**Supplementary information for**

# Cytotoxicity of trifluridine correlates with the thymidine kinase 1 expression level

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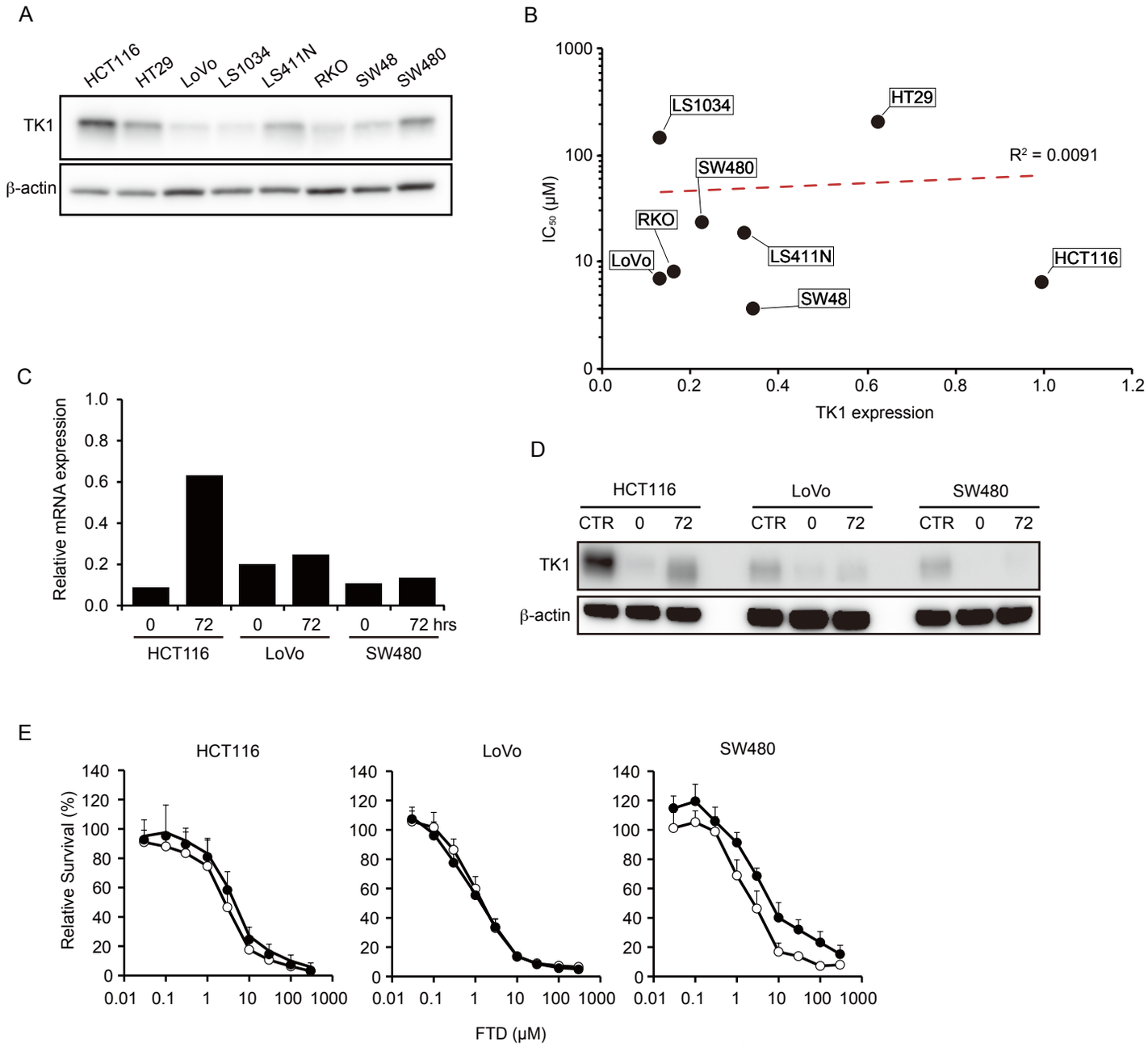
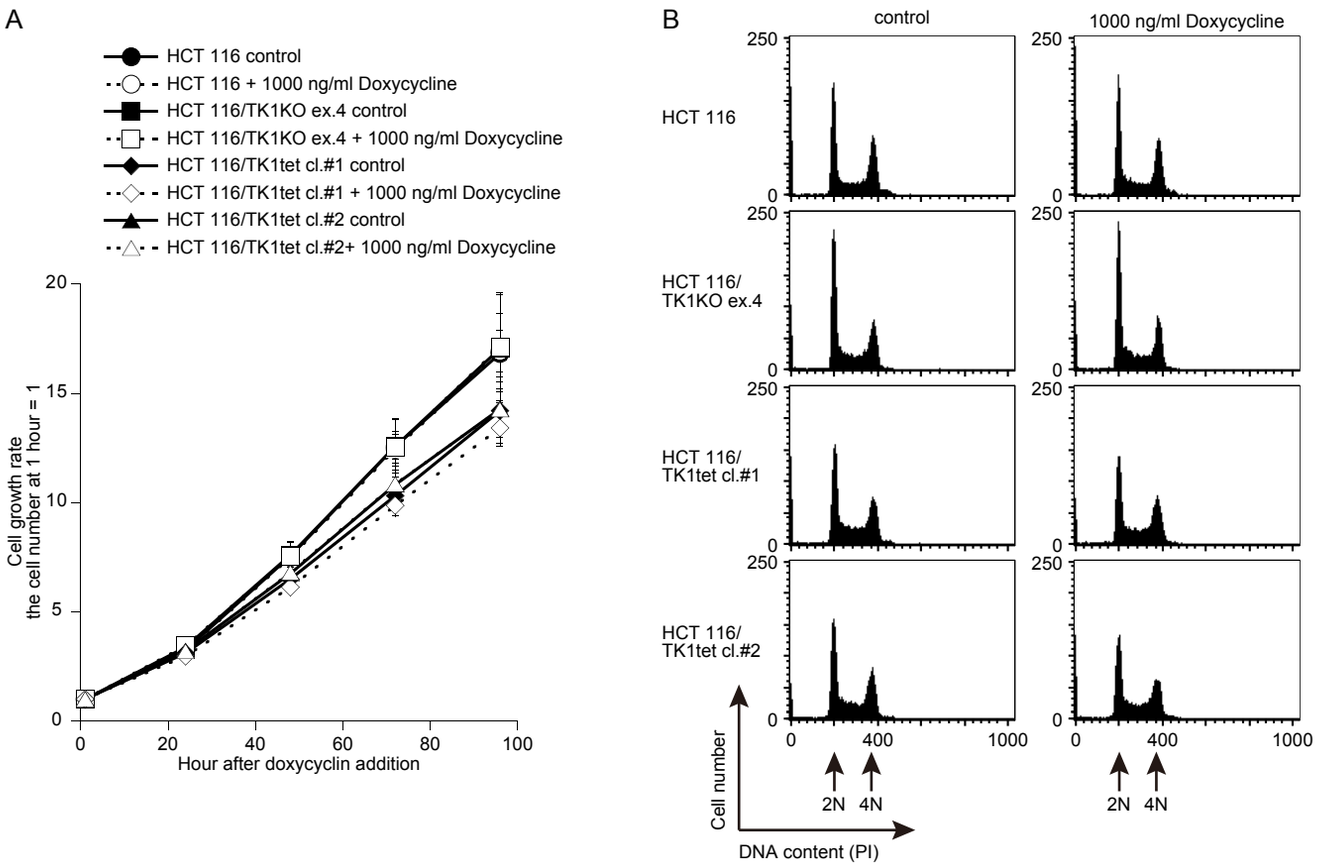


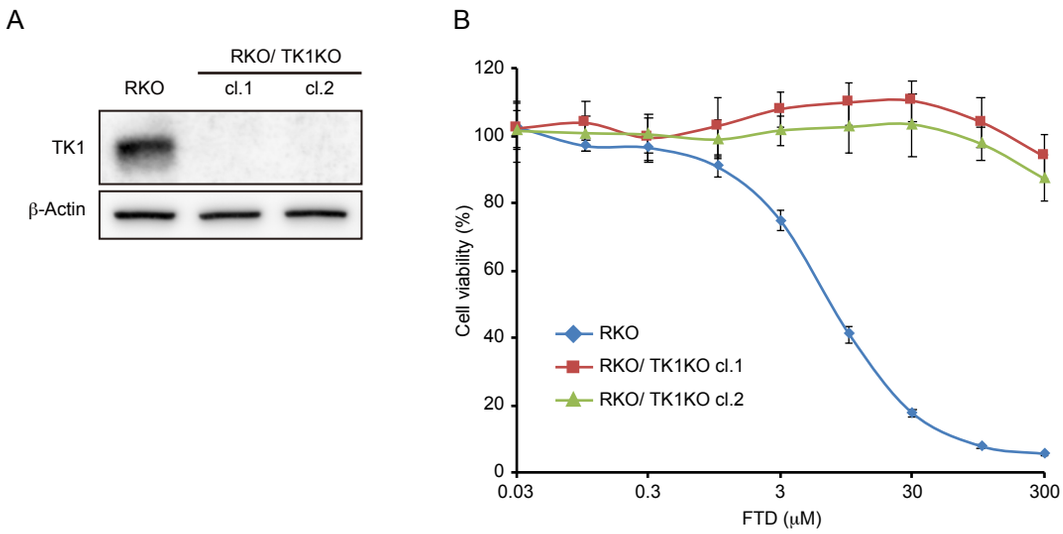

**Supplementary figure 1. FTD cytotoxicity in TK1-knock-down colorectal cancer cells.**

(**A**,**B**) Colorectal cancer cell lines were immunoblotted with the indicated antibody. Correlation analysis between FTD cytotoxicity and the TK1 expression level in A. (**C**,**D**) Cells were transfected with *TK1*-targeting siRNA. At 48 and 120 h after transfection, which are indicated as 0 and 72 h, respectively, cells were harvested and the mRNA (**C**) and protein (**D**) levels of TK1 were measured. (**E**) Influence of *TK1* knock-down on cytotoxicity of FTD. At 48 h after transfection with control (open circles) or *TK1*-targeting (closed circles) siRNA, cells were treated with FTD for 72 h and their viability was determined. All data are expressed as means ± s.d. (**F**) Summary of the IC50 values in each cell line in (**E**).



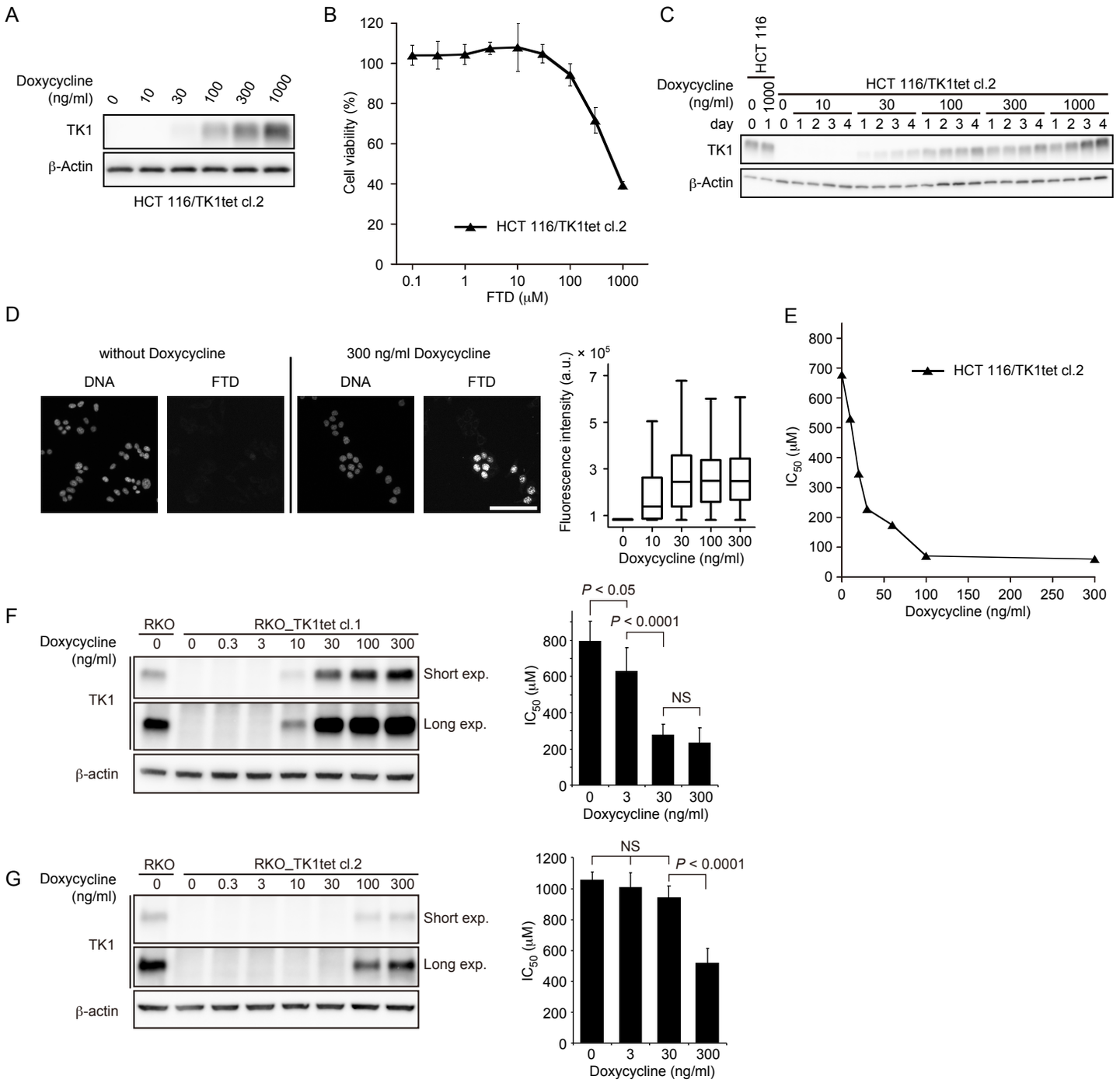

**Supplementary figure 2. Cell growth rates and cell cycle distributions.**

(**A**) Growth curve of each cell line treated with 0 or 1000 ng/ml doxycycline. Cell viability was evaluated at 1, 24, 48, 72 and 96 h after plating, and the cell growth rate relative to that at 1 h was calculated. (**B**) Cell cycle distribution. Cells were treated with 0 or 1000 ng/ml doxycycline for 1 day, fixed and stained with propidium iodide. The DNA content was quantitated using a FACSCalibur instrument.




**Supplementary figure 3. FTD cytotoxicity in *TK1*-knock-out RKO cells.**

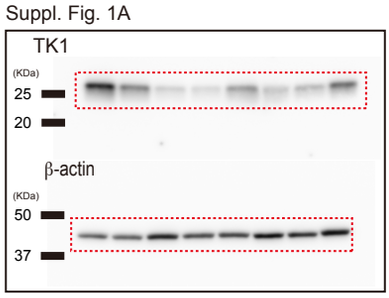
(**A**) Western blot analysis of TK1 proteinin RKO parental and *TK1*-knock-out cells. (**B**) Cell viability assay. Cells were treated with nine points of dilution series of FTD for 3 days and then their viability was determined. The viability of cells not treated with FTD was defined as 100%. Data are means ± s.d. of three independent experiments.




**Supplementary figure 4. Response of HCT 116/TK1tet cl. 2 and RKO/TK1tet cells to doxycycline and FTD.**

(**A**) Western blot analysis of TK1. HCT 116/TK1tet cl. 2 cells were treated with the indicated concentration of doxycycline for 1 day and then the level of TK1 was analysed. (B) Sensitivity of HCT 116/TK1tet cl. #2 cells to FTD. Cells were treated with the indicated concentration of FTD for 3 days and then their viability was evaluated. Relative cell viability was calculated by setting that of cells not treated with FTD to 100%. (**C**) Western blot analysis of TK1. Cells were treated with the indicated concentration of doxycycline for the indicated number of days. (**D**) Immunofluorescence images of FTD-incorporated cells (left). Cells were treated with the indicated concentration of doxycycline for 1 day and then with 6.4 μM FTD for 1 h, fixed and immunostained with an anti-BrdU antibody. Fluorescence intensities of FTD incorporated into genomic DNA were quantified (right). Scale bar, 100 μm. (**E**) FTD sensitivity of HCT 116/TK1tet cl. 2 cells. Cells were treated with the indicated concentration of doxycycline for 1 day and then with nine points of dilution series of FTD for 3 days. The IC50 values in HCT 116/TK1tet cl. #2 cells treated with the indicated concentrations of doxycycline were calculated and plotted. (**F, G**) Western blot analysis of TK1(left) and FTD sensitivity (right) . RKO/TK1tet cells were treated with the indicated concentration of doxycycline for 1 day and then the level of TK1 and IC50 values were analysed. Data are means± s.d. from six independent experiments. Two-tailed t-test, NS, not significant.

Suppl. Fig. 1A




Suppl. Fig. 1B

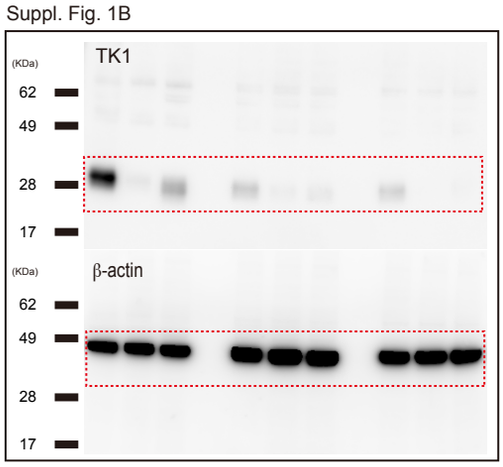
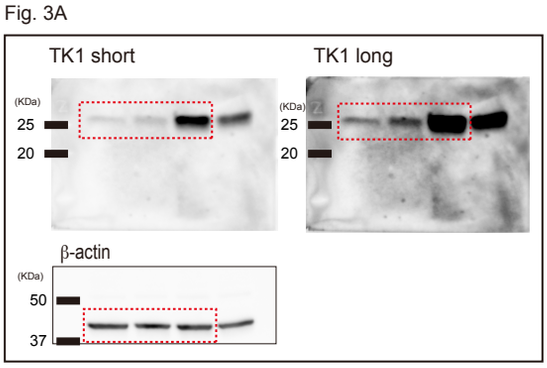



Fig. 3A




Suppl. Fig. 3A

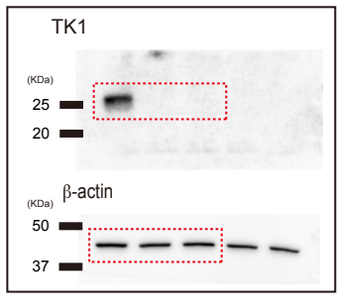



Fig. 1C

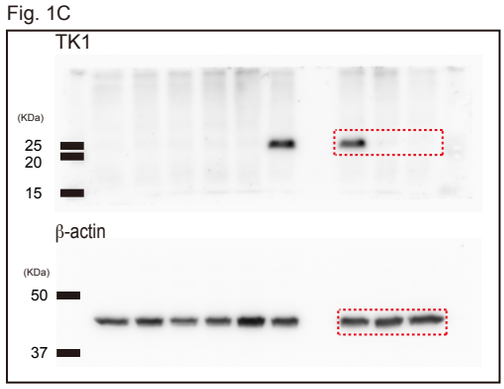
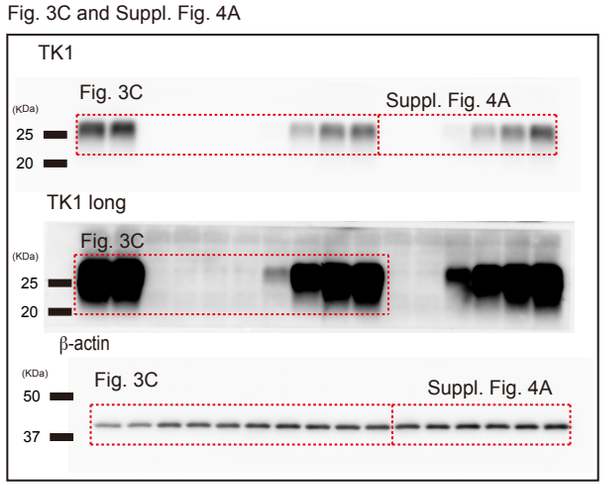
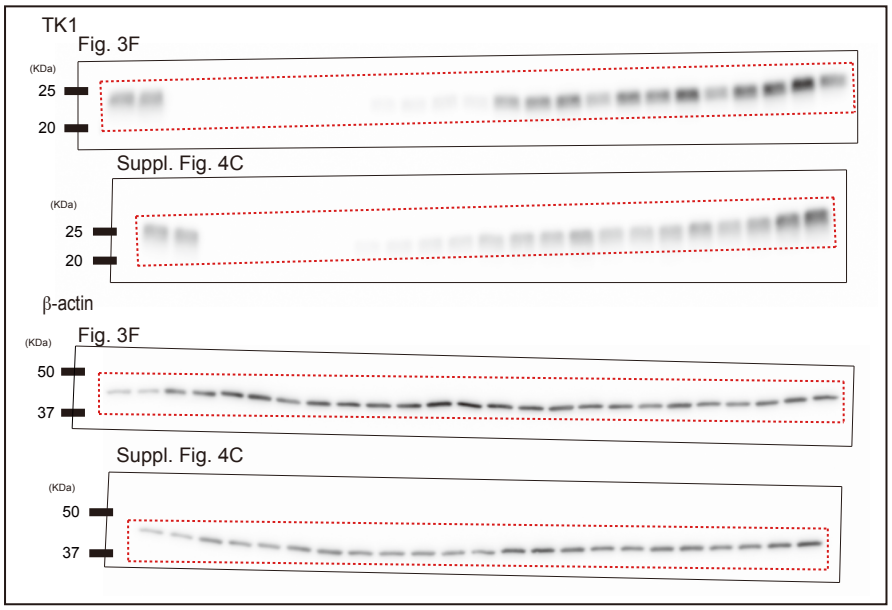



Fig. 3C and Suppl. Fig. 4A

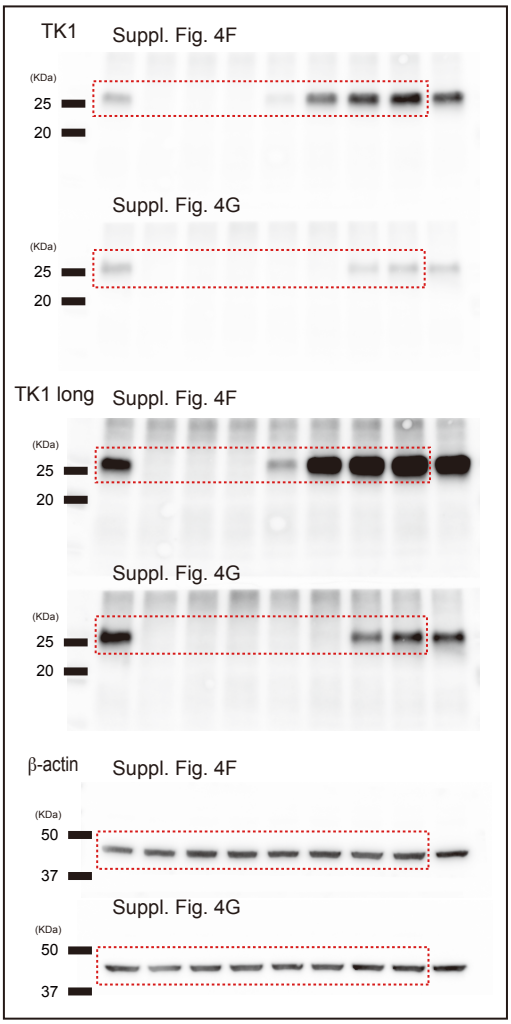



**Supplementary figure 5. Uncropped images of immunoblot.** Red boxes show cropped regions.

Fig. 3F and Suppl. Fig. 4C




Suppl. Fig. 4F,G




**Supplementary figure 5.** Continued

Supplemental Table 1. $IC_{50}$ value of FTD in each cell line

FTD $IC_{50}$ (Average ± SD, μM)

| | Doxycycline (ng/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Cell line | 0 | 10 | 20 | 30 | 60 | 100 | 300 |
| HCT 116 | 6.4 ± 1.6 | - | - | - | - | - | 6.0 ± 1.3 |
| HCT 116/TK1KO ex.4 | 710 ± 47 | - | - | - | - | - | 670 ± 81 |
| HCT 116/TK1tet cl.1 | 710 ± 50 | 470 ± 30 | 390 ± 36 | 240 ± 100 | 99 ± 14 | 41 ± 4.6 | 18 ± 4.7 |
| HCT 116/TK1tet cl.2 | 680 ± 150 | 530 ± 64 | 350 ± 42 | 230 ± 53 | 170 ± 40 | 71 ± 10 | 60 ± 18 |

Supplemental Table 2. Incorporation of FTD into DNA

| Cell line | HCT 116 | | | HCT 116/TK1KO ex.4 | | | HCT 116/TK1tet cl.9 | | | | | | HCT 116/TK1tet cl.16 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FTD (μM) | 0 | 6.4 | | 0 | 6.4 | | 0 | 6.4 | | | | | 0 | 6.4 | | | | |
| Dox (ng/ml) | 0 | 0 | 300 | 0 | 0 | 300 | 0 | 0 | 10 | 30 | 100 | 300 | 0 | 0 | 10 | 30 | 100 | 300 |
| all data | 4262 | 5131 | 5817 | 3245 | 3995 | 3817 | 2611 | 2542 | 2414 | 2295 | 2431 | 2425 | 3432 | 3553 | 3515 | 3462 | 3346 | 2285 |
| usable data | 4258 | 2746 | 3239 | 3242 | 0 | 0 | 2604 | 4 | 32 | 197 | 812 | 1065 | 3411 | 8 | 81 | 474 | 1056 | 863 |
| lower quartile | 44769 | 171743 | 179664 | 42188 | - | - | 44013 | 85325 | 93386 | 124525 | 163856 | 206428 | 43176 | 82053 | 87248 | 138799 | 159392 | 168398 |
| median | 54079 | 235900 | 244067 | 51660 | - | - | 52835 | 85490 | 111675 | 210197 | 280886 | 303336 | 50885 | 83127 | 139099 | 245179 | 249001 | 247708 |
| upper quartile | 62630 | 293967 | 299302 | 60686 | - | - | 61065 | 85600 | 164557 | 317609 | 382829 | 391801 | 58651 | 83930 | 261412 | 357046 | 337297 | 344684 |
| IQR | 17861 | 122223 | 119638 | 18498 | - | - | 17051 | 275 | 71171 | 193084 | 218973 | 185373 | 15476 | 1877 | 174163 | 218247 | 177905 | 176286 |
| minimum | 19059 | 89408 | 89392 | 19775 | - | - | 20609 | 85075 | 86160 | 84814 | 84910 | 84867 | 20329 | 81493 | 81477 | 81431 | 81533 | 81628 |
| maximum | 89356 | 474389 | 472941 | 87281 | - | - | 84808 | 85682 | 243982 | 571356 | 708414 | 639776 | 81404 | 85250 | 504021 | 677535 | 600956 | 607390 |

Supplemental Table 3. Primer, gRNA and siRNA sequences

| Primer | Sequence (5'-3') | Application |
|---|---|---|
| TK1_KO_exon1_sense | CACCGAATGCAGCTCATTGCGCCTC | gRNA for pX330 |
| TK1_KO_exon1_antisense | AAACGAGGCGCAATGAGCTGCATTC | |
| TK1_KO_exon4_sense | CACCGGCTGTCATAGGCATCGACGA | |
| TK1_KO_exon4_antisense | AAACTCGTCGATGCCTATGACAGCC | |
| TK1_exon1_L-arm_F | GCAGCCCGGGGGATCCCTGGCAGGGTCTACGGATATTATTAGC | PCR to construct donor vectors |
| TK1_exon1_L-arm_R | TATACGAACGGTAGGAAGTTCACGAACCCGAGTACTCTCCAA | |
| TK1_exon1_R-arm_F | TATACGAACGGTAGGAGCTGCATTAACCTGCCCACTGT | |
| TK1_exon1_R-arm_R | CGCGGTGGCGGCCGCCCACGGCTTCAGACTCCTTGGTTT | |
| TK1_exon4_L-arm_F | GCAGCCCGGGGGATCCCAGCTCCTGAACAGTGGAAGAGTT | |
| TK1_exon4_L-arm_R | TATACGAACGGTAGGATGCCTATGACAGCCACGCCCAGG | |
| TK1_exon4_R-arm_F | TATACGAACGGTAGGAGTTTGTAAGTTGGCTTGTCTTGGCA | |
| TK1_exon4_R-arm_R | CGCGGTGGCGGCCGCTGTGCGCTGCTATGACTGGCTAATTTCT | |
| TK1_F | CCCTCGTAAAGAATTCACCATGAGCTGCATTAACCTGCCC | PCR of TK1 for insertion into pTetOne |
| TK1_R | GCAGAGATCTGGATCCTCAGTTGGCAGGGCTGCATTG | |
| TK1_F | GGCAGTTTTTCCCTGACATC | Quantitative RT-PCR |
| TK1_R | CCTCGACCTCCTTCTCTGTG | |
| AFMID_F | ACTGGGAGCAGAGGAAGCCTTGA | |
| AFMID_R | GACATGCAGCAGGCTCTTCCT-3 | |
| β-actin_F | CTGGCACCACACCTTCTACAATG | |
| β-actin_R | GGCGTACAGGGATAGCACAGC | |
| TK1_siRNA | CUCGCUACAGCAGCAGCUUdTdT | RNAi |

**Supplementary methods**

The experiments of supplemental figure 1 were performed as follows.

**Cytotoxicity test**

siRNA-transfected cells were seeded at densities of $1 \times 10^3$ (HCT116 cells) or $3 \times 10^3$ (LoVo and SW480 cells) cells/well in 96-well plates, incubated for 24 h, and then treated with FTD for 72 h. Cell numbers were determined using a simplified crystal violet staining method.

**Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)**

Cells were replated 48 hours after siRNA transfection. FTD was added at 24 hours after replating. mRNA expression was quantified at 0 and 72 hours after FTD addition. RT-qPCR was performed on a PRISM 7900HT sequence detection system using TaqMan Universal PCR Master Mix (Applied Biosystems) with the following amplifications conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The expression level of *TK1* was normalized to those of β-actin (*ACTB*). The primers and TaqMan probes were prepared using Assay-on-Demand gene-expression products (Applied Biosystems). Probe ID was Hs01062123_m1 for *TK1*. The human *ACTB* probe (VIC/MGB Probe; Applied Biosystems) was used as an endogenous control.