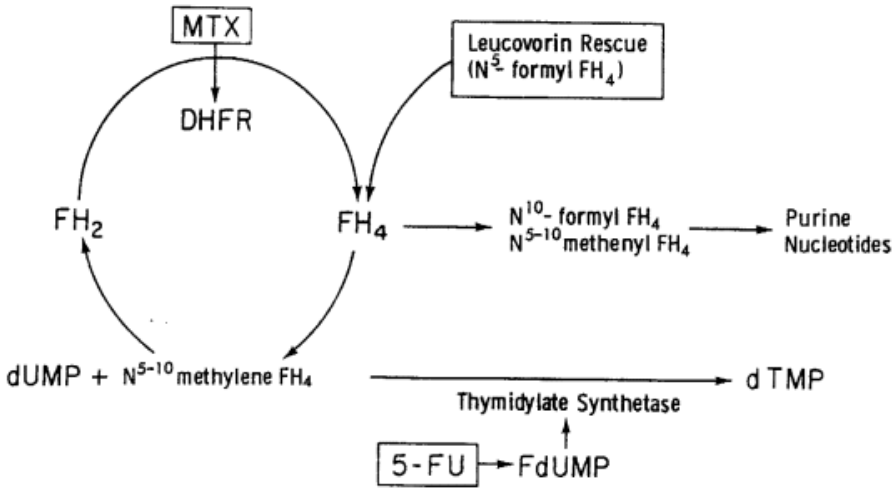


Figure III-1. The mechanism of dihydrofolate reductase

It has been reported that the clinical utility of TS and DHFR as therapeutic targets is hindered by the high incidence of resistance observed in many cancers (Ladner et al., 2000; Mader et al., 1998). Moreover, overexpression of dUTPase is significantly associated with poor prognosis in colorectal cancer patients treated with 5-FU (Conley et al., 1998; Kawahara et al., 2009). There's also a similar phenomenon in hepatocellular carcinoma tissue, and considering that dUTPase is a potent biomarker for predicting prognosis in HCC patients (Takatori et al., 2010). For the improvement of the therapeutic efficacy, dUTPase is a potential chemotherapeutic target for monotherapy or the combination with thymidylate synthase inhibition(McIntosh and Haynes, 1997).

Part IV: Experimental rationale

Because the expression of dUTPase is up-regulated in many types of cancer, the role of dUTPase in tumorigenesis is one of the exciting topics of tumor biology. In addition, it has been reported that the drug resistance of chemotherapy and the poor prognosis are associated with the expression level of dUTPase. In this study, I aim to understand the role of dUTPase in the cell cycle and the relationship between the expression level of dUTPase and methotrexate therapeutic potency.

Materials and Methods

Plasmids

pGEX-2T-dUTPase and pCMV-Flag-dUTPase plasmids were obtained from Dr. Chun-Mei Hu. dUTPase^{shRNA} and Lac*Z*^{shRNA} plasmids were from National RNAi Core Facility, Academia Sinica.

Antibodies

Anti-TK1 polyclonal antibody was prepared as described previously (Chang et al., 1994). Anti-β-tubulin, anti-β-actin, and anti-FLAG (M2) antibodies were obtained from Sigma Chemicals Co., and anti-cyclin B1, anti-R2 antibodies were from Santa Cruz Biotechnology. Anti-TS antibody (clone 4H4B1), and anti-p53 monoclonal antibody (ab-6) were purchased from Zymed and Calbiochem, respectively. Anti-dUTPase antibody was prepared by immunizing rabbits with purified GST-hdUTPase.

Database searching

The Oncomine Cancer Microarray database (http://www.oncomine.org) was used to search the gene expression of *DUT* in 20 cancer types as compared with their normal counterparts. All data were log transformed, median centered per array, and the standard deviation was normalized to one per array.

Cell culture

293T, 293, and HeLa cells were cultured at 37℃ in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum plus 100 μg/ml streptomycin and 100 U/ml penicillin (Life Technologies).

Synchronization and Fluorescence Activated Cell Sorting (FACS) Analysis

$2.5x10^5$ cells were plated onto 60-mm plates and allowed to grow for overnight prior to synchronization. For G1/S block, aphidicolin (Sigma) at a final concentration of 2 μmol/L or hydroxyurea (Sigma) at a final concentration of 10 mmol/L were added to 293 or 293T or HeLa cells for 20 hours. After washing twice with phosphate-buffered saline (PBS), fresh medium was added to release cells.

For FACS analysis, cells trypsinized with Tris-EDTA were fixed with 300 μl of ice chilled PBS and 700 μl of 100% ethanol (final concentration 70% v/v)(Merck), and then stored at -20℃ for at least overnight. Fixed cells were harvested by centrifugation at 2.6k rpm for 3 minutes, and washed with ice chilled PBS. 1 ml of PI staining solution [0.1% Triton-X100(Serva), 0.2 mg/ml RNaseA, 20 μg/ml propidium iodide (Sigma)] was added to sample and suspended the cells gently. After incubation at room temperature for 30 minutes, the cell cycle profile was examined by FACS analysis using Becton Dickison FACScan flow cytometer and CellQuest software.

Immunoblot analysis

Cells were lysed by sonication in RIPA buffer [25 mM Tris pH7.4 (Merck), 150 mM NaCl (Merck), 1% (w/v) sodium deoxycholate (Merck), 0.5 mM Dithiothreitol (DTT, Promega), 1% protease inhibitor (Sigma) and 1% protein phosphatase inhibitor], and protein concentration was determined by using the Bio-Rad protein assay system. 15 μg of protein extracts were diluted with 5X sample loading buffer (175 mM Tris-HCl pH7.0, 5 mM EDTA, 10% SDS, 20% sucrose, 0.01% bromophenol blue, 28.8 mM β-mercaptoethanol) and heated at 95℃ for 5 minutes prior to loading. Samples were separated by 10% SDS-PAGE at 50 volts for staking gel and 80 volts for running gel in SDS-PAGE running buffer (50 mM Tris, 380 mM glycine, 0.1% SDS), and transferred to PVDF membranes (Millipore). The

membranes were blocked in TBST [10 mM Tris (pH8.0), 150 mM NaCl, and 0.1% Tween 20(Sigma)] containing 5% skim milk at room temperature for 1 hour. Western blots were probed overnight at 4℃ with primary antibodies. The membranes were washed for 3 times at room temperature, and probed for 1 hour at room temperature with appropriate secondary antibodies [goat-anti-mouse and goat-anti rabbit horseradish peroxidase (Santa Cruz)]. Visualization of the protein bands was performed using the ECL chemiluminescent western blotting detection system.

Cell growth analysis

$2x10^3$ cells per well were seeded into 96-well plate. After incubation for the indicated days, 100 μl of medium containing CellTiter 96 Aqueous One Solution (Promega)(medium: reagent=5:1) was added to each well, then incubated at 37℃ for 1.5 hours. The absorbance at 490 nm was then measured using an ELISA plate reader (MTS assay).

Cytotoxicity assay

$4x10^3$ cells per well were seeded into 96-well plate and allowed to grow for overnight prior to drug treatment. Cells were exposed to increasing concentrations (0, 0.05, 0.1, 0.2, 0.5, 1 μM) of methotrexate (Sigma) for 20 hours. Cells were incubated at 37℃ for an additional 24 hours after removing drug by replacing fresh medium. Cell viability was then measured by MTS assay according to the manufacturer's instruction.

Transfection

$2.5x10^5$ cells were plated onto 60-mm plates and allowed to grow for overnight prior to transfection. After washing twice with PBS and adding adequate medium, cells were transfected with a mixture containing 2 μg of DNA and 6.4 μl of nanofectin

(PAA)(DNA:nanofectin=1:3.2). The mixture was mixed well and incubated for 25 minutes at room temperature, and added into the dish.

Immunofluorescence

Cells seeded on coverslips were washed with PBS twice, and fixed in 3% paraformaldehyde (Sigma) solution in PBS for 25 minutes at room temperature. Cells were then washed twice with TBST [50 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1% Triton X-100] and permeabilized with 0.3% Triton X-100/PBS for 5 minutes at room temperature. Samples were blocked in 5.5% normal goat serum/TBST for 1 hour at room temperature and incubated with antibody against γH2AX (Cell Signaling) [1:200 diluted in 3% BSA/TBST] at room temperature for 4 hours. Cells were washed for 3 times with TBST, and then incubated with TRITC-conjugated goat anti-mouse (1:200) (Sigma), and Hochest 33342 (1:500) for 1 hour at room temperature. After washing with TBST for three times, cell-contained coverslips were mounted on slides with mounting oil. The coverslips were examined by a fluorescence microscope (Carl Zeiss, AxioObserver A1). Images were captured using a cooled CDD camera operated by AxioVision image software and arranged using Photoshop (Adobe) software.

Apoptosis analysis

An Annexin V-FITC apoptosis kit (Oncogene Research Products) was used to detect apoptosis. Cells were trypsinized and suspended in PBS at a final concentration of $1x10^6$ cells/mL prior to staining with Annexin V-FITC. After staining for 15 minutes, Annexin V-FITC signals were detected by fluorescence microscopy.

Expression and purification of recombinant human dUTPase proteins

pGEX-2T-dUTPase (wild type and mutant) vectors were transformed in to *E. coli* strain JM109 using standard bacterial transformation procedures and a single amp^r colony was inoculated into 500 ml of LB containing 100 μg ml^{-1} ampicillin. When culture grew to an A_{600} of 0.5, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was added to induce protein expression. After 3.5 hours of incubation, cells were harvested by centrifugation. 10 ml of lysis buffer [50 mM Tris-HCl ,pH7.5, Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$(Sigma) ,1 mM DTT, 1 mM PMSF(Sigma) ,0.5 mM CHAPS(Sigma)] was added to cells, and cell lysate was vortexed vigorously. After sonication on ice in short bursts for 4 cycles ,60 times/cycle, cell debris was removed by centrifugation at 10000xg for 15 minutes and the supernatant was incubated with glutathione-4B sepharose (Amersham Pharmacia) at 4℃ for 1.5 hours. The suspension was centrifuged at 1200rpm for 5 minutes, and the supernatant was removed. The beads were washed with 10X volumes of washing buffer [50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.5 mM CHAPS, 30% glycerol(Merck)] for five times. The beads were then incubation with elution buffer [50 mM Tris-HCl, 10 mM glutathione] at 4℃ for 30 minutes, and the purified protein was stored at -80℃ with 10% glycerol.

dUTPase activity assay

The EnzChek Pyrophosphate Assay kit was used for measuring the dUTPase activity following the manufacturer's instruction. In brief, the indicated amount of dUTPase was added to 200 μl of the reaction buffer [34 mM Tris-HCl pH8.3, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.25 mg/ml bovine serum albumin(BSA,Sigma)] containing 100 mM dUTP. Reaction mixtures were incubated at 37℃ for 3 minutes. Samples (15 μl each) were added to the reaction mixtures [715 μl dH_2O, 50 μl 20X reaction buffer, 200 μl MESG substrate, 1 U

purine nucleoside phophorylase, 0.03 U inorganic pyrophosphatase] and incubated at 22℃ for 45 minutes. The absorbance at 360nm was measured using an ELISA plate reader.

Construction of expression plasmids and site-directed mutagenesis

EGFP-dUTPase was generated by inserting the dUTPase cDNA fragment in-frame into the EcoRI-BamHI (NEB) site of pEGFP-C. Mutant version of pEGFP-dUTPase was derived from pEGFP-dUTPase wild type plasmid by using the QuickChange XL site-directed mutagenesis kit with specific designed primers. The primers were as follows:

Forward: 5' GTAGGAGCTGGTGTCATAAATGAAGATTATAGAGGAAAT

Reverse: 5'ATTTCCTCTATAATCTTCATTTATGACACCAGCTCCTAC

The cycling parameters of PCR were outlined in the table as below:

Segment	cycles	Temperature	Time
1	1	95℃	5 minutes
2	18	95℃ 55℃ 68℃	1 minute 1 minute 15 minutes
3	1	68℃	15 minutes

One point mutation was introduced as indicated. The mutated plasmids were confirmed by DNA sequencing.

Preparation of competent cells

The *E. coli* strain of XL-1 blue was streaked on a Luria-Bertani (LB) agar plate and incubated at 37℃ overnight. On the following day, a single colony was inoculated into 20 ml TYM broth [2% Bacto-Tryptone (Difco), 0.5% yeast extract (Merck), 0.1 M NaCl, 10 mM

$MgSO_4 \bullet 7H_2O$] in a 250 ml flask and incubated at 37℃ with vigorous shaking until $O.D_{600}$ of 0.6. Then transferred the cells to 100 ml of TYM broth in a 2 L flask, and incubated at 37 ℃ with vigorous shaking until $O.D_{600}$ of 0.6 again**.** The cells were then transferred to 500 ml of TYM broth in a 2 L flask. After $O.D_{600}$ reached 0.55, cells were harvested by centrifugation at 4000 r.p.m for 15 minutes at 4℃. The supernant was then removed and the pellets were resuspended in 100 ml of cold TfBI buffer (30 mM KOAc (Merck), 50 mM $MnCl_2$ (Sigma),100 mM KCl (Merck), 10 mM $CaCl_2$ (Merck) and 15% (v/v) glycerol). After centrifugation at 4000 r.p.m for 10 minutes at 4℃, the pellets were resuspended in 20 ml of cold TfBII (10 mM Na • MOPS ,pH7.0, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol) by gentle shaking on ice. The cells were aliquot in 0.4 ml to pre-chilled tubes and stored at -80℃.

Transformation

For transformation, 200 µl of prepared competent cells were thawed at room temperature. After adding desired DNA, the cells were placed on ice for 25 minutes, and incubated at 37℃. The cells were then spread on LB plate containing appropriate antibiotics for selection, and incubated at 37℃ overnight.

Small-scale isolation of plasmid DNA

Single colony of transformed cells was inoculated in 2 ml LB containing appropriate antibiotics and incubated at 37℃ overnight. The cells were harvested by centrifugation at 12K rpm for 30 seconds at 4℃, and the pellet was suspended with 100 µl of pre-chilled Solution I [50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA (BDH)]. Then added 200 µl of fresh prepared solution II (0.2 N NaOH, 1% SDS), inverted the mix gently until it became clear, and stored it on ice for 4 minutes. 150 µl of pre-chilled solution III [3 M potassium acetate (Merck), 5 M glacial acetic acid (Merck), pH4.8] was added. After shaking

it gently for 10 seconds, mix was stayed on ice for 4 minutes. The supernant was transferred to a new tube after centrifugation at 12K rpm for 5 minutes at 4℃. An equal volume of phenol:chloroform:IAA(25:24:1) was added and votexed vigorously, and then centrifuged at 12K rpm for 2 minutes. The aqueous layer was transferred to a new tube before adding 2 volumes of 100% ethanol, and then incubated at room temperature for 2 minutes. The DNA pellet was precipitated by centrifugation at 12K rpm for 5 minutes at 4℃. The pellet was washed by 200 μl of 70% ethanol, and dried by the vacuum desiccator, and then dissolved in 50 μl of TE buffer [10 mM Tris-HCl, pH8.0 and 1 mM EDTA, pH 8.0] containing 20 μg/ml DNase-free RNaseA.

Enzyme digestion and agarose gel electrophoresis

One μg of plasmid DNA was digested with 1 μl of BamHI and EcoRI. The mixture was incubated at 37℃ for 1.5 hours. The product was analyzed by 1% agarose gel (Promega) containing ethidium bromide (EtBr,Sigma) in 0.089 M Tris-Borate-EDTA (TBE) buffer at room temperature, and detected by UVP bioimage system. Linear molecular weight standards were λ/*HindIII* fragments and commercial 100 bp DNA ladders.

Results

The association of dUTPase levels with tumors

By searching publicly available gene expression database, ONCOMINE cancer microarray database, a cell line based study showed that *DUT* is highly expressed in leukemia (P=1.06E-6) by comparing 6 tumor types, corresponding to 2 hematological malignancies and 4 solid tumors (Fig.1A). Next, the database for the expression of *DUT* RNA in clinical samples was searched and showed that overexpression of *DUT* RNA in hepatocellular carcinoma compared to normal liver tissue (P=6.29E-9) (Fig.1B); in cervical cancer compared to cervix uteri, oral cavity, palate, and tonsil (P=3.95E-9) (Fig.1C); in cutaneous melanoma compared to melanoma precursor (P=6.03E-14) (Fig.1D). In sum, among 20 types of cancer in the database, up-regulation of *DUT* is statistically significant in 4 types of tumors, including leukemia, hepatocellular carcinoma, cervical cancer, and cutaneous melanoma.

Next, I compared expression levels of dUTPase protein in different cell lines to analyze the endogenous level of dUTPase. Cell extracts were collected from different human cell lines and then subjected to Western blot analysis. As shown in Figure 2, I found that dUTPase is highly expressed in 293T and HeLa cells. In contrast, the expression of dUTPase is relatively low in 293, LoVo and U2OS cells. It is interesting that cell lines from hematological malignancies, such as K562, U937, and THP1, have high expression level of dUTPase.

To further investigate the functional significance of dUTPase in cells, I chose 293 and 293T cells as the materials because of the differential expression levels of dUTPase. It was reported that nuclear dUTPase is under cell cycle control (Ladner and Caradonna, 1997; Strahler et al., 1993). To confirm this observation, I examined the cell cycle oscillation of dUTPase levels in 293T and 293 cells, which were synchronized by aphidicolin treatment and then released from G1/S block at indicated time intervals by Western blot analysis. As shown

in Figure 3, the protein expression level of dUTPase was up-regulated in S phase, and reached maximum at G2/M phase.

Effect of silencing of dUTPase expression on cell growth rate and cell cycle progression.

It was reported that overexpression of thymidylate synthase results in a substantial increase in the growth rate of cells(Rahman et al., 2004). I raised the question whether the amount of dUTPase, which supplies substrate, dUMP, for thymidylate synthase, would affect cell growth. To address the question, dUTPase in 293T cells was depleted by transfection with dUTPase shRNA plasmid and LacZ shRNA vector as the control. Cell viability was measured by MTS assay after 72 hours of transfection. As shown in Figure 4A, silencing of dUTPase slightly reduced the growth rate. The efficiency of dUTPase depletion in three independent experiments analyzed by Western blot analysis was shown in Figure 4B, respectively.

As the expression of dUTPase is increased in the S phase progression, I then tested the effect of dUTPase depletion in cells synchronized at G1/S phase by aphidicolin treatment. After releasing from G1/S block by replacing fresh medium, cells were fixed at the indicated time intervals for FACS analysis. As shown in figure5A, a large sub G1 population was seen in cells depleted of dUTPase after 10 hours releasing from G1/S block in either 293T or HeLa cells. The induction of sub-G1 can be rescued by the addition of 10 μM thymidine, but not adenine, indicating that dUTPase depletion may result in the high dUTP/dTTP ratio to cause cell death during release from G1/S block. Figure 5C showed that the expression levels of TS, TK1 and R2 were not affected by this treatment.

In addition, I also measured the cell survival of dUTPase-depleted cells releasing from G1/S block by MTS assay. As shown in figure 6, cell survival in G1/S block by aphidicolin treatment required dUTPase. However, the effect was not so significant by hydroxyurea

treatment, indicating that ribonucleotide reductase may play a role in the effect. (Figure 7).

Effect of dUTPase depletion on DNA damage and apoptotic induction in cells S phase progression.

Next, I investigated whether the reduction of viability in cells depleted of dUTPase is associated with DNA damage. The 293T cells transfected with dUTPase shRNA or LacZ shRNA were synchronized by aphidicolin treatment, and released from G1/S block for 10 hours. Cells were then fixed for immunofluorescence staining with antibody of γH2AX. γH2AX staining is a marker for DNA double-strand breaks(Lowndes and Toh, 2005; Paull et al., 2000). As shown in Figure 8, strong γH2AX staining was detected in cells depleted of dUTPase after 10 hours releasing from G1/S block (lower panel).The treatment of aphidicolin (2 μmole/L) was unable to cause γH2AX formation both in dUTPase depleted or control cells. Because dUTPase depletion by itself did not induce γH2AX formation in 293T cells (upper panel), these results suggest that dUTPase depletion will cause severe DNA damage when cells progressed from G1/S phase to G2 phase. In addition, the accumulation of γH2AX staining can be diminished by the addition of thymidine. This indicates that DNA damage signal results from the imbalanced dUTP/dTTP pool, and can be rescued by the supply of dTTP from the *salvage* pathway. These results demonstrate the essential role of dUTPase in prevention of dUTP misincorporation into DNA during S phase progression. Nevertheless, it was noted that the addition of 10 μM thymidine by itself also induce certain level of DNA damage signal as indicated by γ-H2AX staining. It was reported that the elevated level of dTTP allosterically stimulates the substrate specificity of ribonucleotide reductase (RNR) for rGDP, and produces more dGDP, which can further be converted to dGTP, resulting in imbalanced dNTP pool and replication stress (Chimploy and Mathews, 2001).

I also examined whether the DNA damage induced by dUTPase depletion will result in

cell apoptosis. By FITC-labeled Annexin V staining, neither the aphidicolin treatment nor did the dUTPase depletion cause cell apoptosis. As shown in figure 9, only dUTPase-depleted cells during S phase progression revealed Annexin V positive staining, indicating that dUTPase plays an important role in preventing apoptosis when cells progress through S phase.

Role of dUTPase in methotrexate sensitivity

Previous study in our laboratory demonstrated that the depletion of thymidylate synthase expression results in DNA damage. Given that inhibitors of thymidylate biosynthesis, such as 5-Florouracil (5-FU) and methotrexate, have been used in anti-cancers treatment, I raised the question whether dUTPase plays a crucial role in modulating sensitivity to thymidylate synthase inhibition. Firstly, I tested the relationship between the expression level of dUTPase and inhibition of thymidylate synthase by methotrexate. HCT116, 293T, MDA-MB231, and 293 cells were treated with 10 μM of methotrexate for 20 hours and subjected to Western blot analysis. In figure 10, we observed that the expression level of dUTPase in 293T cells was increased when treated with methotrexate. TS and TK1 were up-regulated after methotrexate treatment in four different cell lines.

We compared methotrexate sensitivity in 293 and 293T cells. These cells were treated with methotrexate at various concentrations, from 0.05 to 1 μmol/L for 20 hours, and cell viability was measured by MTS assay. As shown in Figure 11, the IC50 of methotrexate was 0.3 and 0.6 μmol/L in 293 and 293T cells, respectively.

Sensitization of 293T cells to methotrexate by dUTPase depletion

To assess the importance of dUTPase in methotrexate killing, 293T cells transfected with dUTPase shRNA or LacZ shRNA were treated with various concentrations of

methotrexate for 20 hours, and cell viability was detected by MTS assay. As shown in Figure 12A, dUTPase-depleted cells were more sensitive to the methotrexate treatment, and the IC50 for methotrexate was 0.075 and 0.35 μmol/L in dUTPase-depleted and control cells, respectively. The efficiency of dUTPase depletion in three independent experiments was demonstrated in Figure 12B.

Enhancement of methotrexate-induced DNA damage response by dUTPase depletion

To further examine the role of dUTPase in the methotrexate-induced DNA damage response, 293T cells transfected with dUTPase shRNA or LacZ shRNA were treated with or without 10 μM methotrexate for 20 hours. By examining histone γH2AX foci formation, I found that the depletion of dUTPase by itself didn't induce DNA damage (Figure 13; upper panel). In contrast, under methotrexate treatment, cells displayed strong γH2AX foci accumulation, and the intensity of damage signal is higher in dUTPase depleted cells. The result suggests that dUTPase depletion enhances methotrexate-induced DNA damage lesions.

Validation of the enzyme activity of wild type and mutant recombinant human dUTPase

In order to elucidate whether the catalytic activity of dUTPase is essential for the resistance to methotrexate, I generated the catalytic-dead mutant plasmid by site directed mutagenesis. Because the carboxylate side-chain of Aspartate 84 is absolutely required for dUTP hydrolysis, the catalytic function of dUTPase was abolished by substituting aspartate 84 to asparagine, without the disruption of dUTP binding (Harris et al., 1999).

Then, I expressed the recombinant human GST-dUTPase fusion protein in *E coli* to confirm the loss of the catalytic ability of this mutant. As shown in figure14, after induction with 0.1mM of IPTG at 37℃ for 3.5 hours, the recombinant human GST-dUTPase was purified by using affinity chromatography. The purified proteins were separated by

SDS-PAGE and detected by Coomassie blue staining. Because dUTPase is a pyrophosphatase, I used a pyrophosphate assay kit to detect the dUTPase activity of those samples. By detecting the absorbance at 360 nm, we can calculate the concentration of PPi by comparing to the standard curve to check the enzyme activity of the recombinant dUTPase. In figure 15A, there is a linear relationship between the absorbance at 360 nm and the volume of wild type dUTPase. To calculate the enzyme specific activity, 0.25 μg wild type and mutant form dUTPase were used in the reaction. As shown in figure 15B, the specific activity of the wild type dUTPase is about 90 U (mM/mg/min) while mutant form dUTPase has no activity. The recombination proteins eluted from glutathione−sepharose were further detected by Coomassie blue staining (Figure 15C). The molecular weight of recombination proteins is about 49KDa.

Construction of EGFP-dUTPase

After confirming the loss of the catalytic activity of the mutant, I generated the EGFP-tagged mammalian expression plasmids of dUTPase by inserting the dUTPase cDNA fragments from CMV2-Flag-dUTPase into BamHI and EcoRI sites of the pEGFP plasmid (Figure 16). The pEGFP-dUTPase plasmids were checked by EcoRI and BamHI digestion and then analyzed by agarose gel electrophoresis. The size of EGFP vector is about 4.7 Kb, and dUTPase cDNA fragment is about 495 bp.

Expression of dUTPase diminishes the damage signal resulted from methotrexate treatment

Finally, I asked that whether overexpression of dUTPase reduces the methotrexate induced DNA damage in 293 cells. Cells transfected with wild type or mutant GFP-dUTPase were treated with 0.1 μM methotrexate for 20 hours. The expression of

GFP-dUTPase and DNA damage signal were detected by fluorescence microscopy. As shown in figure 17A, the cell expressing wild type GFP-dUTPase has no γH2AX foci formation. Apparent damage signals can be detected in cells which didn't express GFP-dUTPase. As expected, cells expressing mutant form of GFP-dUTPase did not reduce methotrexate induced DNA damage. Figure 17B showed the percentage of γ H2AX positive cells without or with methotrexate treatment after GFP-dUTPase (wild type or mutant form) transfection. These results indicate that the amount and the catalytic activity of dUTPase are crucial for protecting cells from methotrexate-induced DNA damage.

Discussion

In this study, the importance of dUTPase is particularly emphasized in 293T and HeLa cells released from the replication stress, since knockdown of dUTPase causes severe DNA damage and apoptosis in cells with aphidicolin synchronization. The biochemical function of dUTPase is to convert dUTP to dUMP, thus preventing uracil misincorporation into DNA. Therefore, it is logical to assume that DNA replication stress induced by aphidicolin leads to the accumulation of dUTP when dUTPase is deficient. Once the DNA replication is resumed from G1/S blockage, dUTP is misincorporated, triggering DNA damaging signal.

The expression level of dUTPase is low during G1, and is progressively increased through S and G2. Likely, the functional role of dUTPase is important in the S phase. Of note, depletion of dUTPase did not cause a significant effect on cell survival in asynchronous population. Thus, dUTPase is more important in cells that have been stressed by stalled DNA replication by treatment of aphidicolin. The expression levels of TS and TK1 were not affected by this treatment (Figure 5C), suggesting that the dTTP supply should maintain intact. So, the question is how dUTP accumulation takes place in the cells after aphidicolin treatment.

Since most of the DNA polymerases can't distinguish dUTP from dTTP during DNA replication(Bessman et al., 1958; Brynolf et al., 1978; Dube et al., 1979; Yoshida and Masaki, 1979), it is possible that the expansion of dUTP pool let DNA polymerase took dUTP instead of dTTP for DNA polymerization. Although I do not have direct evidence that dUTP gets accumulated, exogenous addition of thymidine, which increased dTTP supply, reduced the extent of DNA damage signal induced by dUTPase depletion. This result support the hypothesis the high dUTP/dTTP ration contributes to the DNA damage signal induced by dUTPase knockdown.

Up-regulation of *DUT* is statistically significant in some types of tumors such as

hepatocellular carcinoma, cervical, melanoma, and leukemia. Earlier studies demonstrated that *DUT* indeed overexpressed in hepatocellular carcinoma (Takatori et al., 2010) and cervical cancer (Pyeon et al., 2007). Because of the high proliferation rate of tumor cells, a larger population of S phase cells is usually found in tumor cells than in normal cells. Therefore, higher level of dUTPase gives tumor growth advantage by preventing uracil misincorporation. Relevantly, there are reports that some rapidly proliferating tumors exhibit very low levels of dCMP deaminase, which is responsible for dCMP conversion to dUMP (Blocker and Roth, 1977; Sneider et al., 1969). Thus, dUTPase avoids dUTP formation while providing dUMP to cooperate with TS to form dTTP in these tumor cells for fidelity of DNA replication.

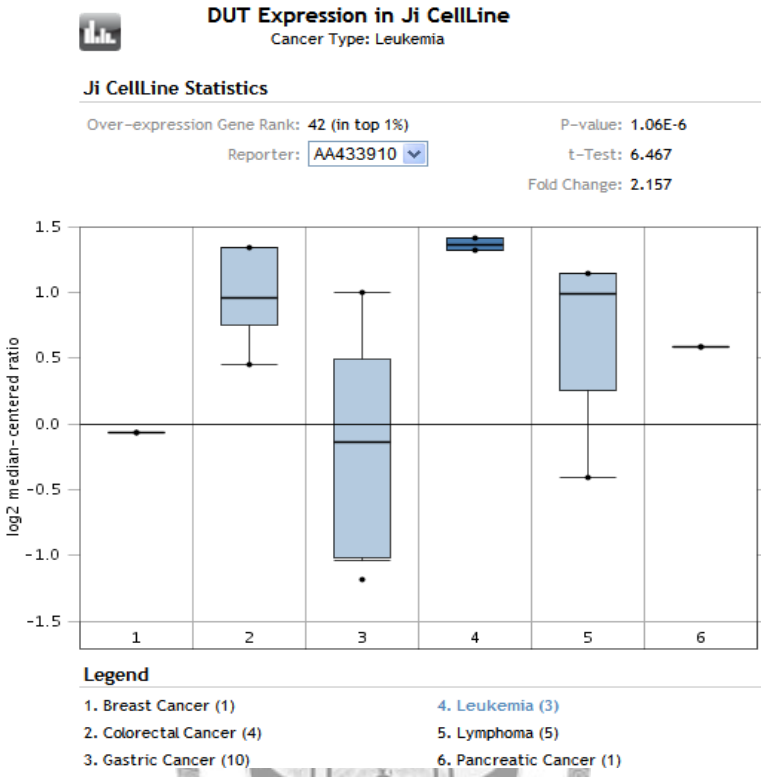
Nucleotide analogs such as 5-fluorouracil (5-FU) or 5-fluorodeoxyuridine (FdUrd), or Capecitabine, a novel 5-FU pro-drug are used for treatment of colon, breast, head, neck, and skin cancers (Heidelberger et al., 1957; Miwa et al., 1998). By forming a ternary complex with the methyl donor (MTHF) and the TS enzyme, these drugs can inhibit TS action (Wilson et al., 2008). A retrospective clinical study showed a negative correlation between the expression level of dUTPase and the response to 5-FU therapy in colorectal cancer patient (Kawahara et al., 2009; Ladner et al., 2000). In this study, I examined the effect of dUTPase on methotrexate sensitivity. In 293T cells, depletion of dUTPase enhances susceptibility to methotrexate-induced DNA damage and cell death. On the other hand, enforced expression of dUTPase indeed diminishes methotrexate-induced DNA damage in 293 cells. Thus, higher level of dUTPase also renders tumor more resistant to genotoxic insults by anti-cancer treatment using methotrexate or 5-FU.

In conclusion, I demonstrated the crucial role of dUTPase in cells resumed from replication stress and in resistance to anti-cancer treatment by blocking *de novo* dTTP synthesis. Therefore, my results suggest that dUTPase inhibitor in conjunction with blockers

of dTTP synthesis has a therapeutic potential in treating cancer cells, and supports the notion that dUTPase can be a biomarker to predict 5-FU and methotrexate therapeutic response.

Figures and Legends

A.



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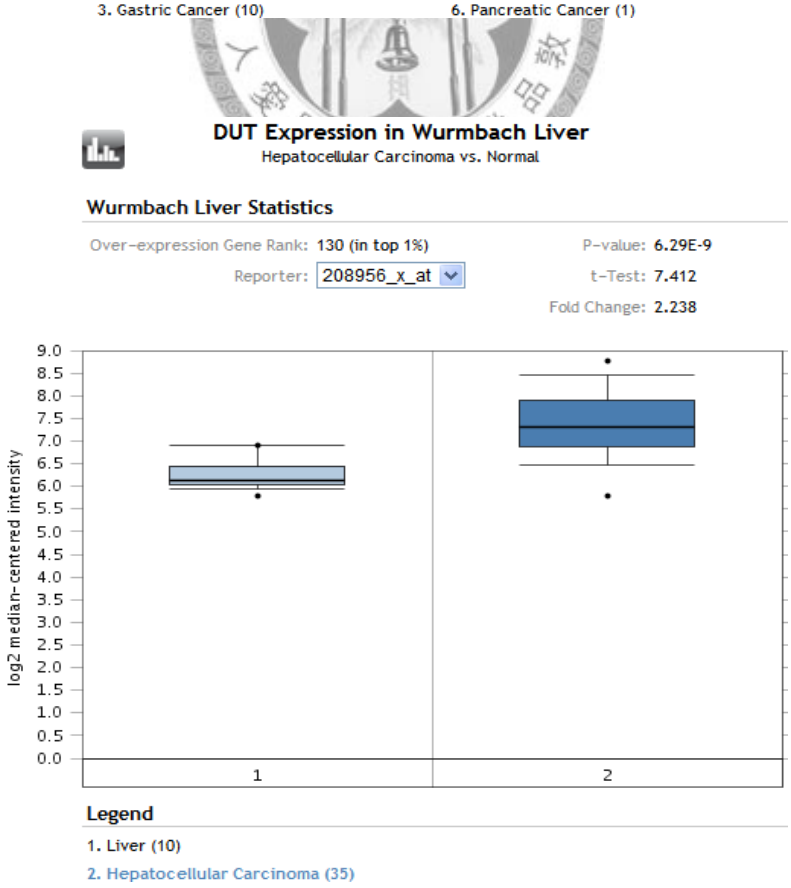

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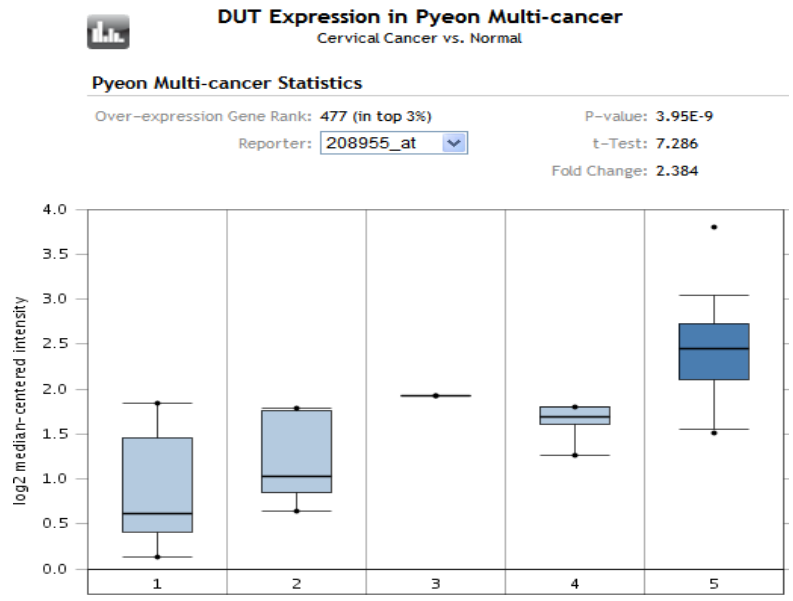

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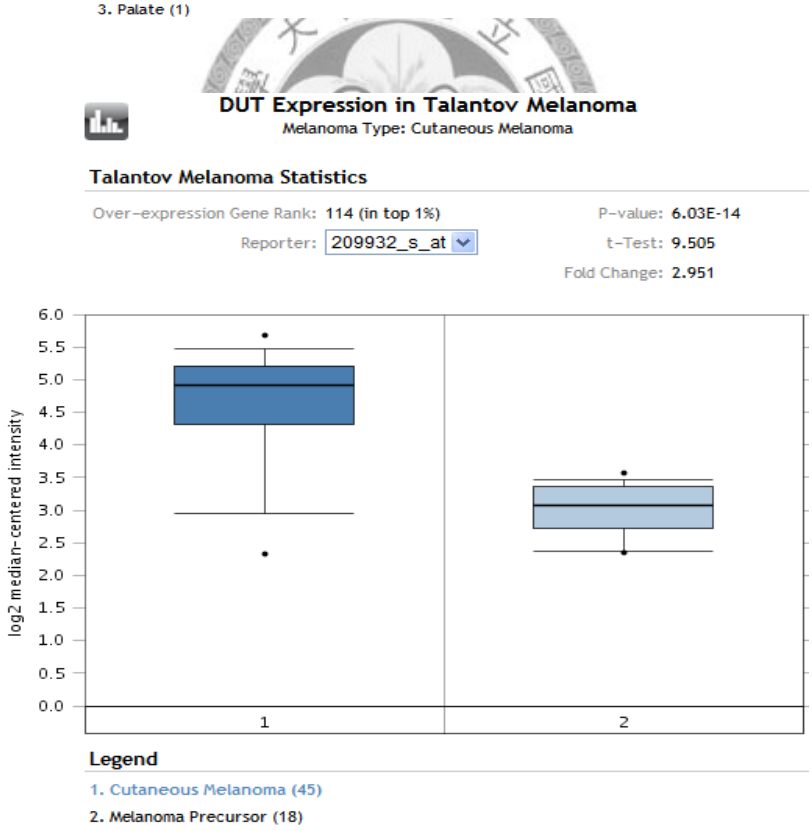


Figure 1 **The relative expression levels of dUTPase RNA in various cancers.**
Gene expression studies comparing normal and cancer tissues were analyzed for *DUT* using the Oncomine database. *DUT* was found to be overexpressed in Leukemia (A), Hepatocellular Carcinoma (B), Cervical Cancer (C), and Cutaneous Melanoma (D).

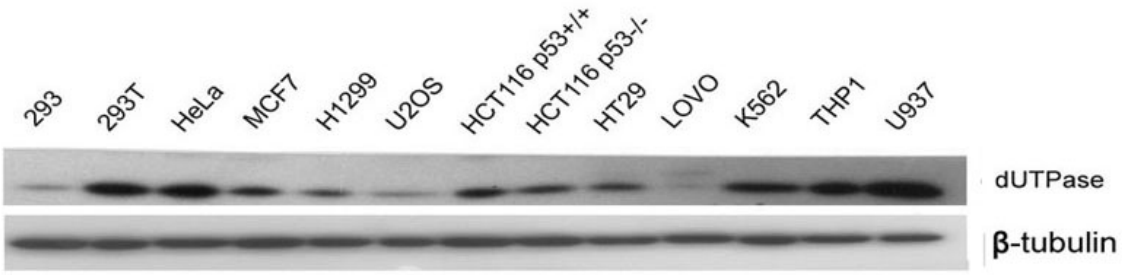
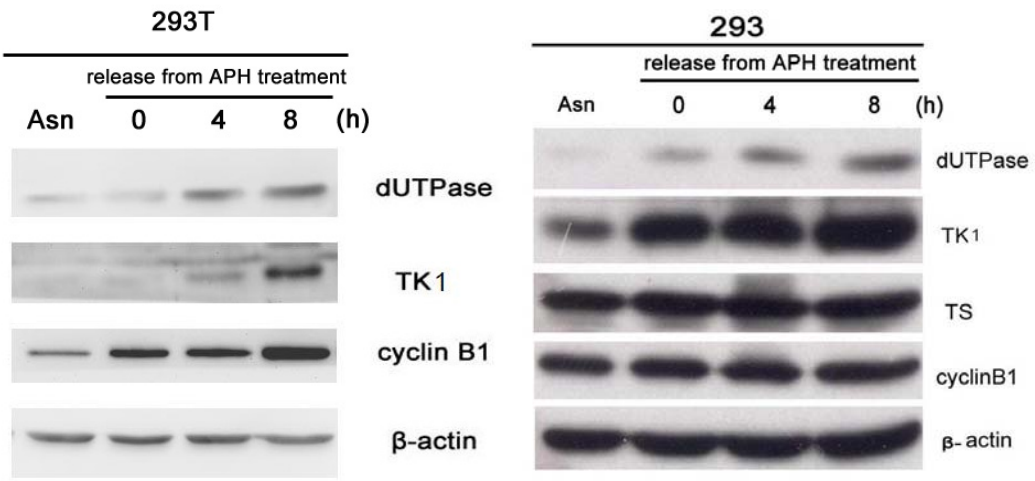


Figure 2 **Endogenous expressions of dUTPase in different human cell lines.**
Cell extracts (25 μg) from different cell lines were separated by 10% SDS-PAGE, and dUTPase and β-actin were detected with dUTPase and β-actin antibodies by Western blot analysis.

A.



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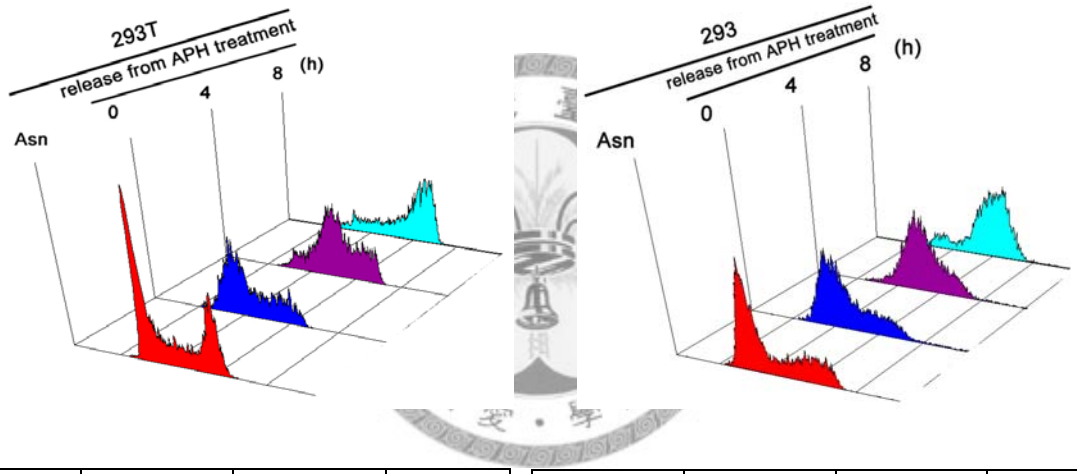


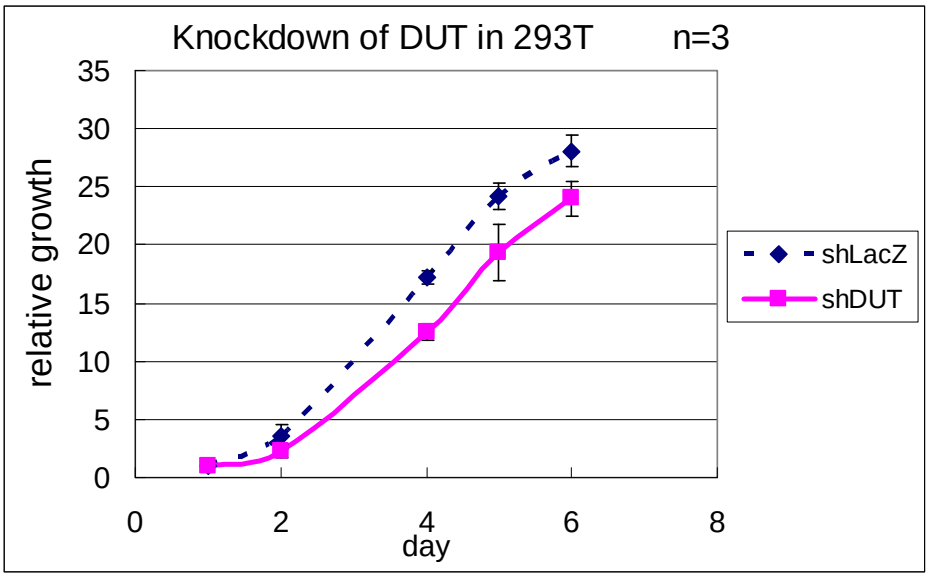
293T	G1	S	G2/M
Asn	45.53	31.51	23.91
R0	39.78	48.04	12.9
R4	11.4	62.92	25.76
R8	12.08	35.07	53.58

293	G1	S	G2/M
Asn	55.07	30.04	15.39
R0	38.62	50.03	11.47
R4	7.93	76.45	15.68
R8	11.01	56.74	32.43

Figure 3 **Cell-cycle dependent oscillation of endogenous dUTPase expression in 293 and 293T cells**

293 and 293T cells synchronized with aphidicolin for 20 hours were released from G1/S block. (A) Cells were then harvested at the indicated time points for Western blotting with anti-dUTPase, TK1, TS, cyclin B1, and β-actin antibodies. (B) A parallel set of cells was fixed and stained with propium iodide for flow cytometric analysis.

A.



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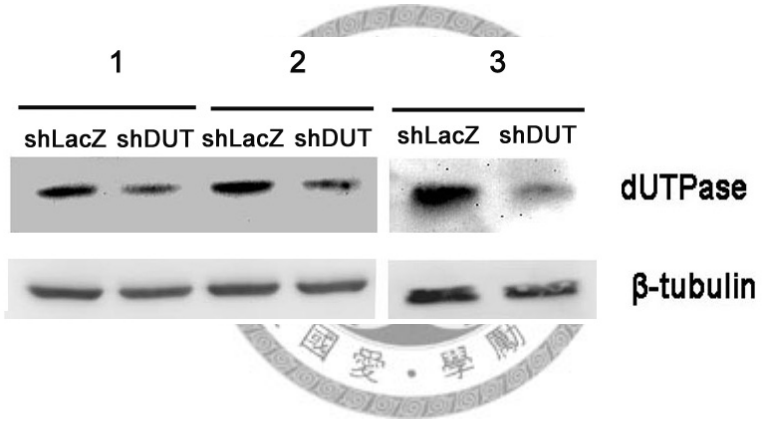
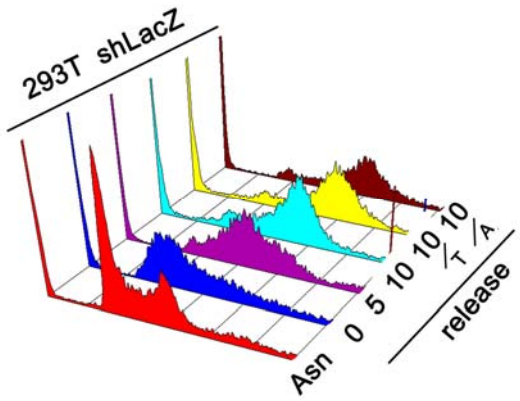
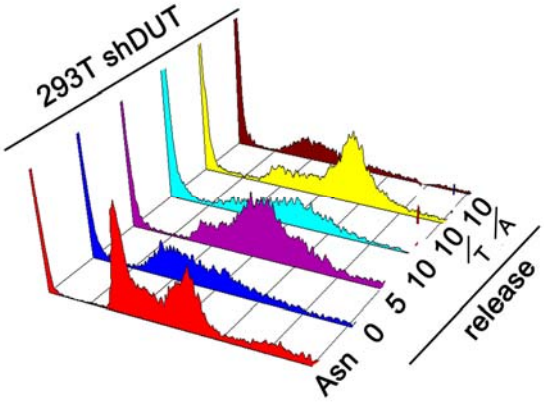


Figure 4 **Effect of depletion of dUTPase by dUTPase^{shRNA} on growth rate in 293T cells.** 293T cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. (A) After 3 days, cells were plated into a 96-well at 2X10^3 cells per well. Following overnight culture, the growth of cells at each day was measured by MTS assay. Values are the average of three independent determinations. The error bars represent standard deviations. (B) A parallel set of cells was subjected to Western blot analysis with anti-dUTPase, and β-tubulin antibodies.

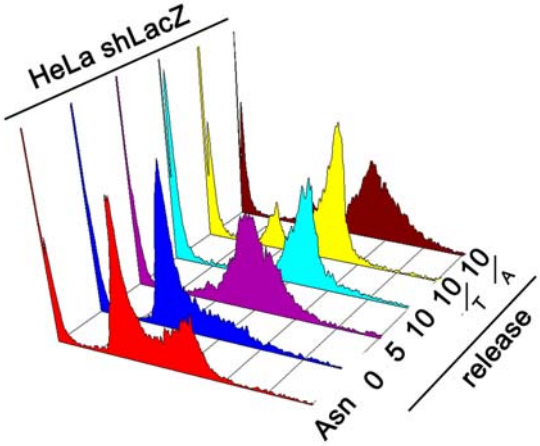
A.

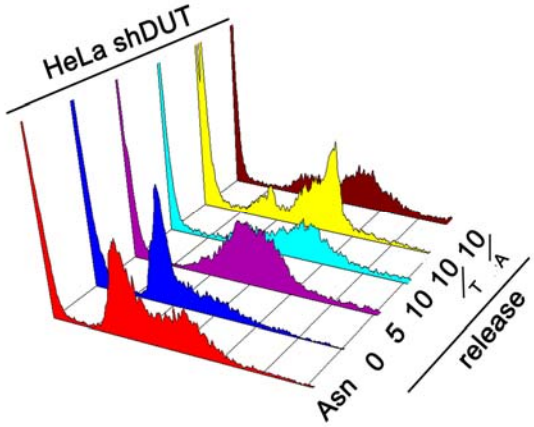




	SubG1	G1	S	G2/M
Asn	19.25	38.86	28.92	12.96
R0	18.73	32.63	30.82	17.82
R5	21.54	7.29	31.62	39.55
R10	26.52	7.82	18.04	47.62
R10+T	32.04	6.32	13.91	47.73
R10+A	37.28	6.96	11.42	44.33

	SubG1	G1	S	G2/M
Asn	19.59	31.11	26.03	23.27
R0	32.74	27.08	20.53	9.65
R5	26.07	8.91	30.12	34.90
R10	69.05	5.37	12.70	12.88
R10+T	31.57	7.90	15.49	45.04
R10+A	79.49	7.66	8.76	4.09

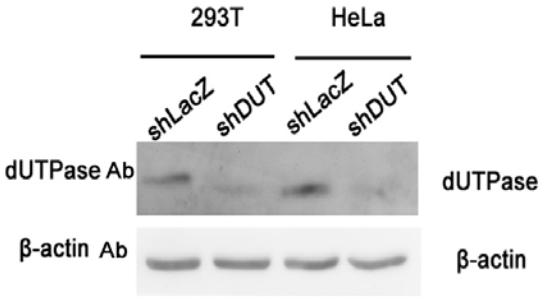




	Sub	G1	S	G2/M
Asn	22.60	37.22	23.22	16.96
R0	26.87	41.47	23.51	8.15
R5	24.02	5.59	42.59	27.80
R10	32.61	11.42	18.14	37.83
R10+T	27.38	12.30	16.40	43.91
R10+A	27.14	11.74	21.73	39.39

	Sub	G1	S	G2/M
Asn	32.59	29.22	24.19	14.00
R0	31.04	47.18	16.33	5.45
R5	34.11	6.13	34.33	25.43
R10	47.71	8.48	25.14	18.68
R10+T	37.82	10.51	19.61	32.06
R10+A	45.82	8.81	25.34	20.03

B.



C.

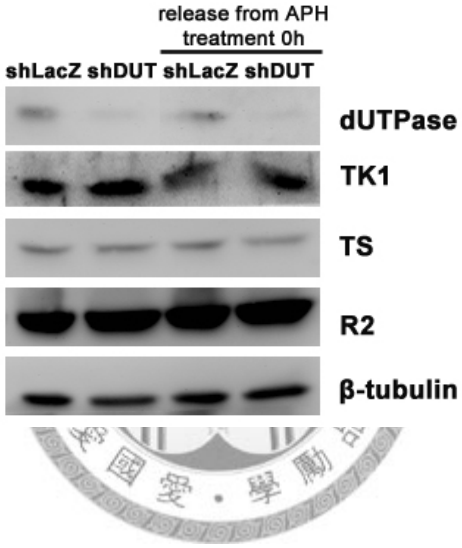
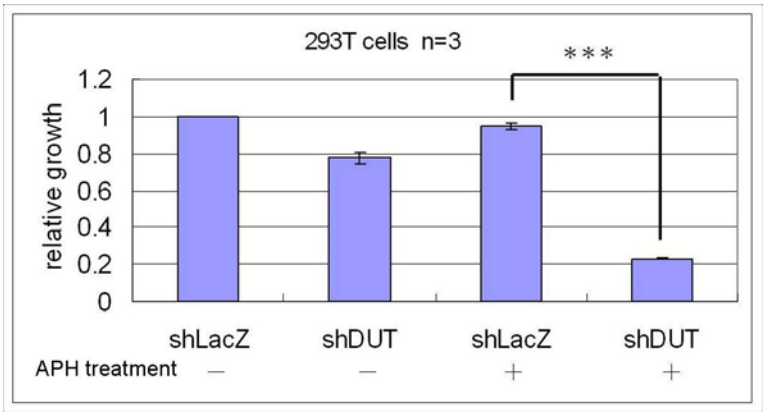


Figure 5 **Effect of depletion of dUTPase by dUTPase^{shRNA} on cell cycle progression in 293T and HeLa cells.**

293T and HeLa cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. After 52 hours, cells were synchronized by aphidicolin for 20 hours, followed by replenishment of fresh medium to release cells from the G1/S block in the presence or absence of thymidine (10 μM) or adenine (10 μM) for 10 hours. (A) The cell cycle profile of corresponding cells was analyzed by flow cytometry. (B) Cell extracts from HeLa and 293T cells at the time point 0h were subjected to western blot analysis. (C) Total lysates from the HeLa cells with or without synchronization were subjected to Western blot analysis with anti-dUTPase, TK1, TS, R2 and β-tubulin antibodies.

A.



B.

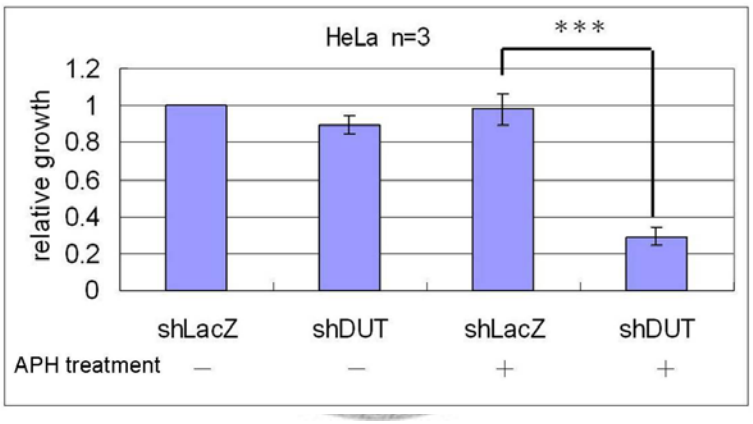
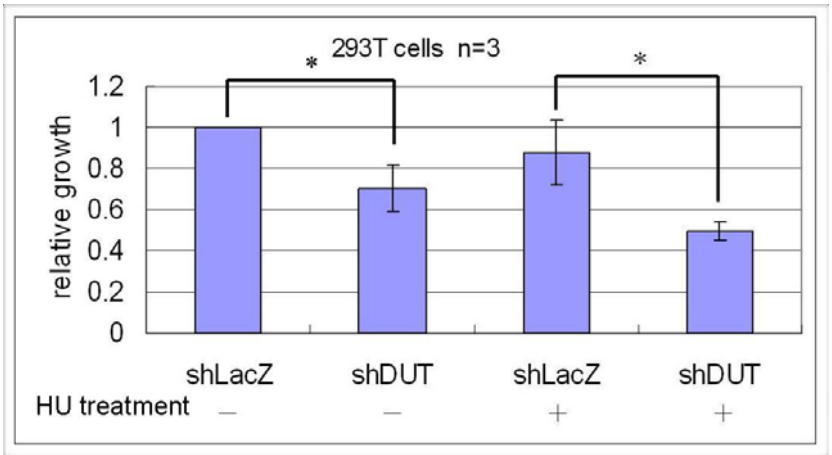


Figure 6 **Effect of depletion of dUTPase by dUTPase^{shRNA} on cell viability in 293T and HeLa cells.**

293T and HeLa cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. After 52 hours, cells were synchronized by aphidicolin for 20 hours, followed by replenishment of fresh medium to release cells from the G1/S block. (A)293T cells or (B)HeLa cells with or without synchronization were subjected to MTS assay to measure the cell viability. Values are the average of three independent determinations. The error bars represent standard deviations. ***, $P < 0.0005$ based on a two-tailed Student's t test.

A.



B.

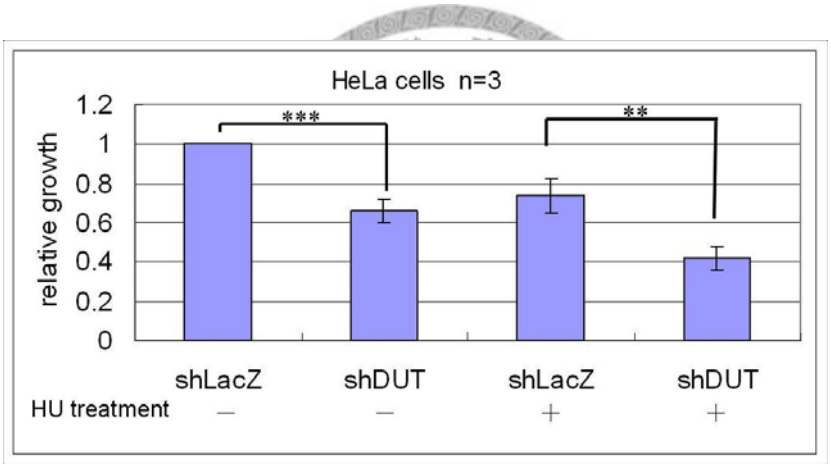


Figure 7 **Effect of depletion of dUTPase by dUTPase^{shRNA} on cell viability in 293T and HeLa cells.**

293T and HeLa cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. After 52 hours, cells were synchronized by hydroxyurea for 20 hours, followed by replenishment of fresh medium to release cells from the G1/S block. (A) 293T cells and (B) HeLa cells with or without synchronization were subjected to MTS assay to measure the cell viability. Values are the average of three independent determinations. The error bars represent standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ based on a two-tailed Student's t test.

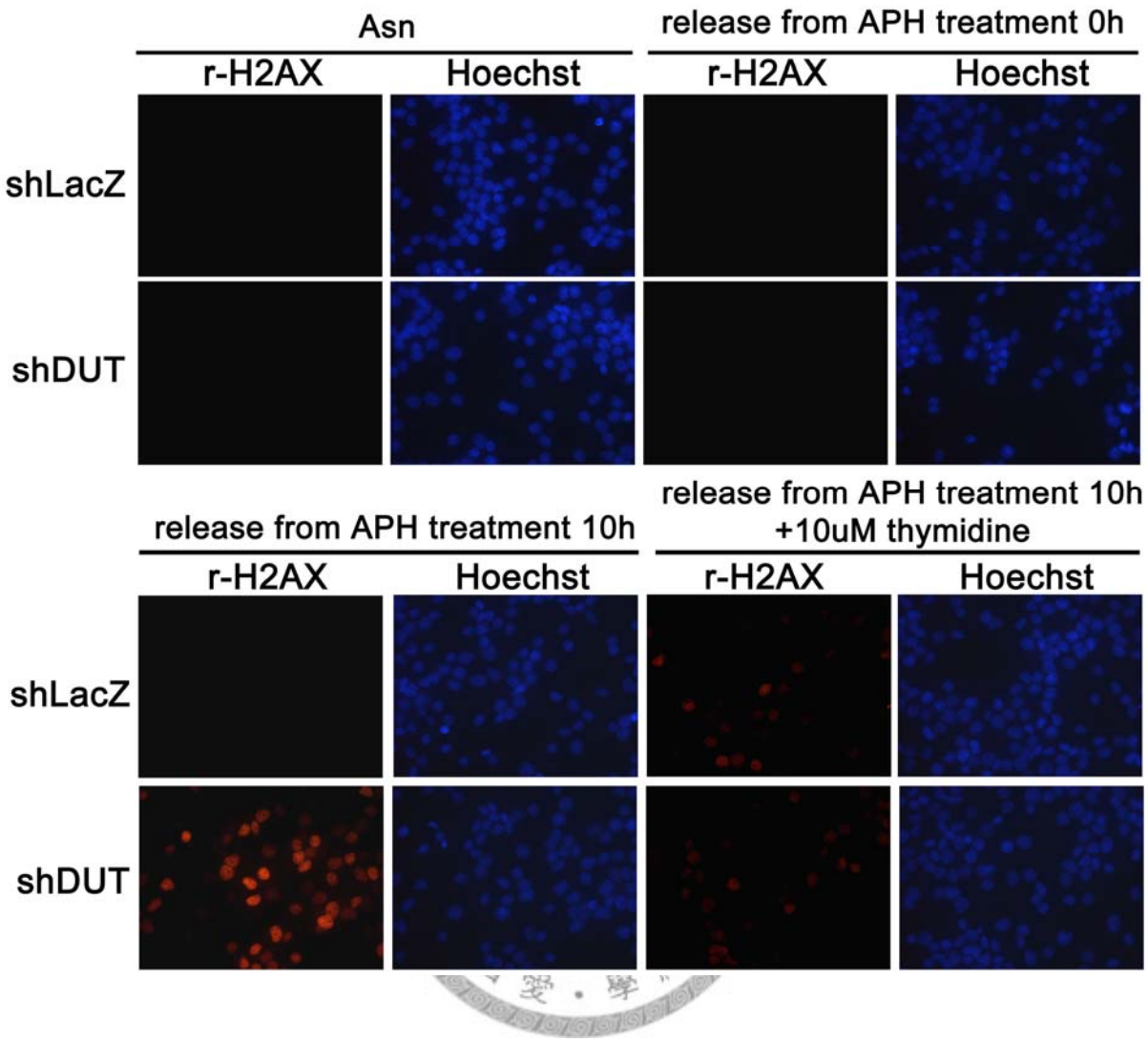


Figure 8 **DNA damage results from dUTPase depletion can be rescued by the thymidine treatment.**

293T cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. After 52 hours, cells were synchronized by aphidicolin for 20 hours, followed by replenishment of fresh medium to release cells from the G1/S block in the presence or absence of thymidine (10 μM) for 10 hours. Cells were then fixed with 3% paraformaldehyde at the time point as indicated, and immunostained with anti- γ H2AX antibody (1:200), Hoechst (1:500) and anti-mouse TRITC (1:200). The formation of γ H2AX foci was observed by fluorescent microscopy.

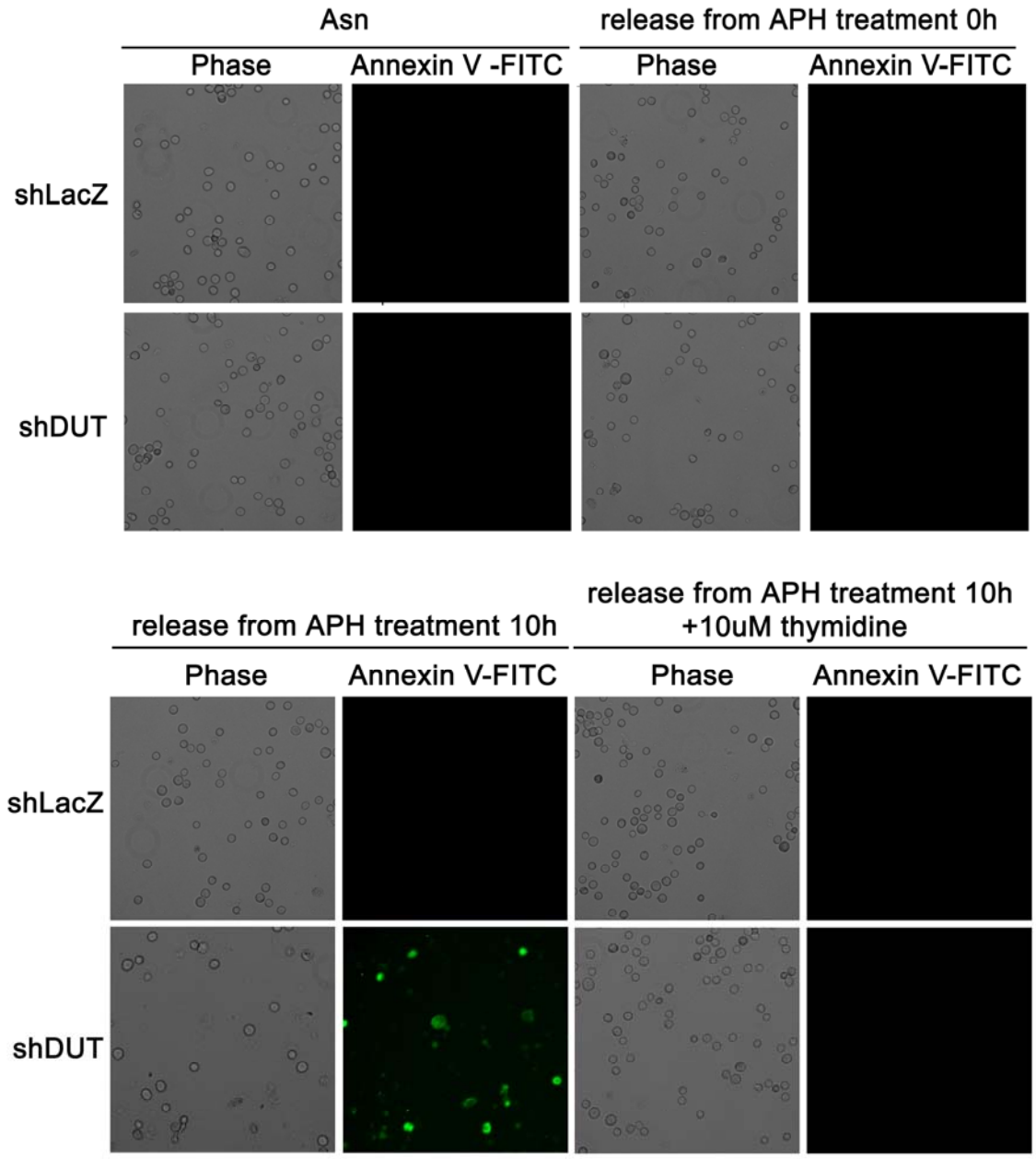


Figure 9 **dUTPase depletion induced apoptosis in 293T cells progressed through S phase.**

293T cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. After 52 hours, cells were synchronized by aphidicolin for 20 hours, followed by replenishment of fresh medium to release cells from the G1/S block in the presence or absence of thymidine (10 μM) for 10 hours. Cells were then subjected to FITC-labeled Annexin V apoptosis assay.

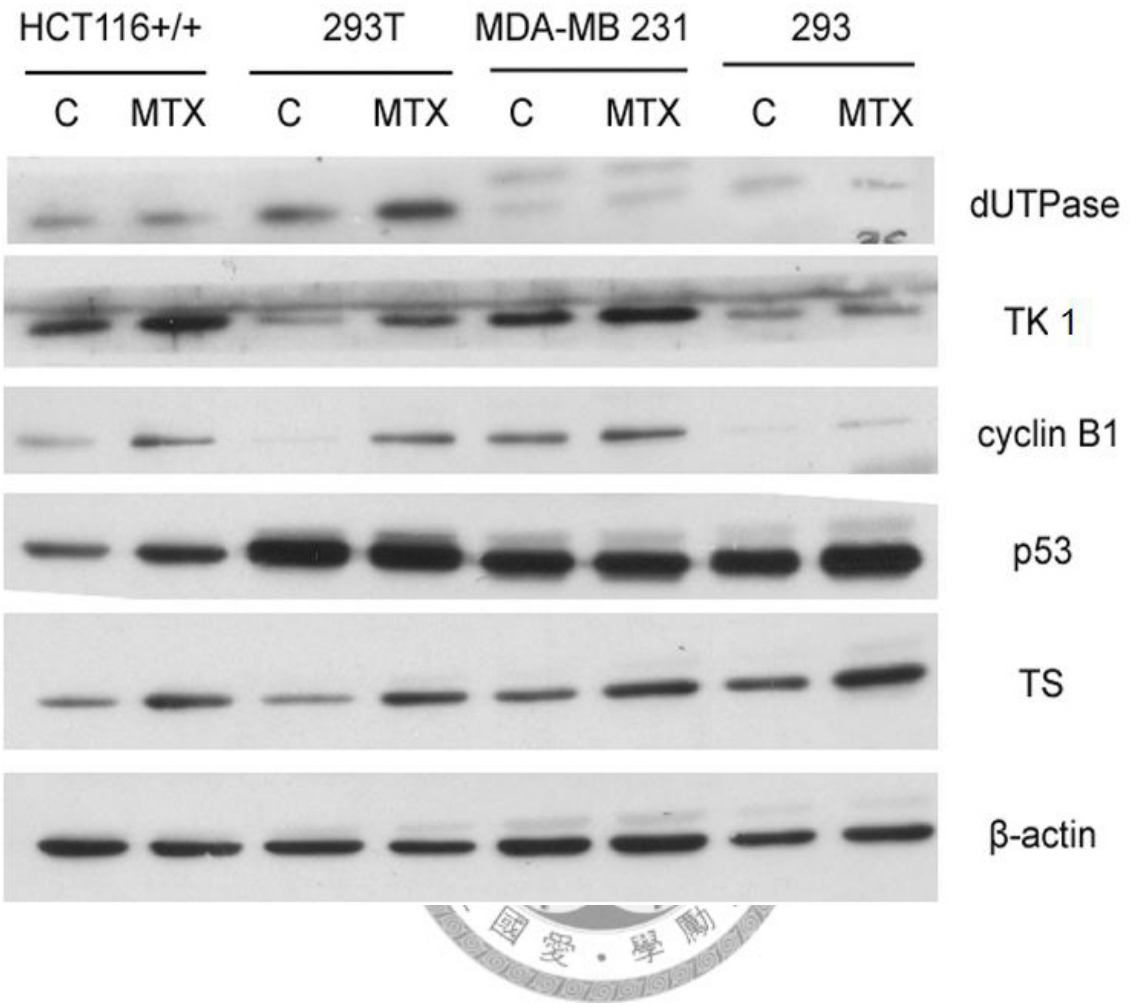


Figure 10 **Effect of the methotrexate treatment in different cell lines.**
HCT116, 293T, MDA-MB231, and 293 cells treated with or without 10 μM methotrexate for 20 hours were harvested and subjected to Western blot analysis with anti-dUTPase, TS, TK, p53, cyclin-B1 and β-actin antibodies.

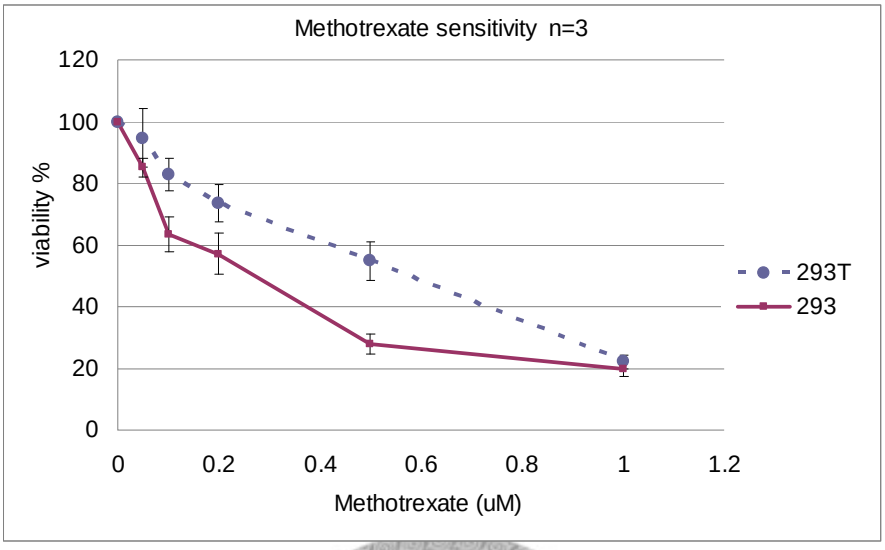
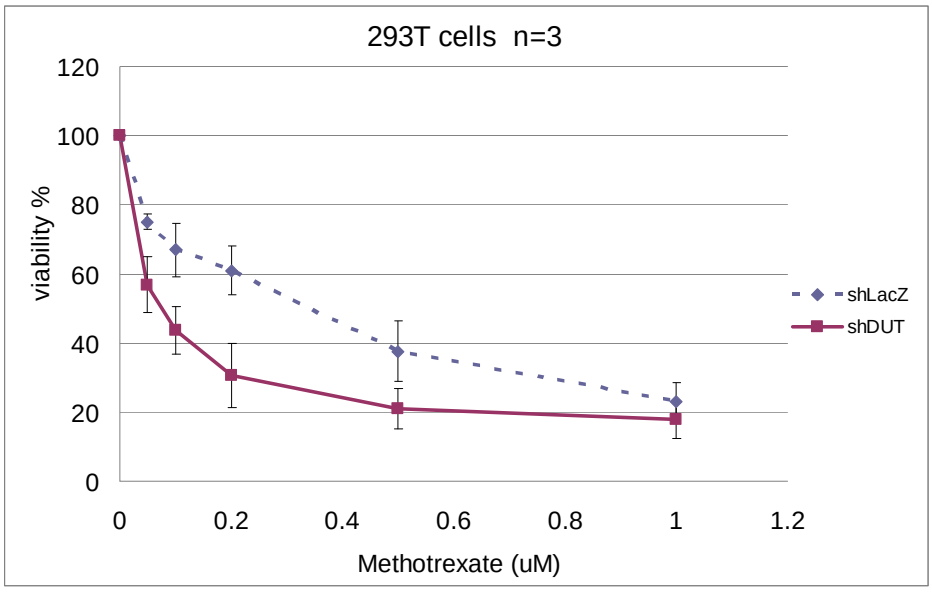


Figure 11 **Methotrexate sensitivities of 293 and 293T cells.**

293 and 293T cells were plated into a 96-well plate. (A) On the following day the growth of cells at each day was measured by MTS assay. (B) For the measurement of methotrexate sensitivity, cells were treated with various concentration of methotrexate as indicated for 20 hours, and the cell viability was then measured by MTS assay. Values are the average of three independent determinations. The error bars represent standard deviations.

A.



B.

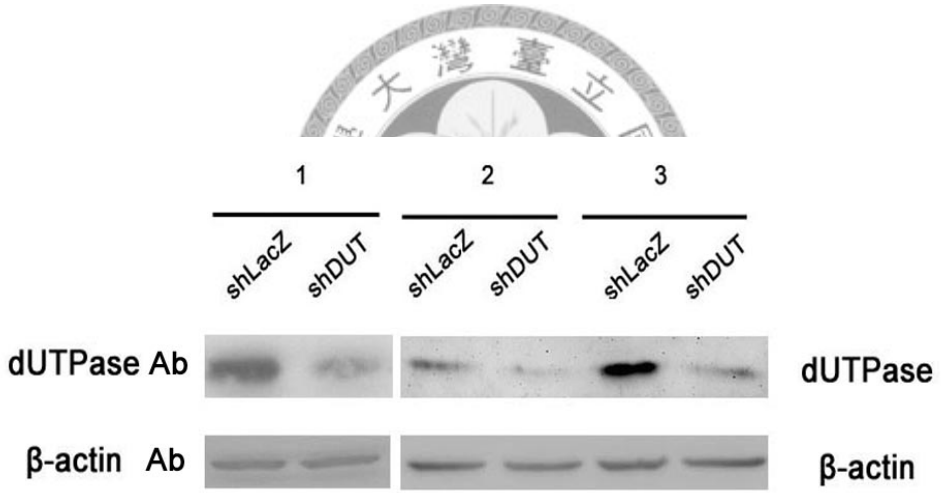


Figure 12 **Depletion of dUTPase enhances susceptibility to methotrexate-induced cell death in 293T cells.**

293T cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. (A) After 48 hours, cells were plated into a 96-well plate at 4X10^3 cells per well. Following overnight culture, cells were treated with various concentrations of methotrexate as indicated for 20 hours, and cell viability was measured by MTS assay. Values are the average of three independent determinations. The error bars represent standard deviations. (B) After 72 hours, cell extracts were subjected to Western blot analysis with anti-dUTPase, and β-tubulin antibodies.

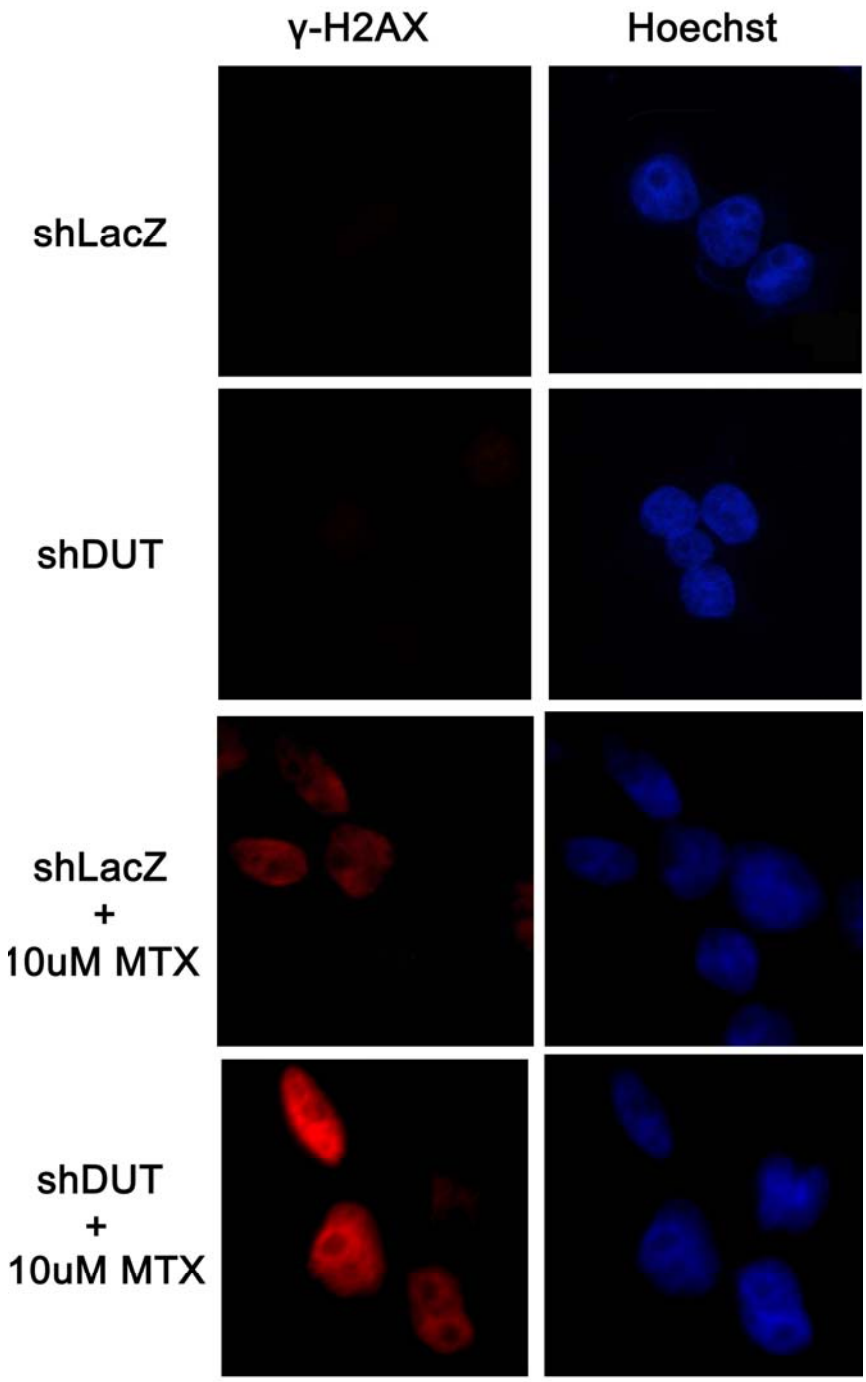


Figure 13 **Depletion of dUTPase enhances methotrexate-induced DNA damage.** 293T cells were transfected with dUTPase^{shRNA} or LacZ^{shRNA}. After 72 hours, cells were treated with or without 10 μM methotrexate for 20 hours. Cells were then fixed with 3% paraformaldehyde at the time point as indicated, and immunostained with anti-γH2AX antibody (1:200), Hoechst (1:500) and anti-mouse TRITC (1:200). The formation of γH2AX foci was observed by fluorescent microscopy.

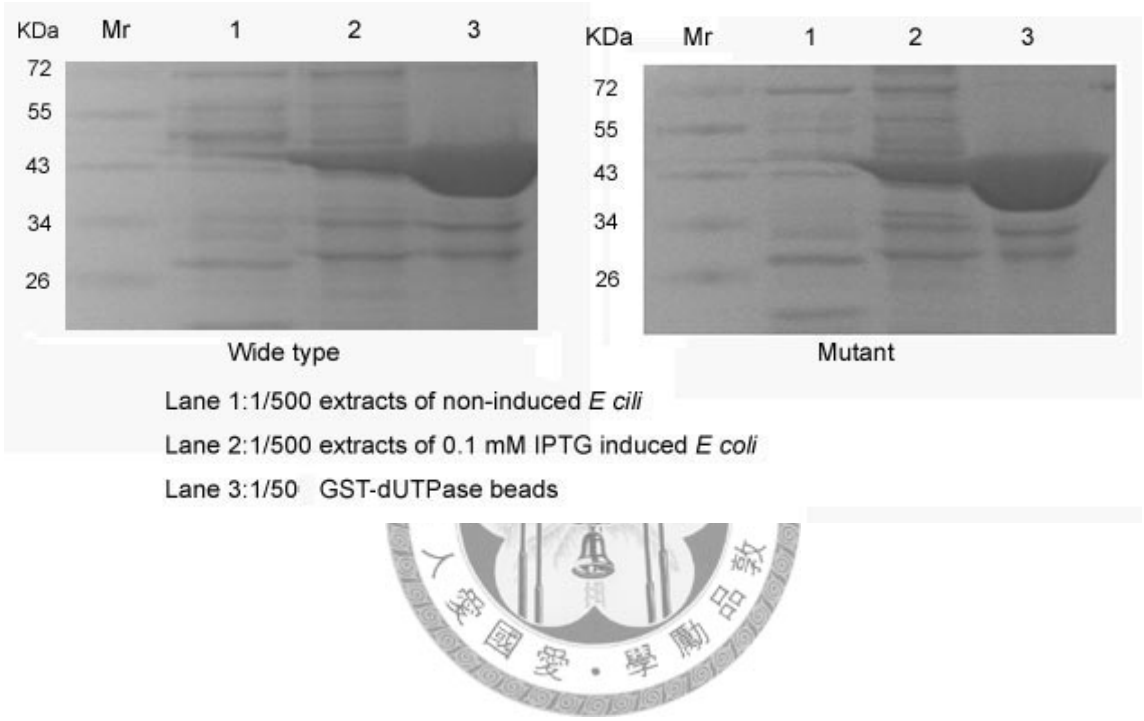
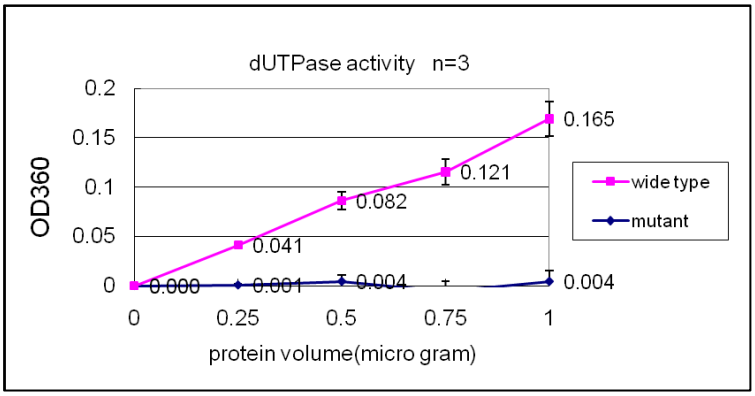


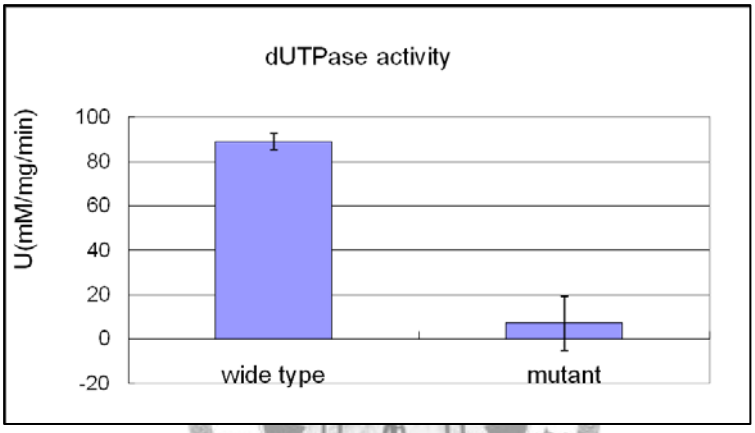
Figure 14 **Purification of recombinant human dUTPase protein.**

Wild type (left panel) or mutant from (right panel) of recombinant human GST-dUTPase protein was purified by affinity chromatography on glutathione−sepharose from *E. coli* after induction with 0.1 mM of IPTG at 37℃ for 3.5 hours. Proteins were separated on a 10% SDS PAGE and stained with coomassie blue.

A.



B.



C.

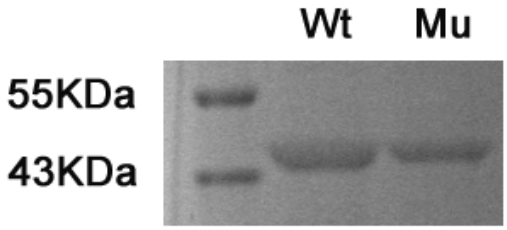


Figure 15 **Measurement of recombinant human dUTPase activity.**
(A) Measurement of dUTPase activity. Reaction products from different amounts of dUTPase were subjected to phosphate assay as substrates, and the accompanying change in the absorbance at 360 nm was recorded. (B) The enzyme specific activity was calculated by measuring the concentration of PPi when the enzyme reaction containing 0.25 μg dUTPase. Values are the average of three independent determinations. The error bars represent standard deviations. (C) Recombinant human GST-dUTPase fusion protein was eluted from glutathione−sepharose with glutathione after affinity chromatography. Proteins were separated on a 10% SDS PAGE and stained with coomassie blue.

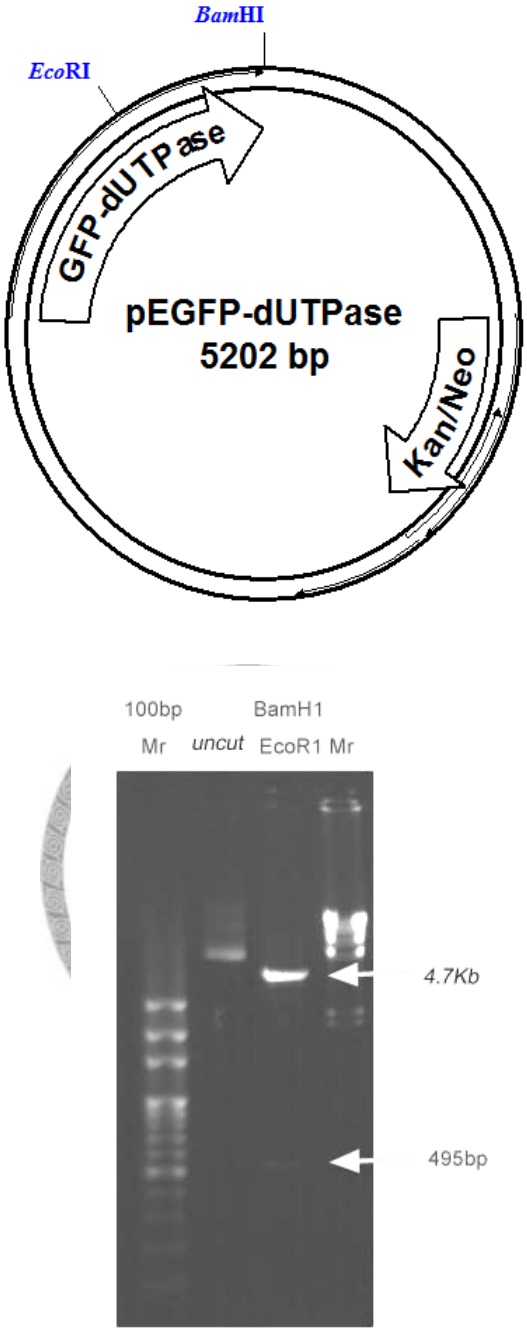
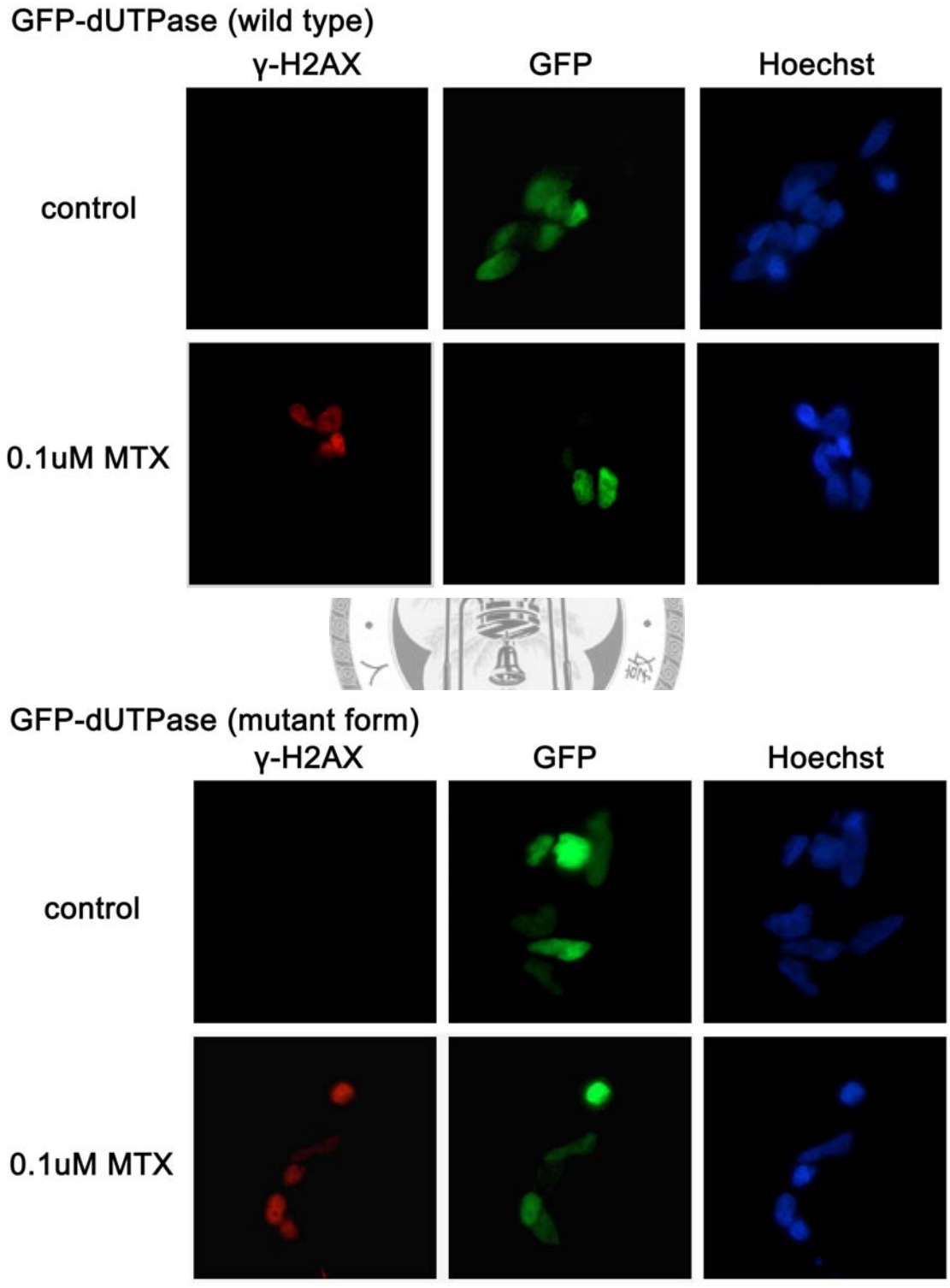


Figure 16 **Construction of pEGFP-dUTPase.**

The fragment of dUTPase cDNA was obtained from CMV2-Flag-dUTPase plasmid digested with EcoRI and BamHI and then inserted in-frame into BamHI and EcoRI sites of pEGFP-C1. (A) The map of pEGFP-dUTPase. (B) pEGFP-dUTPase plasmid was checked by BamHI-EcoRI digestion. pEGFP-dUTPase plasmid (1μg) was digested with BamHI and EcoRI enzyme at 37℃ for 1 hour. The products were then analyzed on a 1% agarose gel and visualized by ethidium bromide staining.

A.


GFP-dUTPase (wild type)
γ-H2AX
GFP
Hoechst
control
0.1uM MTX
GFP-dUTPase (mutant form)
γ-H2AX
GFP
Hoechst
control
0.1uM MTX

B

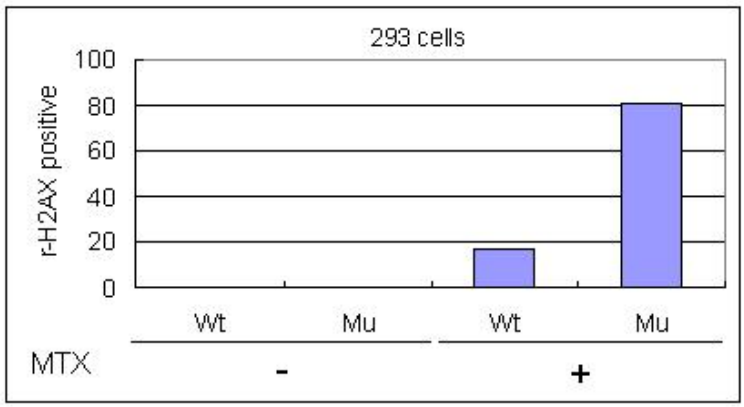


Figure 17 **Ectopic expression of dUTPase diminishes methotrexate-induced DNA damage.**

293 cells expressing wild type (upper panel) or mutant (lower panel) GFP-dUTPase were treated with 0.1 μM methotrexate for 20 hours. (A) Cells were then fixed with 3% paraformaldehyde , and immunostained with anti-γ H2AX antibody (1:200), Hoechst (1:500) and anti-mouse TRITC (1:200). The formation of γ H2AX staining was observed by fluorescent microscopy. (B) The graph represents percentage of γ H2AX positive cells with GFP-dUTPase (wild type or mutant form) expression (n=100).

Appendix

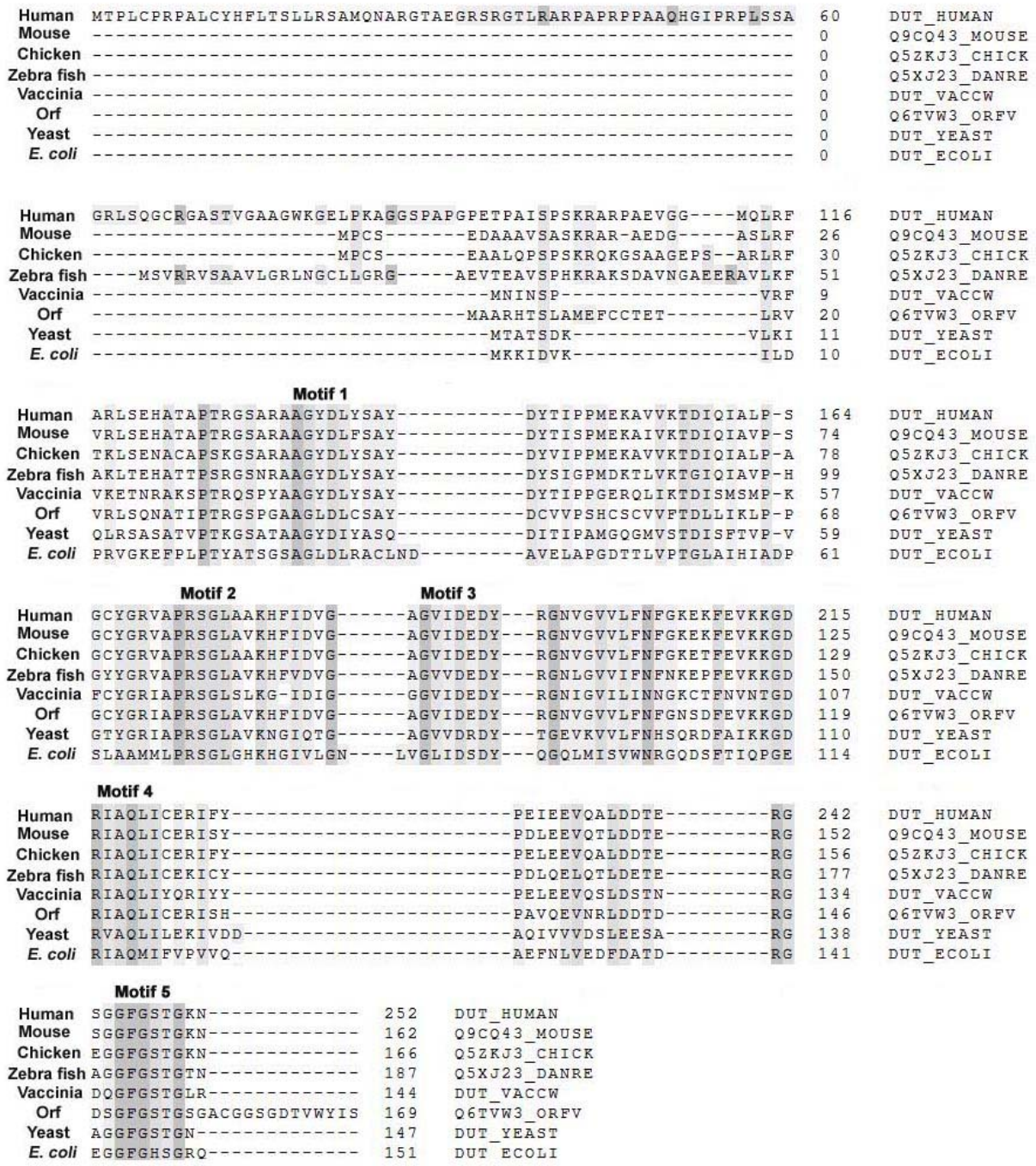


Figure S1 **Multiple alignment of sequences from viral and non-viral dUTPase.**

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