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INTRODUCTION

Cytosolic thymidine kinase (TK1), the key enzyme for dTTP synthesis via the *salvage* pathway, catalyzes the transfer of the terminal phosphate of ATP to the 5' hydroxyl group of thymidine to form dTMP. During cell cycle progression, the expression of TK1 reaches its maximum in the S phase to provide dTTP for DNA replication. Mammalian cells contain another TK isoform, TK2, which is located in mitochondria (1, 2). Unlike TK1, TK2 expression is independent of cell cycle and is required for mitochondrial biogenesis in terminally differentiated and resting cells (3, 4). Multiple levels of control mechanism, including transcriptional (5-8), post-transcriptional (6, 9, 10) and translational (11-13), contribute to up-regulation of TK1 during S phase progression. It has been well documented that TK1 expression is under stringent control in normal cells, whereas in cancer cells high expression of TK1 is often found, which contributes to uncontrolled proliferation (14-17).

After DNA replication is completed, dTTP formation is no longer in great demand in G2/M phase, where TK1 become phosphorylated with a low catalytic efficiency (18, 19). Mitotic degradation of TK1 mediated by its carboxyl-terminal region provides an additional means to terminate TK1 function in the mitotic phase (20, 21). Our laboratory has recently shown that TK1 is targeted for ubiquitination/proteasomal degradation at mitotic exit by anaphase-promoting complex/cyclosome (APC/C), so that the level of hTK1 protein is kept low in the early G1 phase. APC/C-mediated ubiquitination and degradation require two different activators, Cdc20 and Cdh1. APC/C is activated by its association with Cdc20 during mitosis through direct binding to the D box of the substrate protein, whereas APC/C-Cdh1 is activated in mitotic exit by recognizing either a D- or a KEN box. Our previous study has shown that TK1 is recognized by Cdh1 through binding to the KEN box located in the carboxyl terminal region of TK1 (22). Similarly, TMPK, an enzyme that catalyzes dTDP formation from dTMP, is targeted by APC/C-Cdc20 and APC/C-Cdh1 for degradation during G2/M transition to mitotic exit (23). Disruption of this proteolytic control causes elevation of the dTTP pool size in the early G1 phase. Since dTTP is an allosteric regulator of ribonucleotide reductase (RNR) by inhibiting and activating the reduction of rCDP and rGDP, respectively (24-27), too much dTTP production in the G1 phase induces a severe dNTP imbalance. As a result, the gene mutation rate is significantly increased (23). Thus, the balanced dNTP pool is also controlled by APC/C-mediated protein degradation.

While thymidine treatment has been used to synchronize cells in the S phase, it has been also shown that incubation of Chinese hamster cells with a high concentration of thymidine (10 mM) increases the gene mutation rate (28). In addition, it has been reported that thymidine is able to counteract the growth-arrest-specific decrease of TK1 expression in LM-TK⁻ cells (29, 30). These experimental observations led us to raise the question of whether thymidine supply can also act as a factor regulating mitotic proteolysis of hTK1, thus affecting the balance of four dNTP pools in the G1/S phase. Here, we provide the first evidence that binding of thymidine to TK1 interferes with its interaction with Cdh1, abolishing APC/C-mediated degradation of TK1. As a consequence, the increased dTTP pool induces nucleotide imbalance in the early G1 phase of thymidine-treated cells, leading to S phase retardation. Our results illuminate the importance of the thymidine supply in controlling mitotic degradation of TK1, which in turn affects the dNTP balance and the S phase progression.

MATERIALS AND METHODS

Materials

Deoxythymidine, deoxycytidine, nocodazole, anti- α -tubulin, anti-FLAG and anti-Cdc27 antibodies were purchased from Sigma (St. Louis, MO). Anti-hTK1 polyclonal antibody was prepared as described previously (31). Anti-cyclin B, anti-p27 and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against HA and GST were obtained from Roche and Amersham Pharmacia, respectively. LLnL was from Calbiochem, and G418 and zeocin were purchased from Invitrogen.

Cell Culture, synchronization, and flow cytometric analysis

LM-TK⁻ and HeLa cells were maintained in DMEM supplemented with 10% FBS. K562 cells were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (HyClone). NIH3T3 fibroblasts stably expressing hTK1/hTMPK were grown in DMEM supplemented with 10% FCS, zeocin (800 μ g/ml) and G418 (600 μ g/ml). All culture media contain 100 U/ml streptomycin and 100 U/ml penicillin (Invitrogen Life Technologies) and cells were grown at 37°C under 5% CO₂. For G2/M arrest, nocodazole was added to HeLa and K562 cells at a final concentration of 1 μ g/ml for 20 hr. To release cells from G2/M arrest, cells were washed with PBS and incubated in fresh medium containing 10% of dialyzed FBS. The synchronized cells were fixed in 70% ethanol and stained with propidium iodide for cell cycle analysis using fluorescence-activated cell sorter (FACS) with CellQuest software.

Plasmids

Mutation of the thymidine binding site of hTK1 (converting RKPF at 131/134 position to AKPA) was introduced to pCDNA3.1-hTK1 with a Quick-Change Site-directed Mutagenesis kit (Stratagene) using specific mutated primers to generate the pCDNA3.1-hTK1(RFN) mutant, which was further subcloned to construct GST-hTK1(RFN). pHA-Cdh1 plasmids were provided by Kristian Helin (European Institute of Oncology, Milan, Italy). Mouse E1 cells, UbcH10 expression plasmids and pFASTBacHis-Cdh1 plasmids were provided by Tim Hunt (ICRF Clare Hall Laboratory, South Mimms, UK), Joan V. Ruderman (Harvard Medical School, Boston, MA), and Jan-Michael Peters (Research Institute of Molecular Biology, Vienna, Austria), respectively.

In vitro degradation, polyubiquitylation and binding assays

In vitro degradation was performed as described previously (32). G2/M arrested HeLa cells were released by washing four times in PBS and resuspended in fresh medium for 2hr to enter early G1 phase, and harvested for extract preparation. One μ l of *in vitro*-translated ³⁵S-methionine-labeled hTK1 and GST- Δ N90-Cyclin B was added to 10 μ l of early G1 extracts supplemented with an energy-regeneration system (100 μ g/ml cycloheximide, 1.5 mM ATP, 40 mM phosphocreatine, creatine kinase 80 μ g/ml, ubiquitin 1.25 mg/ml) and incubated at 30°C. Sample aliquots (2 μ l) were taken at the indicated time points for SDS-PAGE and autoradiography. *In vitro* polyubiquitylation assays were performed as described previously (33-35). The activated APC/C beads were isolated from cell extracts by using anti-Cdc27 antibody beads, and His-Cdh1 protein was prepared using a baculovirus expression system as described previously. APC/C and His-Cdh1 were added to a reaction mixture of 200 μ g/ml mouse E1, 100 μ g/ml UbcH10, 1.25 mg/ml ubiquitin, 100 ng of GST-hTK1 or GST-hTK(RFN), and energy-regeneration system in a total volume of 5 μ l. All reaction mixtures were incubated at 37°C for 1 hr, after which reaction mixtures were analyzed by SDS-PAGE on 5-15% gradient gels and Western blotting with anti-GST antibody. *In vitro* binding assay of GST-hTK1 with His-Cdh1 beads was performed according to previous methods (22).

Metabolic labeling and half-life determination of hTK1

For metabolic labeling, NIH-3T3 fibroblasts stably expressing hTK1 were washed twice with PBS and incubated in 5 ml of methionine-free DMEM for 1 hr. Cells were then incubated in 2 ml of fresh methionine-free DMEM containing 10% dialyzed FBS and ³⁵S-methionine (500 μ Ci,

Amersham Pharmacia) for 30 min, after which the medium was removed and replenished with complete DMEM medium without FBS. Cells were then harvested at the time indicated for immunoprecipitation using hTK1 antibody, followed by SDS-PAGE separation and autoradiography.

Whole-cell dNTP pool extraction and pool size determination

5×10^6 cells were washed twice with 10 ml of cold PBS and extracted with 1 ml of ice-cold 60% methanol at -20°C for 1 hr, followed by centrifugation for 15 min at $14,000 \times g$. The supernatant was transferred to a fresh tube and dried under vacuum. The residue was dissolved in sterile water and store at -20°C for later analysis. Analysis of the dNTP pool size in each extract was based on the method of Sherman and Fyfe (36).

RESULTS

To investigate thymidine-regulated stability of TK1, we first compared the effect of thymidine incubation on the half-life of hTK1 expressed in NIH-3T3 fibroblasts under serum-deprived conditions. To this end, NIH-3T3 fibroblasts stably expressing TK1 were pulse-labeled with [³⁵S]-methionine for 1 hr, after which cells were chased in serum-free complete medium in the presence or absence of thymidine and were harvested for hTK1 immunoprecipitation. The half-life of [³⁵S] pre-labeled hTK was found to be less than 12 hr during serum starvation. Inclusion of thymidine in serum-deprived medium prolonged the half-life to longer than 24 hr (Fig. 1). Thus, elevation of thymidine concentration in culture medium stabilizes cellular hTK1 during growth arrest.

To directly address the question of whether hTK1 degradation is influenced by thymidine binding, we performed *in vitro* degradation assays using hTK1 as the substrate with G1 extracts of HeLa cells. GST-ΔN-cyclin B protein devoid of the D-box was included in the degradation reaction to indicate the specificity of protein degradation. As shown in Fig. 2A, both the wild-type and the thymidine-binding-defective-mutant of hTK1 (RFN) were rapidly destroyed in G1 extracts. RFN mutant is enzymatic inactive because the thymidine binding site of this mutant is disrupted by the replacement of arginine-131 and phenylalanine-134 with alanine residues (30, 37). In the degradation reaction, APC/C resistant GST-ΔN-cyclinB protein remained unaffected, indicating the assay is specific to the protein containing the degradation signal. Preincubation of wild-type hTK1 with thymidine inhibited its *in vitro* degradation, while deoxycytidine had no effect. In contrast, RFN mutant protein was degraded in this *in vitro* degradation assay regardless of thymidine preincubation. To verify whether inhibition of hTK1 degradation by thymidine *in vitro* was due to interference with APC/C-mediated ubiquitination of hTK1, we then performed *in vitro* ubiquitination of hTK1 using a reconstituted E1/E2/APC/C-Cdh1 system. GST-hTK1 was polyubiquitinated in a reaction mixture containing E1/E2/APC/C in the presence of Cdh1, but this was not dependent on the presence of Cdc20. Consistently, GST-hTK1 carrying a KEN box mutation could not be polyubiquitinated by APC/C-Cdh1 (Figure 2B, left panel). Inclusion of thymidine in the E1/E2/APC/C-Cdh1 mixture prohibited the polyubiquitination of wild-type hTK1 but not that of RFN mutant *in vitro* (Fig. 2B, right panel). Similar to what had been observed in the *in vitro* degradation assay, deoxycytidine had no effect on *in vitro* ubiquitination of hTK1.

Since hTK1 is targeted by APC/C/Cdh1 for ubiquitination through its interaction with Cdh1, we then examined the effect of thymidine on the binding of Cdh1 with hTK1. To this end, we purified GST-hTK1 and GST-RFN to perform a pull-down assay with His-Cdh1 protein using Ni-NTA beads. As shown in Fig. 3A, the presence of thymidine inhibited the pull-down of GST-hTK1 by His-Cdh1. Consistent with the above results, GST-RFN mutant protein was still capable of binding with Cdh1 regardless of thymidine incubation. We next tested the concentration of thymidine required to inhibit hTK1 binding to Cdh1. It appeared that a thymidine concentration of 10 μM was sufficient to completely block the interaction between hTK1 and Cdh1 (Fig. 3B). These results suggested that thymidine binding prevents hTK1 from interacting with Cdh1, thereby prohibiting its *in vitro* ubiquitination-dependent degradation via the APC/C/Cdh1 pathway.

To test the *in vivo* effect of thymidine on Cdh1-sensitive control of hTK1 expression, LM-TK⁻ cells were transfected with expression vector of wild-type or RFN mutant of hTK1 together with pHA-Cdh1 or the control vector. Because Cdh1 is a limiting factor for APC/C-mediated proteasomal control of hTK1 expression, the overexpression of Cdh1, as expected, decreased ectopic expression levels of both wild-type and mutant TK1 in LM-TK⁻ cells. In this experiment, all cells were co-transfected with pEGFP as an internal control to monitor transfection efficiency in different transfected cells. Addition of thymidine or LLnL, a proteasome inhibitor, in the culture medium increased expression of wild-type hTK1 in cells

ectopically expressing Cdh1. In contrast, thymidine treatment had little effect on the expressed level of hTK1(RFN) mutant in cells co-expressing Cdh1, while LLnL treatment was still able to restore RFN mutant levels (Fig. 4A). Moreover, thymidine treatment markedly decreased *in vivo* polyubiquitination of hTK1 in these transfected cells (Fig. 4B). These results further reinforced the notion that thymidine binding negatively modulates Cdh1-dependent ubiquitination of hTK1.

Next, we examined the dose effect of thymidine on hTK1 stabilization in HeLa and K562 cells, both of which contained an elevated level of endogenous TK1 protein. After nocodazole (Noc) treatment overnight to induce mitotic arrest, TK1 was found to accumulate in mitosis-blocked HeLa cells. Upon removal of Noc to allow cells to transit the mitotic exit, TK1 expression was decreased due to APC/C-Cdh1-mediated proteolysis. Addition of 1 mM thymidine during Noc release slightly reduced hTK1 degradation, while 10 mM thymidine completely abolished TK1 degradation during mitotic exit. For K562 cells, a chronic myeloid leukemia cell line, mitotic arrest by Noc treatment also resulted in elevation of TK1 expression. Following release from mitotic block for 6hr, level of TK1 expression was clearly reduced due to its degradation in the early G1 phase. In contrast to HeLa cells, treatment of K562 cells with thymidine at a concentration of 100 μ M during mitotic release was sufficient to stabilize TK1 protein (Fig. 5). In both cell lines, thymidine treatment had no effect on downregulation of cyclin B, an APC/C-Cdh1 substrate, further indicating the specific effect of thymidine on protecting hTK1 protein. The difference in thymidine sensitivity could be due to differences in the efficiency of thymidine uptake or in the amount of enzymes involved in thymidine metabolism present in these two different cell types.

To evaluate the effect of TK1 stabilization by thymidine treatment in early G1 phase on nucleotide balance, we measured the levels of four dNTPs in K562 cells that had been released from mitotic block for 6 hr. The results showed that the presence of thymidine during release gave rise to a 7-fold increase of the dTTP pool accompanied by a 2-fold reduction and 3-4-fold increase of dCTP and dGTP level, respectively (Fig. 6A). Thus, nucleotide imbalance was induced in the early G1 phase by treating K562 cells with thymidine during Noc release. By flow cytometric analysis, thymidine treatment during mitotic release did not exert a significant effect on S phase entry during the first 6 hr of release. After Noc release for 24 hr, thymidine-treated cells were mainly arrested in the S phase. In contrast, cells released from mitotic block without thymidine treatment were mainly distributed in both G2/M and G1 phases. Addition of deoxycytidine to release medium containing thymidine restored the G2/M phase progression, implicating the dCTP depletion in thymidine-induced S phase arrest (Fig. 6B). Knockdown expression of TK1 by siRNA prevented thymidine-induced S phase retardation, suggesting the essential role of TK1 in mediating the thymidine effect (Fig. 7A, B). In conclusion, thymidine treatment removes mitotic control of TK1 degradation in K562 cells, resulting in over-production of dTTP in the early G1 phase, which in turn induces nucleotide imbalance and retards S phase progression.

DISCUSSION

In this study, we provide evidence that thymidine plays a direct role in negatively regulating the proteolytic control of hTK1 during mitosis. Our *in vitro* experiments demonstrate that thymidine binding of hTK1 interferes with its interaction with Cdh1, protecting hTK1 from APC/C-Cdh1-mediated ubiquitination in mitotic exit. Addition of thymidine to the culture medium not only increased the *in vivo* half-life of hTK1 in growth arrest cells, but also abolished *in vivo* Cdh1-responsive ubiquitination of hTK1. Finally, we show that hTK1 stabilization by thymidine in the early G1 phase induces nucleotide imbalance, resulting in S phase retardation. Depletion of hTK1 expression by RNA interference abolishes thymidine-induced S phase accumulation. In conclusion, the supply of thymidine is an important factor in controlling mitotic degradation of hTK1 by affecting its susceptibility to APC/C-Cdh1. These findings elucidate a molecular mechanism by which thymidine-inhibited degradation of hTK1 elicits a feed-forward regulation elevating dTTP formation.

Our *in vitro* binding assay indicated that thymidine at a concentration of 10 μM was sufficient to completely abolish interaction between Cdh1 and hTK1. Given that the K_m of hTK1 for thymidine ranges from 0.5 to 14 μM depending on its oligomeric form (19, 38), the inhibitory effect of thymidine on APC/C-Cdh1-mediated degradation is physiologically significant. How does thymidine affect degradation targeting of hTK1 by APC/C-Cdh1? Our previous study has shown that the KEN box located in the C-terminal region of hTK1 is a necessary signal element for Cdh1 recognition in mitotic exit. Since thymidine had no effect on ubiquitination and degradation of either an hTK1(RFN) mutant defective in thymidine binding or of cyclin B, another APC/C-Cdh1 substrate, we ruled out the possibility that the E3 function of APC/C-Cdh1 was affected by thymidine. Our pull-down assays showed that thymidine binding did impair the interaction of Cdh1 with the wild-type but not the thymidine-binding-defective mutant hTK1. Therefore, it is clear that the inhibitory effect of thymidine on hTK1 degradation is through interference with the interaction between TK1 and Cdh1 molecules. Since the KEN box is located in the C-terminal region of hTK1, we propose that binding of thymidine to hTK1 protein changed the conformation of its C-terminal region, hiding the KEN box from Cdh1 recognition.

Crystallographic study of C-terminus-deleted hTK1 in complex with dTTP has shown that active hTK1 has a tetrameric structure (39). By comparing with structure of full-length *Ureaplasma urealyticum* TK (Uu-TK), which has similar enzymatic kinetic properties to hTK1, it has been proposed that the KEN box motif present in the C-terminus of hTK1 is probably located in an exposed subunit-contacting region (39). However, it should be mentioned that a KEN box is not present in the C-terminal region of Uu-TK and the sequence homology between hTK1 and Uu-TK in this region is very low. Based on our current results, it is more likely that the KEN box motif is probably not in the exposed area when TK1 is in the active conformation for thymidine binding. Co-crystal structure analysis of full-length hTK1 in complex with thymidine is needed to validate this point. To the best of our knowledge, this is the first evidence showing that the potential for an enzymatic protein to be marked for APC/C-Cdh1 targeting depends not only on the presence of degradation signal motif but also its substrate binding status. It is likely that the functional engagement of a protein binding to its specific substrate provides a mechanism by which an induced conformation change is able to hide the degradation signal, thus preventing specific proteolytic targeting.

It has been established that addition of excess thymidine to the mammalian cell culture medium significantly increases the gene mutation rate while cell growth is also inhibited due to the occurrence of replication stress in the S phase (26, 40). This is because a high level of dTTP allosterically stimulates the rGDP substrate specificity of ribonucleotide reductase (RNR) and inhibits reduction of CDP, thus resulting in dNTP imbalance due to increase of the dGTP pool and depletion of dCTP in the cells (26, 27). The induced dNTP imbalance in turn not only causes DNA replication stress but also has a mutagenic effect by promoting replication error (41).

During cell cycle progression, mitotic degradation of TK1 and TMPK provides a mechanism by which the dTTP pool is minimized in the early G1 phase of the next cell cycle to avoid dCTP depletion and an elevated level of dGTP. In the late G1 phase, expression levels of TK1 and TMPK rise to allow maximal dTTP production for correct DNA synthesis in the S phase.

It has been shown that overexpression of thymidylate synthase confers oncogenic potential to NIH3T3 fibroblasts (42), indicating that the increase of *de novo* synthesis of dTTP may drive cells toward malignant transformation. Although increased gene mutation is a driving force in tumorigenesis, we were unable to find any cancer-associated mutation disrupting KEN and D box of TK1 and TMPK, respectively, in database searches for sequence variation in TK1 and TMPK. However, a single V106M mutation, which affects the oligomerization status of TK1, was found in SV-40 transformed, HeLa cells and tumor tissues (43, 44). Perhaps, dTTP deregulation is unnecessarily dependent on KEN box mutation of hTK1. Our present findings imply that the amount of thymidine available could be one important factor contributing to control of mitotic degradation of hTK1. According to the results from this study, we suggest that thymidine-mediated inhibition of hTK1 degradation confers a feed-forward regulation to increase the dTTP pool in cells that express high levels of hTK1, perturbing the balance of the dNTP pool and upsetting the optimal genomic stability.

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Figure Legend.

Figure 1. Thymidine treatment prolongs *in vivo* half-life of hTK1.

Proliferating NIH3T3-hTK1 cells were labeled with ^{35}S -methionine followed by a chase period in serum-free medium in the presence or absence of thymidine (100 μM). Cells were harvested for hTK1 antibody immunoprecipitation and the immunoprecipitated samples were separated in 12% PAGE and autoradiography. -: in the absence of thymidine; +dThd: in the presence of thymidine; IgG: immunoprecipitation of the pulse-labeled cell lysates with normal rabbit IgG.

Figure 2. Thymidine binding inhibits *in vitro* APC/C-Cdh1-mediated degradation and ubiquitination of hTK1

(A) *In vitro* degradation of wild-type and thymidine-binding-defective hTK1 was assayed for 0 and 90 min with early G1 extracts as described in Materials and Methods. *In vitro* translated [^{35}S]-methionine-labeled hTK1 was preincubated with 750 μM thymidine (dThd) or deoxycytidine (dCyd) as indicated and added into extracts in assays. ΔN90 cyclin B1 was added to extracts to monitor sample loading and specificity of degradation in the assay. RFN represents a mutant hTK1 defective in thymidine binding site. (B) Left panel: *In vitro* polyubiquitination of GST-hTK1 or GST-hTK(KEN) as the substrate in a reaction mixture containing E1, E2, APC/C, Cdc20 or Cdh1 as indicated. Right panel: *In vitro* polyubiquitination of GST-hTK1 or GST-hTK1(RFN) using E1/E2/APC/C/Cdh1 was performed in the presence of thymidine or deoxycytidine as indicated.

Figure 3. Thymidine binding to hTK1 prohibits its direct interaction with Cdh1

(A) Purified GST-hTK1 and GST-hTK1(RNF) were subjected to *in vitro* binding assay using Ni-NTA beads bound with His-Cdh1 with or without 750 μM dThd and dCyd as indicated. The pulled-down samples and 30% of total input for each binding reaction were analyzed by Western blotting. (B) *In vitro* binding assays of purified GST-hTK1 with Cdh1 were performed in the presence of dThd at different concentration as indicated.

Figure 4. *In vivo* thymidine treatment abolishes Cdh1/ proteasome-sensitive expression and ubiquitination of hTK1.

(A) LM-TK⁻ cells were transfected with either wild-type or RFN mutant of pCDNA3.1-hTK1 together with vector (mock) or pHA-Cdh1 as indicated and pEGFP. Empty vector was added to the lipofectamine/DNA mixture to ensure an equal amount of DNA for each transfection. Cells were treated with thymidine (dThd, 750 μM) or LLnL for 6 hr before harvesting. After transfection for 24 hr, cells were extracted for Western blotting analysis. Expression of GFP indicated the transfection efficiency among different samples. (B) LM-TK⁻ cells were transfected with pCDNA3.1-hTK1 and pHA-Cdh1 together with pMyc-Ub. After transfection, cells were treated with LLnL in the presence or absence of thymidine for 24 hr before harvesting for hTK1 antibody and control IgG immunoprecipitation. The immunocomplexes were separated by SDS-PAGE and analyzed by Western blot using Myc and hTK1 antibodies. Total cell lysates were also subjected to Western blot analysis.

Figure 5. Sensitivity of TK1 stabilization to thymidine in HeLa and K562 cells.

HeLa (A) and K562 (B) cells were arrested by nocodazole (1 $\mu\text{g/ml}$) treatment for 20 hr, followed by PBS wash and replenishment with medium containing 10% dialyzed FBS to release cells from the G2/M arrest for 6 hr. The mitotic release medium contained different concentrations of dThd as indicated. Cells were harvested for SDS-PAGE and Western blotting.

Figure 6. Nucleotide imbalance and S phase retardation induced by thymidine treatment.

K562 cells were mitotic arrested and released as described in the legend to Figure 4.

(A) Cells that were proliferating, mitotic arrested, and released for 6 hr with and without

thymidine were harvested for determination of dATP, dCTP, dGTP and dTTP levels. Each data point represents the mean \pm S.D. of two separate experiments in triplicate (B) Mitotic arrested cells were released in the medium containing 10% dialyzed FBS without or with dThd (100 μ M) or dThd/dCyd (both at 100 μ M). The cell cycle profiles of mitotic arrested cells released at different time points were analyzed by flow cytometry and the results were expressed as percentage of cells in G1, S, and G2/M phases.

Figure 7. TK1 mediates thymidine-induced S phase retardation

K562 cells (5×10^5) were transfected with 1 nmole of control or hTK1 siRNA by electroporation in a final volume of 0.4 ml. (A) After transfection for 48 hr, a portion of cells were analyzed by Western blot using antibodies against hTK1 and α -tubulin. (B) The siRNA-transfected K562 cells were arrested by nocodazole (1 μ g/ml) treatment for 20 hr. Mitotic arrested cells were washed and refreshed with medium containing 10% dialyzed FBS without or with dThd (100 μ M). The cell cycle profiles of mitotic arrested cells released at different time points were analyzed by flow cytometry and the results were expressed as percentage of cells in G1, S, and G2/M phases.

Figure 1.

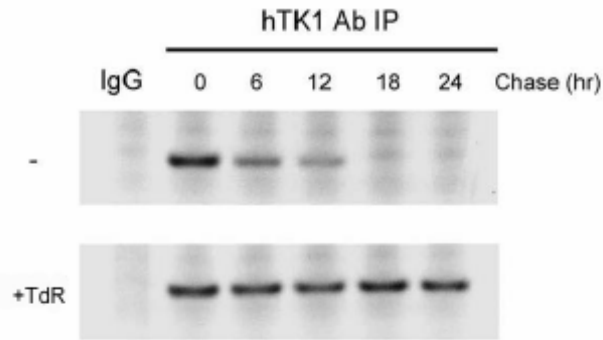
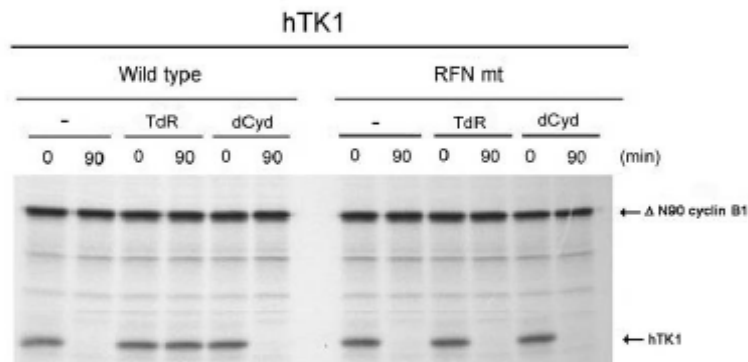


Figure 2.
A.



B.

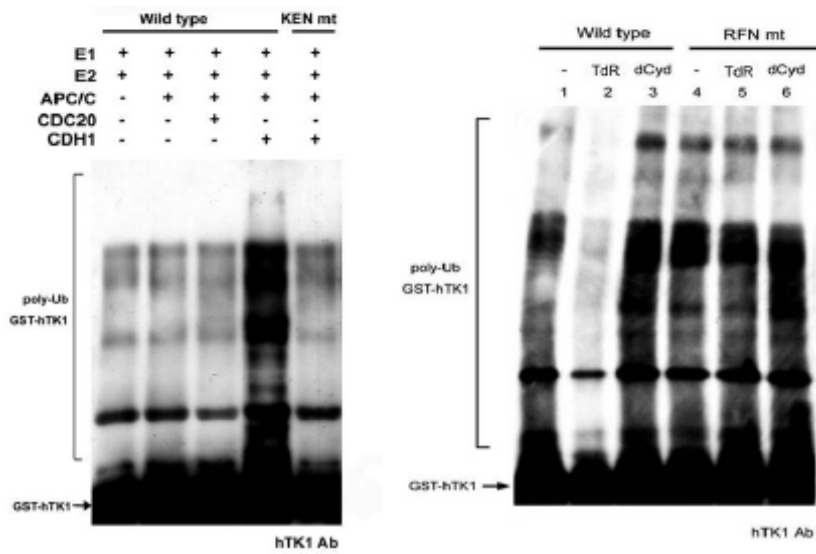
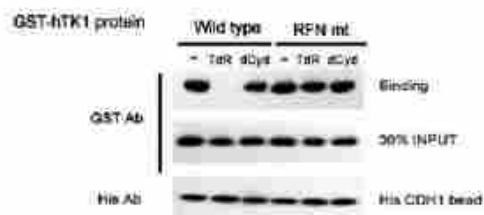


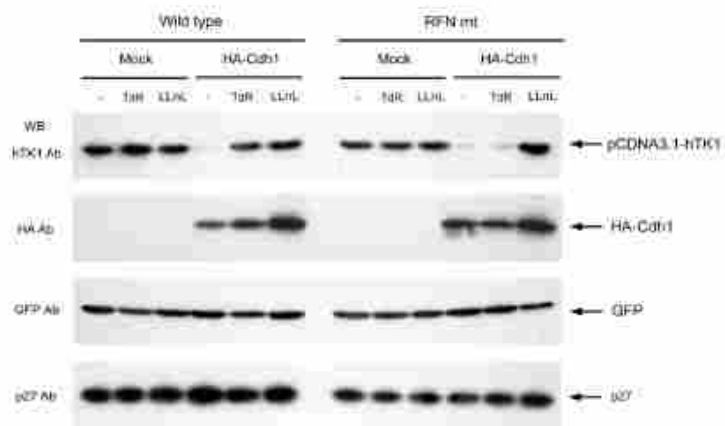
Figure 3.
A.



B.



Figure 4.
A.



B.

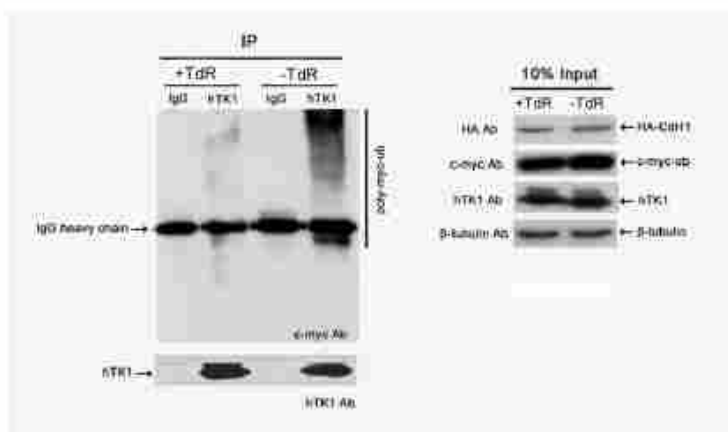
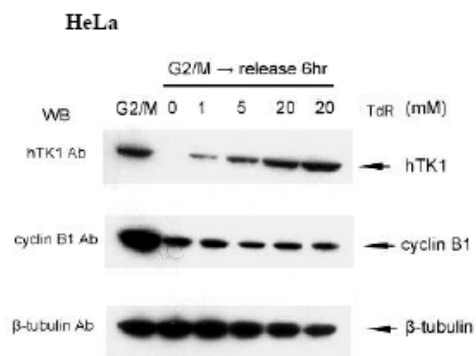


Figure 5.
A.



B.

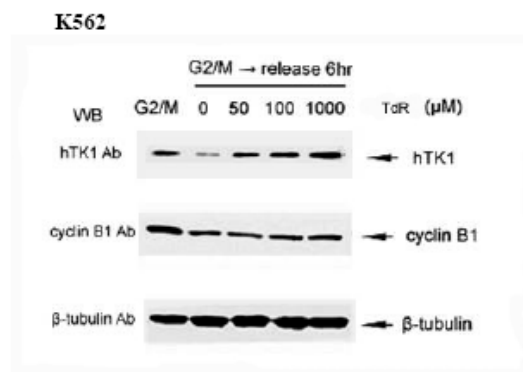
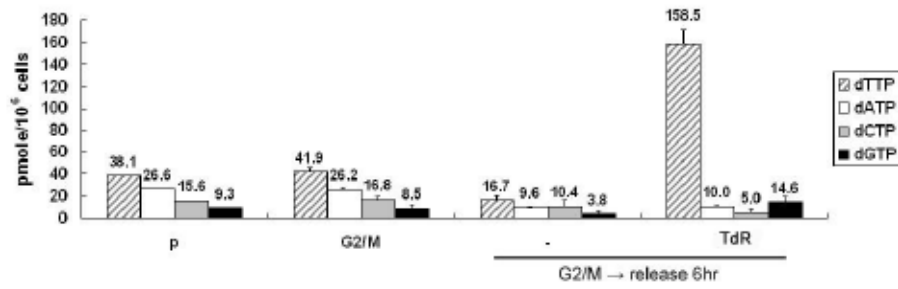


Figure 6.
A.



B.

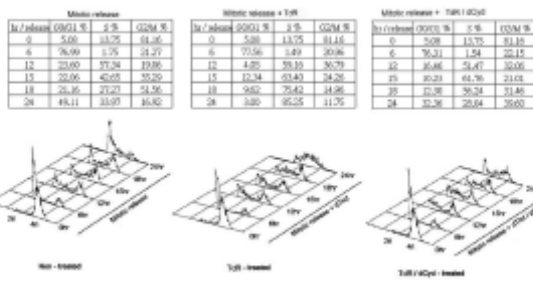


Figure 7.

A.



B.

