5. RESULTS

Part I. Expression of Pm-fortilin/TCTP

1. Expression of Pm-fortilin/TCTP transcripts in infected samples

To investigate whether the gene encoding Pm-fortilin/TCTP was actually inducible by viral infection, we performed RT-PCR analyses and semi-quantitative assay on total RNA extracts from normal and WSSV-injected shrimp using specific primers designed from the Pm-fortilin/TCTP sequences (Bangrak, 2003). RT-PCR of the ß-actin gene was used as an internal control in the RT-PCR semi-quantitative analysis and to insure that the RT-PCR for each sample contained the same amount of total RNA and that the RNA was intact. The purity of the isolated RNA was analyzed for DNA contamination by using RT-PCR in the presence (+RT) and absence (-RT) of reverse transcriptase. The Pm-fortilin/TCTP was observed in six individual normal shrimp (N), early WSSV-infected shrimp (I), and moribund WSSV-infected shrimp (D) as shown in Figure 21A. The amount of Pm-fortilin/TCTP was measured semi-quantitatively by comparing with the amount of the ß-actin in the same samples (Figure 21B). Interestingly, the level of Pm-fortilin/TCTP expression decreased when shrimp showed the mortality characteristic (D).

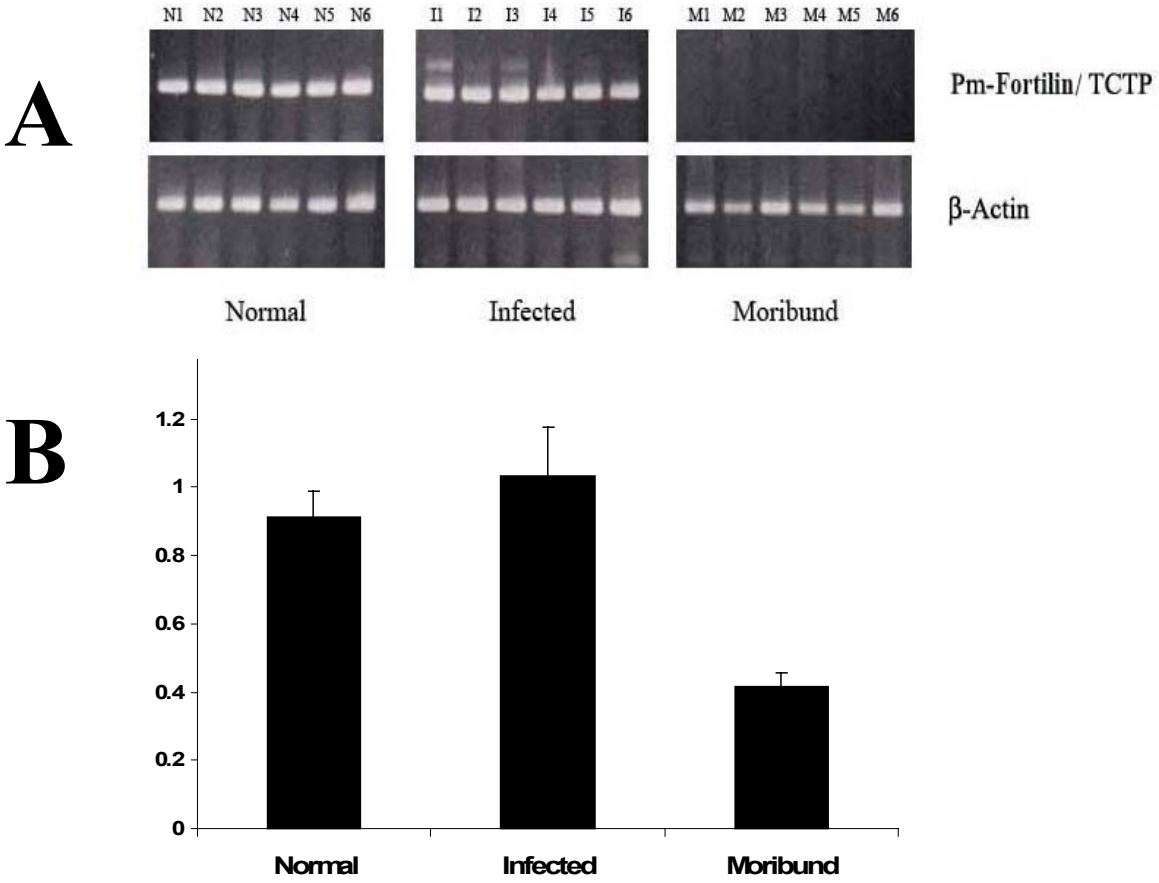


Figure 21. Expression of Pm-fortilin/TCTP specific mRNA in response to viral infection.

A, Expression of Pm-fortilin/TCTP specific mRNA in response to viral infection and expression of ß-actin as an internal control. Total RNA was isolated from haemocytes of 6 individuals of *Penaeus monodon* uninfected and infected with WSSV. RNA isolation, RT-PCR and the primer sequences are as described in Section 2. N1-N6: normal (uninfected samples), I1-I6: infected (24 h WSSV post-injection samples), D1-D6: moribund (WSSV-infected samples shows mortality fate). ***B***, The ratio of Pm-fortilin/TCTP to ß-actin was calculated from the image using Scion Image software. The data represent the average results obtained from six samples in each sample set.

2. Human-fortilin/TCTP and Pm-fortilin/TCTP prevent 5-FU-induced cell death

To determine the function of Pm-fortilin/TCTP in prevention of cell death, 5 x 10^4 U2OS cells line that stably expressed Pm-fortilin/TCTP and human-fortilin/TCTP were seeded onto each well of 24-well plate. Next day, cells were generated and tested for their susceptibility to 10 cell death stimuli in the optimal concentration; 10 μM campothecin, 1 mM cisplatin, 10 μM doxorubicin, 2 mM etoposide, 1 mM 5-fluorouracil, 100 μM H_2O_2, 2 mM hydroxyurea, 0.1 μM staurosporine, 10 μM thapsigargin, 1 nM TNF-α + 10 μM cyclohexamide (data not shown). The dead cell was determined and represented the population of dead cells in total cells, including both adhesive and floating cells, at the termination of experiment. Determination of cell death showed that overexpression of fortilin/TCTP from both shrimp and human suppressed apoptosis induced by etoposide ($U2OS_{Empty1}$ 81.8 ± 1.4%, $U2OS_{Human\text{-}fortilin/TCTP8}$ 72.3 ± 2.0%, $U2OS_{Pm\text{-}fortilin/TCTP10}$ 69.6 ± 1.8%), staurosporine ($U2OS_{Empty1}$ 72.1 ± 2.4%, $U2OS_{Human\text{-}fortilin/TCTP8}$ 62.0 ± 1.6%, $U2OS_{Pm\text{-}fortilin/TCTP10}$ 60.5 ± 1.6%), cisplatin ($U2OS_{Empty1}$ 76.1 ± 0.9%, $U2OS_{Human\text{-}fortilin/TCTP8}$ 73.4 ± 1.2%, $U2OS_{Pm\text{-}fortilin/TCTP10}$ 61.8 ± 1.3%), hydroxyurea ($U2OS_{Empty1}$ 45.3 ± 2.0%, $U2OS_{Human\text{-}fortilin/TCTP8}$ 38.3 ± 0.1%, $U2OS_{Pm\text{-}fortilin/TCTP10}$ 31.1 ± 2.3%), and 5-fluorouracil ($U2OS_{Empty1}$ 56.1 ± 4.5%, $U2OS_{Human\text{-}fortilin/TCTP8}$ 42.8 ± 0.8%, $U2OS_{Pm\text{-}fortilin/TCTP10}$ 42.8 ± 0.8%) (Figure 22).

3. Anti-apoptotic activity of Pm-fortilin/TCTP and human-fortilin/TCTP

To examine the function of Pm-fortilin/TCTP in prevention of cell death, 5 x 10^4 U2OS overexpressing cells that stably expressed Pm-fortilin/TCTP were seeded onto each well of 24-well plate. Next day, cells were challenged and tested for their

susceptibility to 1 mM of 5-FU for 48 h (Figure 23A). The result indicated that high expression level of Pm-fortilin/TCTP, $U2OS_{Pm\text{-}fortilin/TCTP10}$ (23.7 ± 1.2%), made the cells more resistant to 5-FU treatment than a lower expression level, $U2OS_{Pm\text{-}fortilin/TCTP2}$ (40.2 ± 3.2%) and $U2OS_{Empty}$ (43.3 ± 1.7%). Pm-fortilin/TCTP expression level is shown in Figure 23B.

To assess the function of Pm-fortilin/TCTP, the concentration of 5-FU was varied. The dose dependence analysis of the effect of exposure to 5-FU for 48 h on Pm-fortilin/TCTP viability indicated that $U20S_{Pm\text{-}fortilin/TCTP10}$ cells were significantly more resistant to 5-FU-induced cell death than $U20S_{Empty}$ (Figure 24).

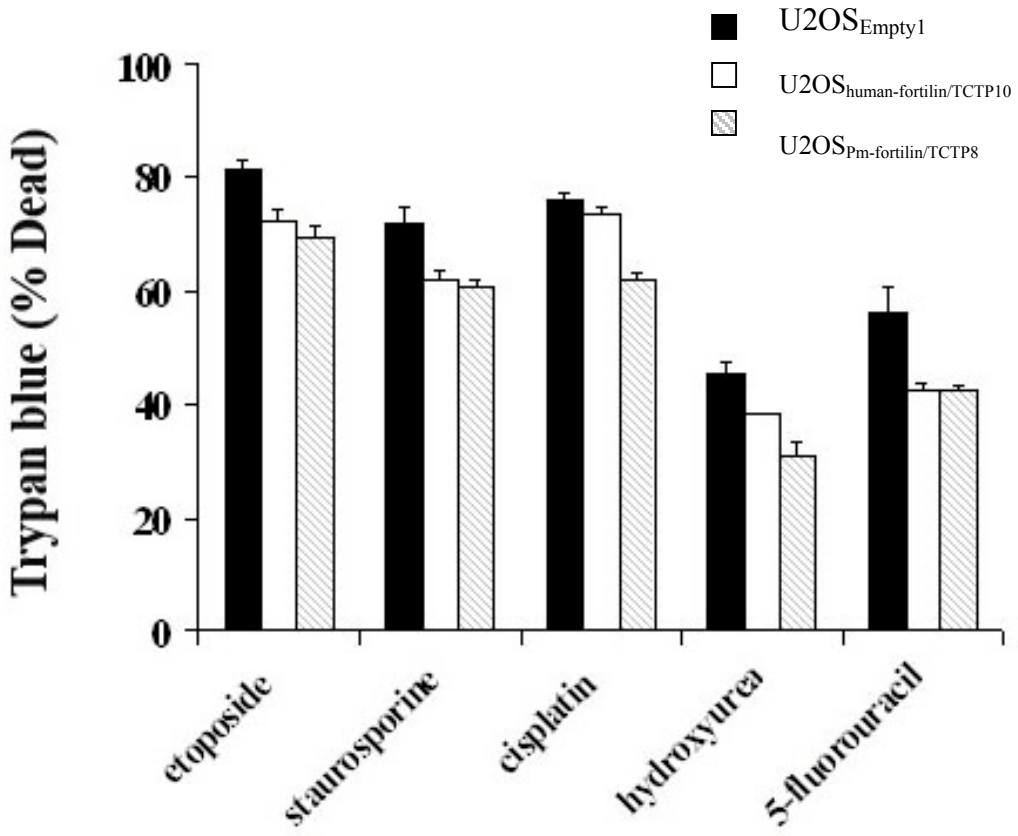


Figure 22. Overexpressing of Pm-fortilin/TCTP and human-fortilin/TCTP prevent cell from undergoing cell death.

U2OS cells stably expressing Pm-fortilin/TCTP (U2OS$_{Pm-fortilin/TCTP10}$) or human-fortilin/TCTP (U2OS$_{human-fortilin/TCTP8}$) and empty plasmid (U2OS$_{Empty1}$) were exposed to 2 mM etoposide and 0.1 μM staurosporine for 5 h; 1 mM cisplatin and 2 mM hydroxyurea for 24 h; and 1 mM 5-fluorouracil for 48 h. Cell viability (% of cell death) was determined using the trypan blue exclusion assay. Trypan blue assays were performed in triplicate.

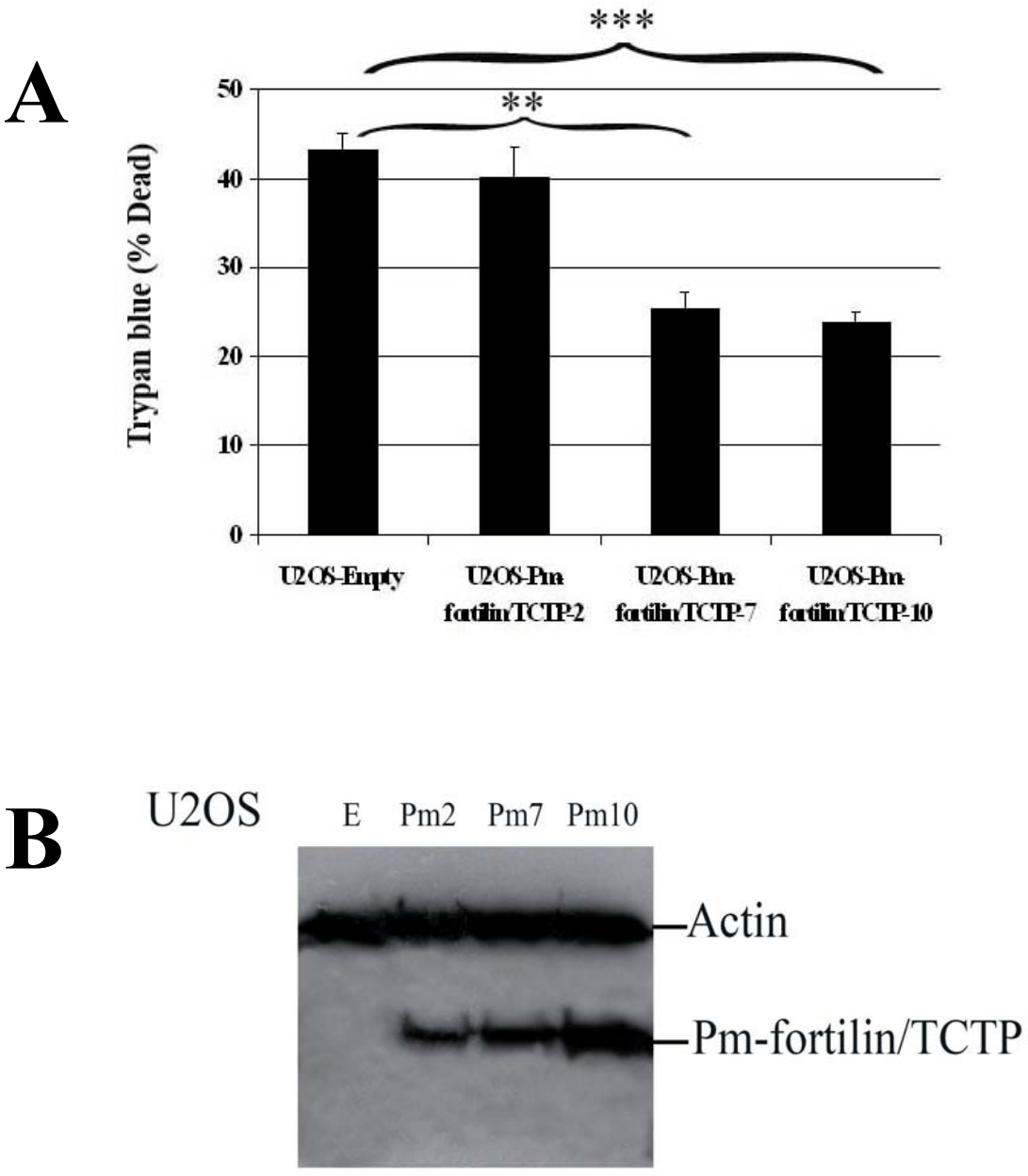


Figure 23. Protection of U2OS cells overexpressing Pm-fortilin/TCTP from 5-FU-induced cytotoxicity.

***A*,** U2OS cells were stably transfected and clonally selected to establish clones harboring empty plasmids (*E*, $U2OS_{Empty}$) or overexpressing fortilin/TCTP. **, $p < 0.01$, comparing $U2OS_{Empty}$ and $U2OS_{Pm\text{-}fortilin/TCTP\text{-}7}$ cells and ***, $p < 0.005$ by ANOVA, comparing $U2OS_{Empty}$ and $U2OS_{Pm\text{-}fortilin/TCTP\text{-}10}$ cells. (*Pm2*, $U2OS_{Pm\text{-}}$

fortilin/TCTP2; *Pm7*, U2OS$_{Pm\text{-}fortilin/TCTP7}$; *Pm10*, U2OS$_{Pm\text{-}fortilin/TCTP10}$). ***B*,** Clones were evaluated by Western blot analyses with anti-Pm-fortilin/TCTP and anti-actin antibodies. Trypan blue assays were performed in triplicate.

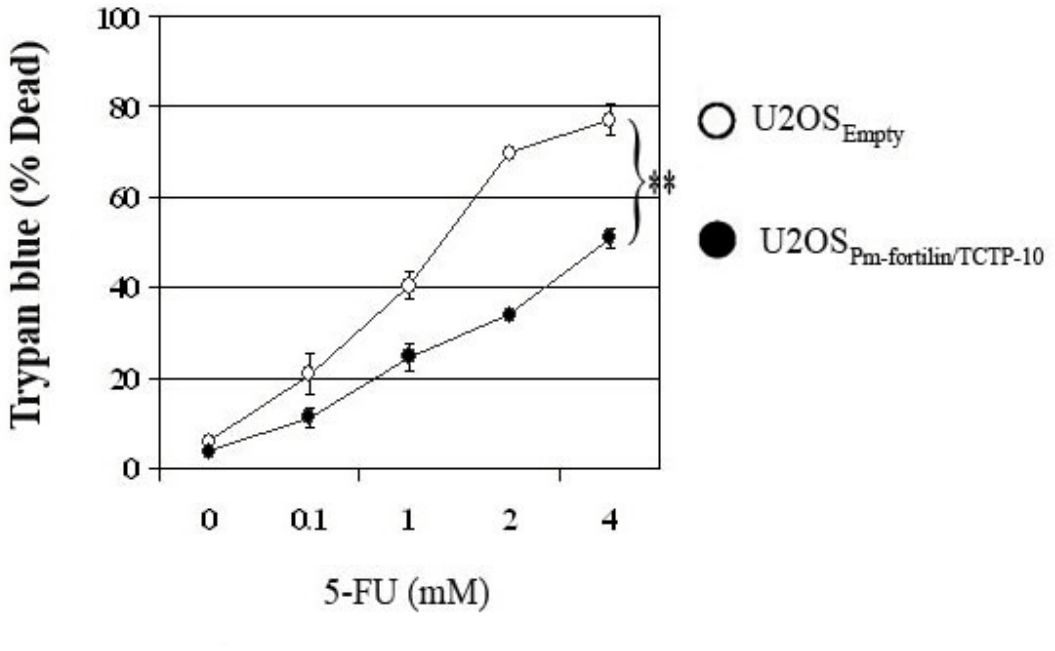


Figure 24. Protection from 5-FU induced cytotoxicity in U2OS cells overexpressing fortilin/TCTP.

Cells were challenged with 0-4 mM 5-FU for 48 h and subjected to trypan blue assay. Overexpression of fortilin/TCTP was associated with significantly higher survival in $U2OS_{Empty}$ cells. **, $p < 0.01$ by ANOVA, comparing $U2OS_{Empty}$ and $U2OS_{Pm\text{-}fortilin/TCTP\text{-}10}$ cells. Trypan blue assays were performed in triplicate.

Part II. siRNA system and apoptosis assay

1. Development of siRNA system to knockdown MCL1 and human-fortilin/TCTP.

To determine the feasibility of creating the microenvironment in which MCL1, human-fortilin/TCTP or both can be selectively depleted, we generated siRNAs against MCL1 ($siRNA_{MCL1}$), fortilin ($siRNA_{human\text{-}fortilin/TCTP}$), and luciferase ($siRNA_{Luciferase}$) (as control) and performed time course, dose dependent, and double knock down experiment.

1.1 Time course experiment

The siRNAs were tested for their ability to knockdown the target proteins. 8.75×10^5 U2OS cells were seeded onto 6 cm dish. Next day, cells were transfected with siRNA against human-fortilin/TCTP or MCL1. When U2OS cells are transfected with $siRNA_{MCL1}$, MCL1 expression was knocked down to an undetectable level within 12 h (Figure 25A). Similarly, $siRNA_{Fortilin}$ was able to knock down human-fortilin/TCTP expression, but it took the siRNA up to 48 h to do it (Figure 25A). Higher $siRNA_{human\text{-}fortilin/TCTP}$ concentrations did not shorten the time to the complete silencing (data not shown). Surprisingly, once knocked down, MCL1 and human-fortilin/TCTP expression remained undetectable for as long as 120 h.

1.2 Dose response experiment

To determined siRNA concentration, 8.75×10^5 U2OS cells were seeded onto 6 cm dish. Next day, cells were transfected with siRNA against human-fortilin/TCTP or MCL1. siRNA against MCL1 ($siRNA_{MCL1}$) and human-fortilin/TCTP

($siRNA_{human\text{-}fortilin/TCTP}$) were used at 25, 50, 100, 150 nM. The results are shown in Figure 26. Treatment of cells with TransIT-TKO transfection reagent did not affect the intracellular levels of MCL1 and human-fortilin/TCTP (Figure 26, N *vs.* T). In addition, in dose-response experiments, we found that 25 nM $siRNA_{MCL1}$ and 25 nM $siRNA_{human\text{-}fortilin/TCTP}$ were sufficient to completely knock-down MCL1 and human-fortilin/TCTP, respectively (Figure 26).

1.3 Double knock down experiment

The $siRNA_{MCL1}$ and $siRNA_{human\text{-}fortilin/TCTP}$ were tested for their ability to knockdown both target proteins. 8.75 x 10^5 U2OS cells were seeded onto 6 cm dish. Next day, cells were transfected with siRNA against human-fortilin/TCTP and MCL1. The result showed that it was possible to silence both MCL1 and human-fortilin/TCTP at the same time by introducing both $siRNA_{MCL1}$ and $siRNA_{human\text{-}fortilin/TCTP}$ into the cells simultaneously (Figure 27). With 25 nM each of $siRNA_{MCL1}$ and $siRNA_{human\text{-}fortilin/TCTP}$, both MCL1 and human-fortilin/TCTP were silenced within 24 h (Figure 27). There data suggested that it is possible to quickly and persistently silence MCL1 and/or human-fortilin/TCTP expression in U2OS cells by the single transfection with $siRNA_{MCL1}$ and/or $siRNA_{human\text{-}fortilin/TCTP}$.

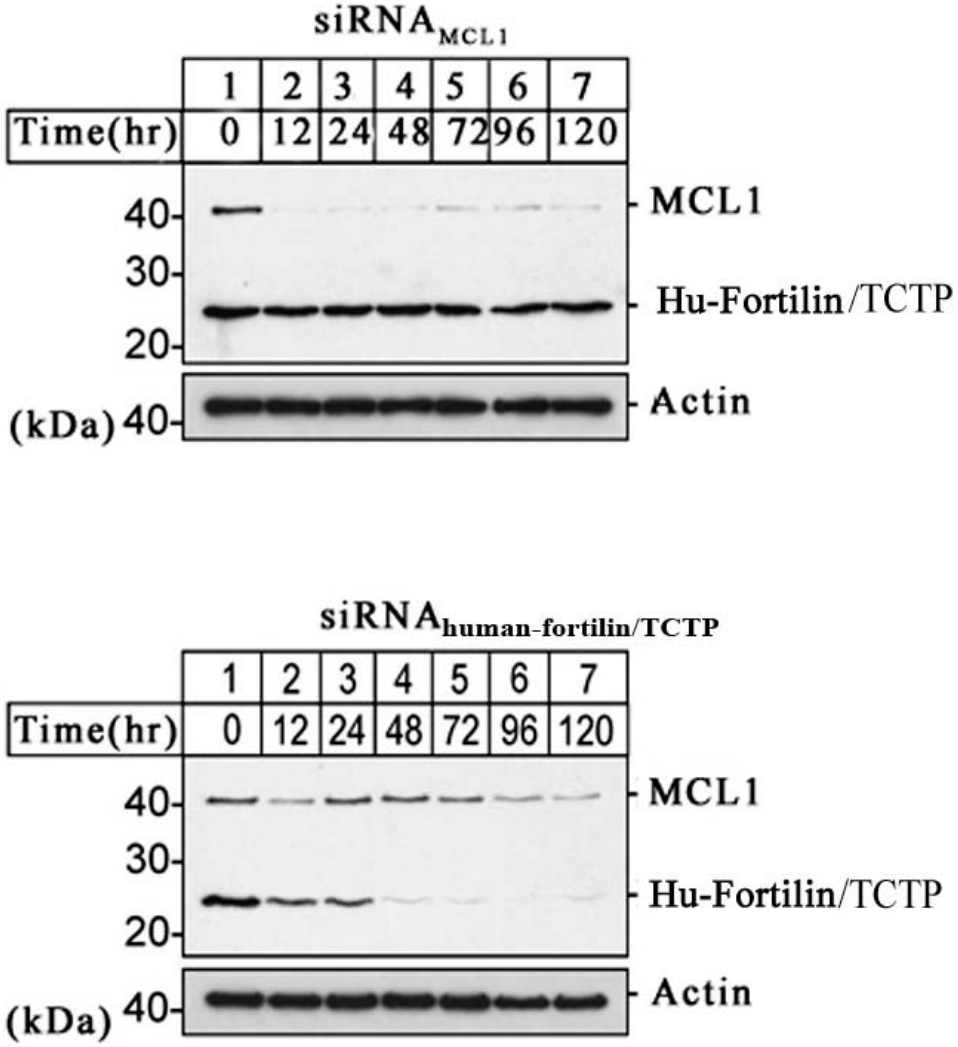


Figure 25. Time course of MCL1 and human-fortilin/TCTP silencing by siRNAs.

Proteins were sized-fractionated by SDS-PAGE and then probed by antibodies to MCL1, human-fortilin/TCTP and actin. MCL1 and human-fortilin/TCTP gene silencing was evident 12 and 48 h after cells were treated with siRNA$_{MCL1}$ and siRNA$_{human-fortilin/TCTP}$, respectively. The silence lasted for at least 120 h after siRNA transfection. Experiments were performed in triplicate.

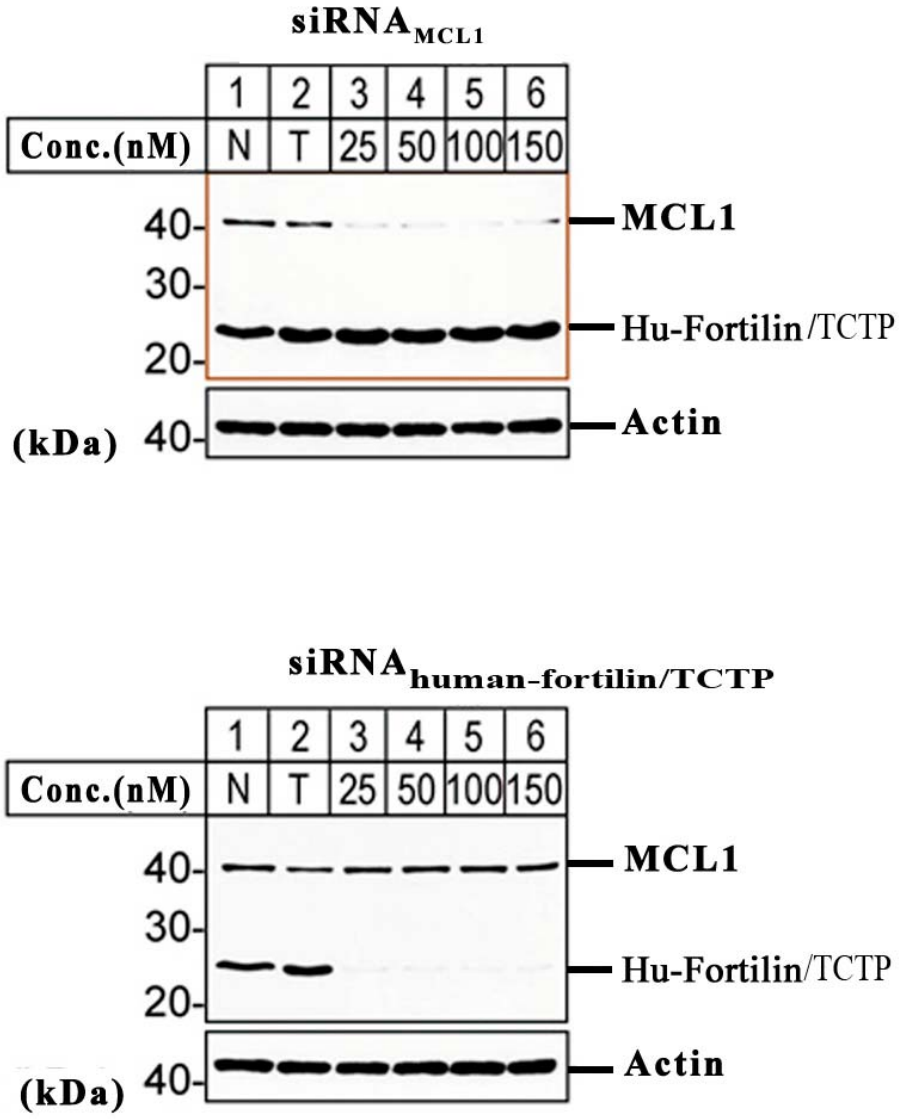


Figure 26. Dose response of MCL1 and human-fortilin/TCTP silencing by siRNAs.

Proteins were sized-fractionated by SDS-PAGE and, then, probed by antibodies to MCL1, human-fortilin/TCTP and actin. MCL1 and human-fortilin/TCTP gene silencing were effectively achieved with 25 nM siRNA$_{MCL1}$ and 25 nM siRNA$_{human-fortilin/TCTP}$, respectively. *Conc*., concentration; *N*, cells not treated with either siRNA or TransIT-TKO transfection reagent; *T*, cells treated with TransIT-TKO transfection reagent only. Experiments were performed in triplicate.

Figure 27. Double silencing of MCL1 and human-fortilin/TCTP silencing by siRNAs.

Proteins were sized-fractionated by SDS-PAGE and, then, probed by antibodies to MCL1, human-fortilin/TCTP and actin. Both MCL1 and human-fortilin/TCTP expression were knocked down to undetectable levels at 24 h after double transfection of cells with 25 nM $siRNA_{MCL1}$ and 25 nM $siRNA_{human\text{-}fortilin/TCTP}$, respectively. Experiment was performed in triplicate.

2. The depletion of human-fortilin/TCTP or MCL1 increases the susceptibility of cells to 5-FU- induced cell death; whereas, the overexpression of human-fortilin/TCTP or MCL1 protects cells from 5-FU-induced cell death

2.1 The depletion of human-fortilin/TCTP or MCL1

To evaluate the viability of cells treated with $siRNA_{human\text{-}fortilin/TCTP}$ or $siRNA_{MCL1}$, 7 x 10^4 U2OS cells were seeded onto each well of 24-well plate. Next day, cells were treated with 25 nM of siRNA, incubated for 48 h, and, then, incubated with 1 mM 5-FU for another 24 h. Then, cells were subjected to trypan blue assay for the assessment of cell viability. The expression levels of human-fortilin/TCTP and MCL1 were examined by western blotting (Figure 28, upper panel). As shown in Figure 28, lower panel, silencing of human-fortilin/TCTP and MCL1 was associated with a significantly greater rate of 5-FU-induced cell death than was treatment with irrelevant $siRNA_{Luciferase}$ ($siRNA_{Luciferase}$ *versus* $siRNA_{human\text{-}fortilin/TCTP}$, 49.0 ± 3.6% *versus* 63.3 ± 2.65%, $p < 0.005$; $siRNA_{Luciferase}$ *versus* $siRNA_{MCL1}$, 49.00 ± 3.6% *versus* 63.3 ± 5.2%, $p < 0.005$, by two sample *t* tests). These data showed that human-fortilin/TCTP and MCL1 are prosurvival molecules whose silencing makes cells less resistant to cytotoxic stimuli.

2.2 The overexpression of human-fortilin/TCTP or MCL1

5 x 10^4 U2OS cells line overexpressing MCL1 or human-fortilin/TCTP were seeded onto each well of 24-well plate. Next day, both U2OS overexpressing cells were established, characterized, and tested them for their susceptibility to 5-FU for 48 h (Figures 29, upper panel and 29, lower panel). Consistent with the results of the siRNA experiments described in Result 2.1, overexpression of either MCL1 and

human-fortilin/TCTP made the cells more resistant to 5-FU treatment ($U2OS_{Empty}$ *versus* $U2OS_{human\text{-}fortilin/TCTP}$, 55.8 ± 4.0% *versus* 31.3 ± 4.3%, $p < 0.001$; $U2OS_{Empty}$ *versus* $U2OS_{MCL1}$, 55.8 ± 4.0% *versus* 31.3 ± 5.6%, $p < 0.001$).

These data confirms that human-fortilin/TCTP and MCL1 are prosurvival molecules whose overexpression makes cells more resistant to cytotoxic stimuli.

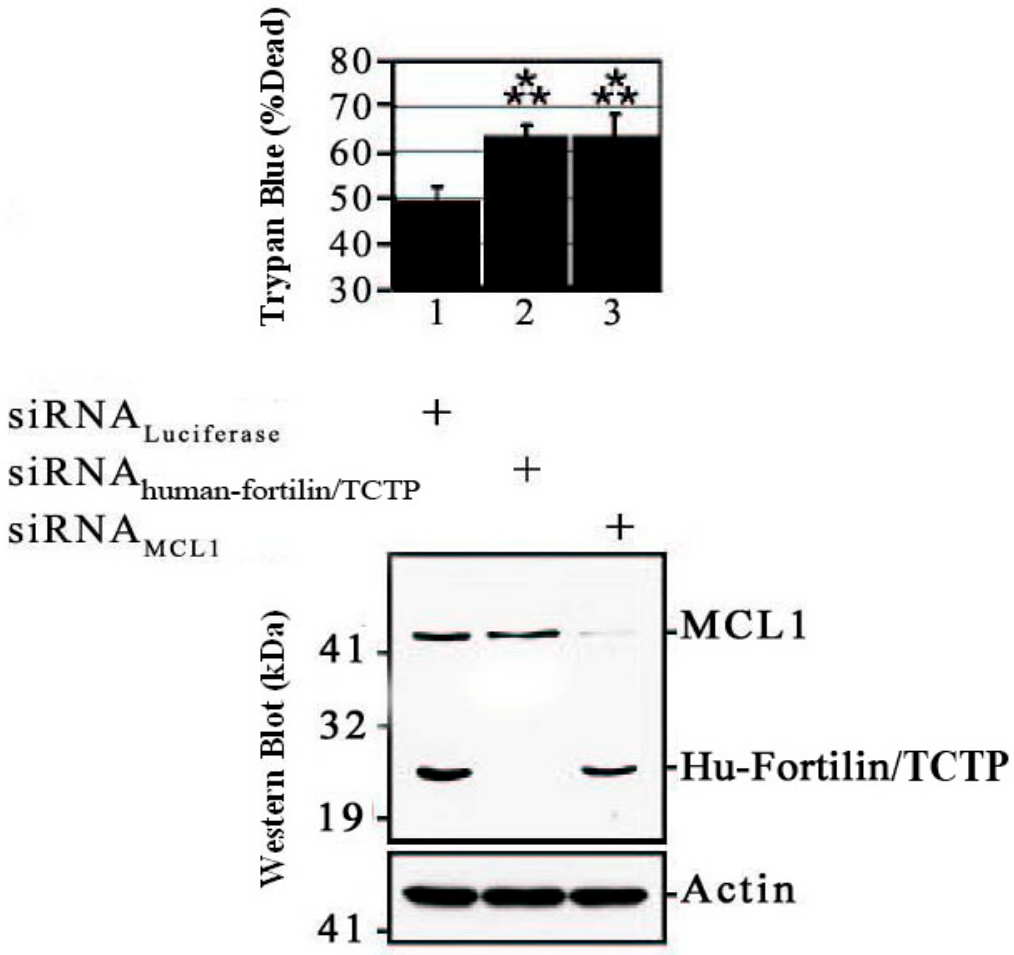


Figure 28. MCL1 and human-fortilin/TCTP prevent 5-FU-induced cell death.

As shown by siRNA gene knockout and overexpression assay, increased 5-FU-induced cytotoxicity in U2OS cells depleted of human-fortilin/TCTP or MCL1. Cells were transfected with $siRNA_{Luciferase}$, $siRNA_{MCL1}$, or $siRNA_{human\text{-}fortilin/TCTP}$; incubated for 24 h; challenged with 1 mM 5-FU; incubated for 48 h; and subjected to trypan blue assay. After counting, cells were subjected to Western blot analysis with anti-MCL1, anti-human-fortilin/TCTP, and anti-actin antibodies. Human-fortilin/TCTP and MCL1 gene silencing were associated with a significant increase in 5-FU-induced cell death. Assays were performed in triplicate. ***, $p < 0.005$ in

comparison with $siRNA_{Luciferase}$ -treated cells. *$siRNA_{Luciferase}$*, siRNA against luciferase, an irrelevant non mammalian protein (control); *Trypan blue*, trypan blue exclusion assay.

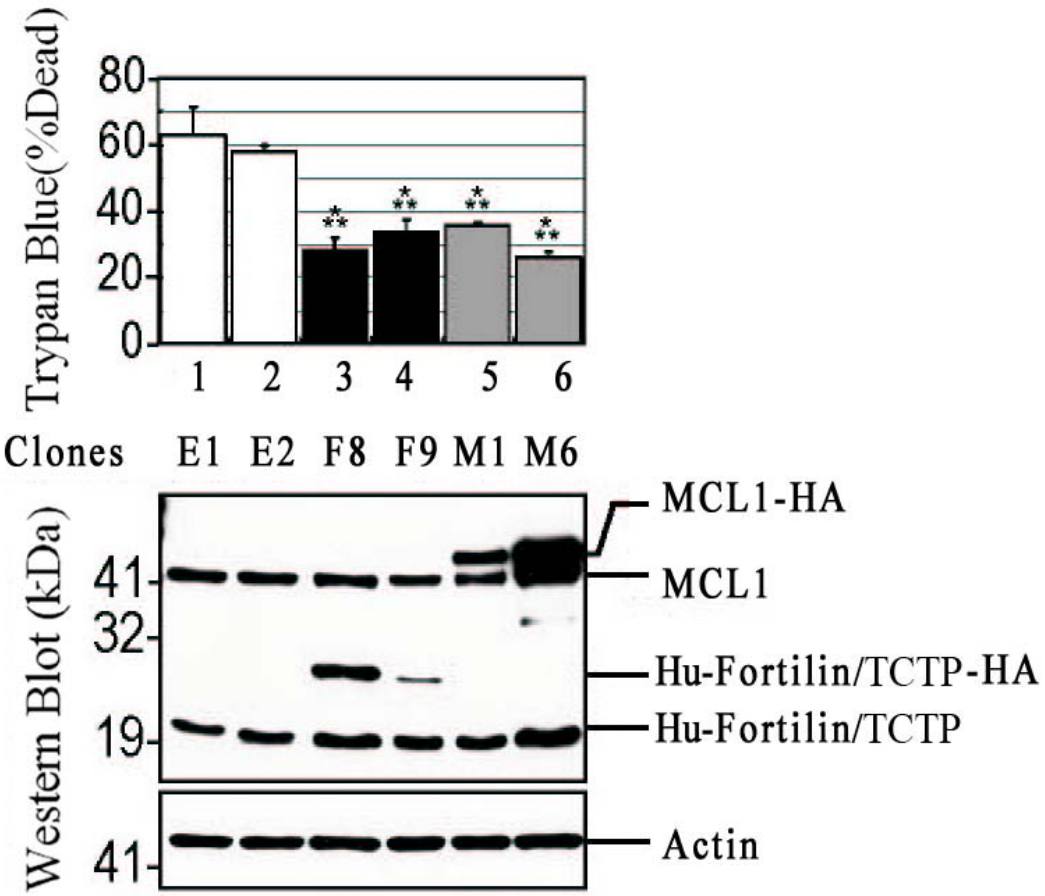


Figure 29. MCL1 and human-fortilin/TCTP prevent 5-FU-induced cell death.

As shown by siRNA gene knockout and overexpression assay, protection of U2OS cells overexpressing human-fortilin/TCTP or MCL1 from 5-FU-induced cytotoxicity. U2OS cells were stably transfected and clonally selected to establish the clones harboring empty plasmids ($U2OS_{Empty}$, E1 and E2) or overexpressing human-fortilin/TCTP ($U2OS_{human\text{-}fortilin/TCTP}$, F8 and F9) or MCL1 ($U2OS_{MCL1}$, M1 and M2). Clones were evaluated by Western blot analysis with anti-MCL1, anti-human-fortilin/TCTP, and anti-actin antibodies. Two clones each from all the appropriate clones were randomly selected (E1, E2, F8, F9, M1, and M6), propagated, challenged with 5-FU for 48 h, and subjected to trypan blue assay. Overexpression of human-

fortilin/TCTP and MCL1 was associated with significantly less 5-FU-induced cell death. Assays were performed in triplicate. ***, $p < 0.005$ in comparison with U2OS cells harboring empty plasmids. $siRNA_{Luciferase}$, siRNA against luciferase, an irrelevant non mammalian protein (control); *Trypan blue*, trypan blue exclusion assay.

3. The depletion of human-fortilin/TCTP and MCL1 does not change the intracellular localization of each partner's protein, MCL1 and human-fortilin/TCTP, respectively.

3.1 Immunocytochemical analysis

To evaluate intracellular localization of human-fortilin/TCTP and MCL1 in the absence of its protein partner, 4 x 10^4 U2OS cells were seeded onto each well of 4-well chamber slide. Next day, U2OS cells were transfected with $pcDNA4_{human\text{-}fortilin/TCTP\text{-}HA}$ or $pcDNA4_{MCL1\text{-}HA}$, and, then, treated with $siRNA_{human\text{-}fortilin/TCTP}$, $siRNA_{MCL1}$ or $siRNA_{Luciferase}$. Cells were stained with anti-HA, anti-human-fortilin/TCTP (in case of human-fortilin/TCTP silencing) or anti-MCL1 (in case of MCL1 silencing), and 4',6-diamidio-2-phenylinole (DAPI). The introduction of $siRNA_{MCL1}$ and $siRNA_{human\text{-}fortilin/TCTP}$ into cells was associated with a significant reduction of MCL1 and human-fortilin/TCTP signals, respectively (Figure 30, *c versus f* and *i versus l*). In this system, the intracellular localization of human-fortilin/TCTP and MCL1, as assessed by signals from anti-HA staining were identical, regardless of the silencing of MCL1 and human-fortilin/TCTP, respectively (Figure 30, *b versus e* and *h versus k*). As shown by immunocytochemistry, human-fortilin/TCTP localized predominantly in the nucleus and a small degree in the cytosol; whereas, MCL1 localized predominantly in cytosol and a small degree in the nucleus.

3.2 Subcellular fractionation

To study intracellular localization of human-fortilin/TCTP and MCL1 in the absence of its protein partner, 1.5 x 10^6 U2OS cells were seeded onto 10 cm dish.

Next day, cells were treated with $siRNA_{human\text{-}fortilin/TCTP}$ or $siRNA_{MCL1}$ or $siRNA_{Luciferase}$ for 48 h. Then, U2OS cells were harvested, and fractionated the lysate from cells into cytosolic and nuclear fractions. As shown in Figure 31, Western blot analyses revealed that α-tubulin was detectable only in the cytosolic fraction, and histone H1 was detectable only in the nuclear fraction which suggests that the cytosolic and nuclear fractions did not cross-contamination each other. In addition, the treatment with $siRNA_{MCL1}$ depleted MCL1 in both nucleus and cytosol (Figure 31, lanes 3 and 6). Similarly, treatment with $siRNA_{human\text{-}fortilin}$ depleted fortilin/TCTP in both the nucleus and cytosol (Figure 31, lanes 2 and 5). MCL1 localized predominantly in the cytosol and a small degree in the nucleus before human-fortilin/TCTP silencing (Figure 31; lanes 1 and 4, MCL1), which is consistent with the immunostaining data in Result 3.1. Upon human-fortilin/TCTP silencing, this pattern of cytosolic predominance persisted (Figure 31; lanes 2 and 5, MCL1). Human-fortilin/TCTP localized in the cytosol more than in the nucleus before MCL1 silencing (Figure 31; lanes 1 and 4, Fortilin). Upon MCL1 silencing, this pattern again persisted (Figure 31; lanes 3 and 6, MCL1). Together, these data suggested that the depletion of MCL1 and human-fortilin/TCTP by siRNA does not change the intracellular localization of their protein partners, namely, human-fortilin/TCTP and MCL1, respectively.

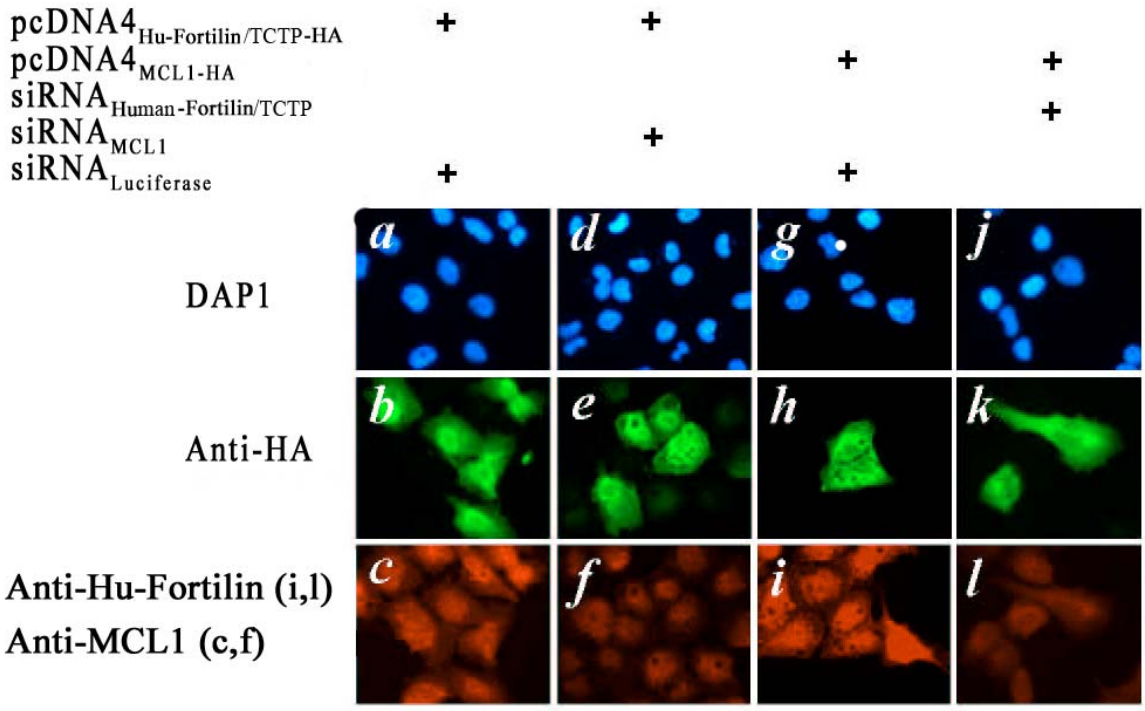


Figure 30. Immunocytochemical analysis.

Depletion of human-fortilin/TCTP or MCL1 does not change the intracellular localization of MCL1 or human-fortilin/TCTP, respectively. U2OS cells were transfected with either pcDNA4$_{\text{human-fortilin/TCTP-HA}}$ (*a-f*) or pcDNA4$_{\text{MCL1-HA}}$ (*g-l*) and with either siRNA$_{\text{human-fortilin/TCTP}}$ (*j-l*), siRNA$_{\text{MCL1}}$ (*d-f*) or siRNA$_{\text{Luciferase}}$ (*a-c* and *g-i*) and subjected to triple immuno staining using 4', 6-diamidino-2-phenylindole (*a*, *d*, *g*, and *j*), anti-HA antibody (*b*, *e*, *h*, and *k*), anti-human-fortilin/TCTP (*I* and *l*), and anti-MCL1 (*c* and *f*). siRNA$_{\text{human-fortilin/TCTP}}$ and siRNA$_{\text{MCL1}}$ were highly effective at silencing human-fortilin/TCTP (*i* versus *l*) and MCL1 (*c* versus *f*) genes, respectively. The intracellular localization of HA-tagged human-fortilin/TCTP (*b* and *e*) and MCL1 (*h* versus *k*) was the same in the presence (*f* and *l*) and absence (*c* and *i*) of MCL1 and human-fortilin/TCTP silencing, respectively. Experiment was performed in triplicate.

pcDNA4$_{human\text{-}fortilin/TCTP\text{-}HA}$, pcDNA4 vector containing the cDNA of HA-tagged human-fortilin/TCTP; *pcDNA4*$_{MCL1\text{-}HA}$, pcDNA vector containing the cDNA of HA-tagged MCL1; *DAPI*, 4', 6-diamidino-2-phenylindole.

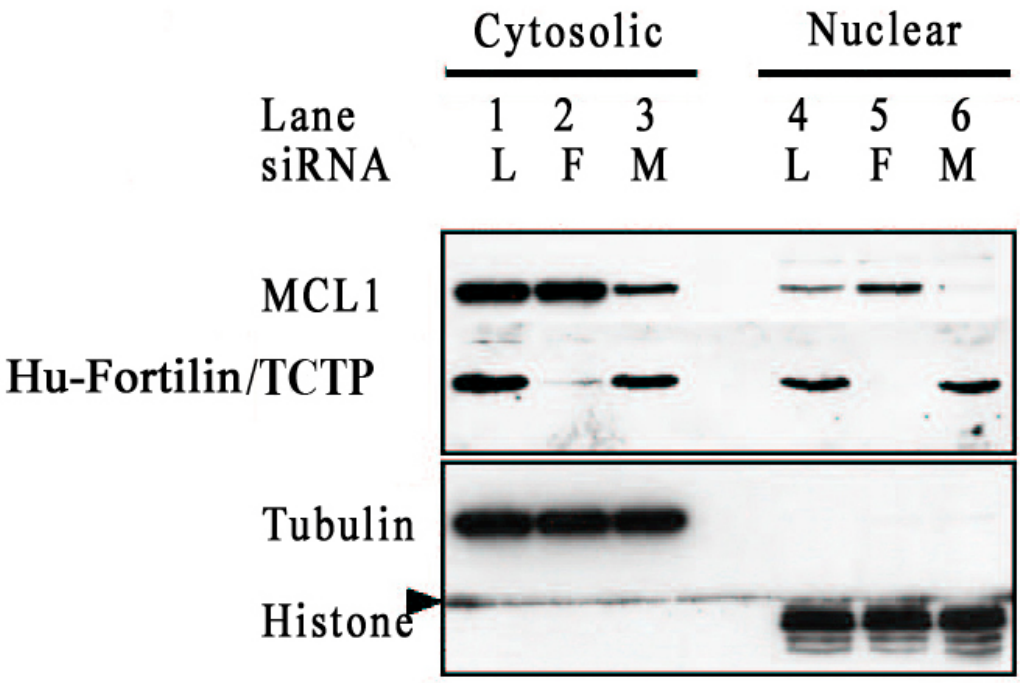


Figure 31. Subcellular fractionation and Western blot analysis.

Depletion of human-fortilin/TCTP and MCL1 does not change the intracellular localization of MCL1 and human-fortilin/TCTP, respectively. U2OS cells were transfected with $siRNA_{human\text{-}fortilin/TCTP}$ or $siRNA_{MCL1}$, lysed, and fractionated into subcellular (cytosolic and nuclear) fractions. After size fractionation of exactly 10 µg of proteins by SDS-PAGE and transfered to nitrocellulose membrane, proteins were probed with anti-MCL1, anti-human-fortilin/TCTP, anti-α-tubulin, and anti-histone H1 (clone AE-4) antibodies. The $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{MCL1}$ robustly depleted human-fortilin/TCTP and MCL1 from both cytosolic (lanes 2 and 3) and nuclear fractions (lanes 5 and 6), respectively. The depletion of human-fortilin/TCTP and MCL1, however, was not associated with any change in the predominant localization of these proteins. Experiment was performed in triplicate. *Cytosolic*, cytosolic fraction; *Nuclear*, nuclear fraction; *L*, $siRNA_{Luciferase}$; *F*, $siRNA_{human\text{-}fortilin/TCTP}$, *M*, $siRNA_{MCL1}$; *arrowhead*, nonspecific Western blot signals at the edges of membranes.

4. Human-fortilin/TCTP prevents cells from undergoing cell death in the absence of its protein partner MCL1.

4.1 U2OS cells overexpressing human-fortilin/TCTP protect cells from 5-FU induced cytotoxicity.

To investigate apoptotic activity of human-fortilin/TCTP, $U2OS_{Fortilin\text{-}8}$ was characterized, one of the clones of U2OS cells overexpressing human-fortilin/TCTP, using $U2OS_{Empty\text{-}1}$ as control. 5 x 10^4 U2OS cells overexpressing human-fortilin/TCTP and Empty were seeded onto each well of 24-well plate. Next day, cells were challenged with 1 mM of 5-FU for 48 h and subjected into trypan blue exclusion assay. Trypan blue assay showed that $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ cells were significantly more resistant to 5-FU-induced cell death than $U2OS_{Empty\text{-}1}$ cells (Figure 32, $p < 0.005$ by ANOVA).

4.2 Dose dependent protection of human-fortilin/TCTP.

To evaluate the principal hypothesis that human-fortilin/TCTP was capable of blocking cell death even in the absence of MCL1. 7 x 10^4 U2OS cells overexpressing human-fortilin/TCTP and Empty were seeded onto each well of 24-well plate. Next day, $U2OS_{Empty\text{-}1}$ and $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ cells were transfected with $siRNA_{human\text{-}fortilin/TCTP}$, $siRNA_{MCL1}$, or $siRNA_{Luciferase}$ for 48 h; challenged the cells with 1 mM 5-FU for 24 h; and finally determined their viability using trypan blue assay. Consistent with data in Figures 29 and 32), human-fortilin/TCTP-overexpressing cells ($U2OS_{human\text{-}fortilin/TCTP\text{-}8}$) were more resistant to 5-FU-induced cell death than were control cells ($U2OS_{Empty\text{-}1}$) (Figure 33; $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ (lane 6) *versus* $U2OS_{Empty\text{-}1}$(lane 5): 33.4 ± 0.6% *versus* 54.5 ± 0.4%, $p < 0.005$ by two sample

t-test). When native human-fortilin/TCTP expression was knocked down by $siRNA_{human\text{-}fortilin/TCTP}$ in $U2OS_{Empty\text{-}1}$ cells (Figure 33, Western blot analysis, lanes 4 *versus* 5, Fortilin/TCTP), the loss of native human-fortilin/TCTP was associated with an increase in cell susceptibility to 5-FU treatment (no human-fortilin/TCTP (lane 4) *versus* native human-fortilin/TCTP (lane 5) in $U2OS_{Empty\text{-}1}$ cells: 61.3 ± 1.1% *versus* 54.5 ± 0.4%, $p < 0.05$ by two sample *t*-test). Thus, intracellular human-fortilin/TCTP levels consistently and significantly correlated with the degree of cell survival (Figure 33, no human-fortilin/TCTP (lanes 4) *versus* native human-fortilin/TCTP (lane 5) *versus* native plus overexpressed human-fortilin/TCTP (lane 6), $p < 0.001$ by ANOVA). In the presence of native MCL1, the higher the intracellular levels of human-fortilin/TCTP were, the lower the susceptibility of cells to 5-FU-induced cell death was (Figure 33, lanes 4-6). Human-fortilin/TCTP had a dose-dependent anticytotoxic effect on 5-FU-challenged U2OS cells.

To examine whether the same dose-dependence would be present in the essential absence of MCL1, $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ and $U2OS_{Empty\text{-}1}$ cells were treated with $siRNA_{MCL1}$ to silence MCL1. In some cases, both MCL1 and human-fortilin/TCTP were simultaneously knocked down by cotransfection of $siRNA_{human\text{-}fortilin}$/TCTP and $siRNA_{MCL1}$ as optimized and shown in Figure 27. Importantly, the total amount of siRNA introduced into these cells was kept constant by the addition of $siRNA_{Luciferase}$, an irreverent control siRNA. As shown in Figure 33, the introduction of $siRNA_{MCL1}$ into the cells caused the intracellular level of native MCL1 to decrease to undetectable levels by Western blot analyses (Figure 33, Western blot analysis, MCL1, lanes 1-3). In case of double knock down, Western blot analysis showed no significant signals of MCL1 or human-fortilin/TCTP proteins (Figure 33, Western blot

analysis, MCL1 and human-fortilin/TCTP, lane 1). However, as assessed by trypan blue assay, the increasing intracellular concentration of human-fortilin/TCTP was associated with increasing resistance to 5-FU-induced cell death, even in the absence of MCL1 (Figure 33, no human-fortilin/TCTP (lane 1) *versus* native human-fortilin/TCTP (lane 2) *versus* native plus overexpressed human-fortilin/TCTP (lane 3): 76.8 ± 0.7% *versus* 64.7 ± 0.5% *versus* 47.4 ± 0.1%, $p < 0.001$ by ANOVA). Human-fortilin/TCTP was equally capable of protecting cells from 5-FU-induced cytotoxicity in both the presence and absence of MCL1 and did it in a dose-dependent fashion (Figure 33, lanes 4-6 *versus* lanes 1-3). Together, these data suggested that human-fortilin/TCTP does not require the presence of MCL1 to exert its antiapoptotic activity.

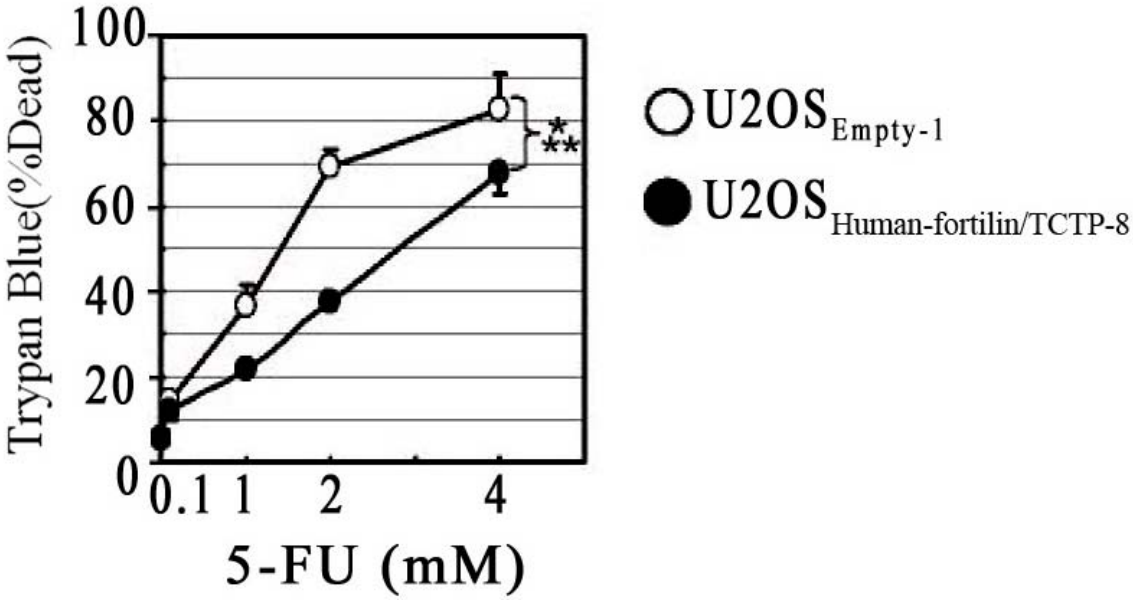


Figure 32. Protection from 5-FU-induced cytotoxicity in U2OS cells overexpressing human-fortilin/TCTP.

Human-fortilin/TCTP protects cells from undergoing cell death in the absence of its protein partners, MCL1. Cells were challenged with 0-4 mM 5-FU and subjected to trypan blue assay. Overexpression of human-fortilin/TCTP was associated with significantly higher survival in U2OS cells. Trypan blue assays were performed in triplicate. ***, $p < 0.005$ by ANOVA, comparing $U2OS_{Empty-1}$ and $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ cells.

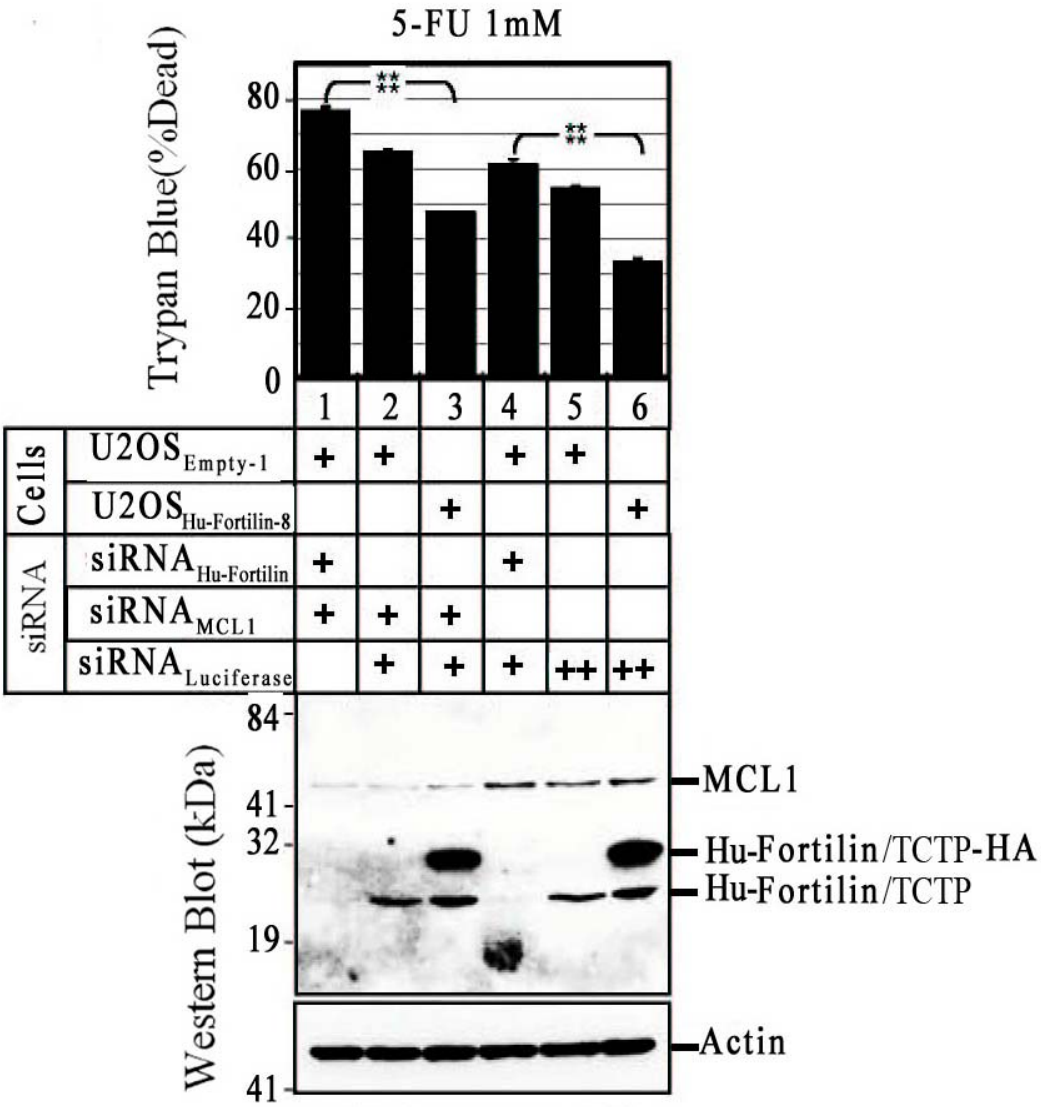


Figure 33. Dose-dependent protection of 5-FU-challenged U2OS cells by human-fortilin/TCTP in the absence of MCL1.

Human-fortilin/TCTP protects cells from undergoing cell death in the absence of its protein partners, MCL1. $U2OS_{Empty-1}$ or $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ cells were transfected with $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{MCL1}$ (lane 1), $siRNA_{MCL1}$ and $siRNA_{Luciferase}$ (lanes 2 and 3), $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{Luciferase}$ (lane 4), or $siRNA_{Luciferase}$ alone (lanes 5 and 6). Western blot analysis showed that both

$U2OS_{Empty\text{-}1}$ and $U2OS_{human\text{-}fortilin/TCTP\text{-}8}$ cells expressed no MCL1 after $siRNA_{MCL1}$ treatment (lanes 1-3). Double transfection of $siRNA_{MCL1}$ and $siRNA_{human\text{-}fortilin/TCTP}$ resulted in the reduction of intracellular MCL1 and human-fortilin/TCTP to undetectable levels (lane 1). In the presence of MCL1 (lanes 4-6), higher intracellular human-fortilin/TCTP levels were associated with lower cytotoxicity as assessed by trypan blue assay. However, in the absence of MCL1 (lanes 1-3), higher intracellular human-fortilin/TCTP levels were still associated with lower cytotoxicity as assessed by trypan blue assay. Experiments were performed in triplicate. ***, $p < 0.001$ by ANOVA.

5. MCL1 prevents cells from undergoing cell death in the absence of its protein partner, human-fortilin/TCTP.

5.1 U2OS cells overexpressing MCL1 protect cells from 5-FU induced cytotoxicity.

The Result 4.2 showed that human-fortilin/TCTP does not require MCL1 in order to be antiapoptotic. To determine whether the reverse was true, $U2OS_{MCL1\text{-}6}$, one of the clones of U2OS cells overexpressing MCL1 was characterized, using $U2OS_{Empty\text{-}1}$ as a control. 5 x 10^4 U2OS cells $U2OS_{MCL1\text{-}6}$ and $U2OS_{Empty\text{-}}1$ were seeded onto each well of 24-well plate. Next day, cells were challenged with 1 mM of 5-FU for 48 h and subjected to trypan blue exclusion assay. Trypan blue assay showed that $U2OS_{MCL1\text{-}6}$ cells were significantly more resistant to 5-FU-induced cell death than were $U2OS_{Empty\text{-}1}$ cells (Figure 34, $p < 0.01$ by ANOVA, when comparing $U2OS_{Empty\text{-}1}$ and $U2OS_{MCL1\text{-}6}$ cells).

5.2 Dose dependent protection of MCL1.

To evaluate one principal hypothesis, namely, that MCL1 is capable of blocking cell death, even in the absence of human-fortilin/TCTP by using the reagent and systems that have already characterized (Figures 25-29, and 34). 7 x 10^4 $U2OS_{Empty\text{-}1}$ and $U2OS_{MCL1\text{-}6}$ were seeded onto each well of 24-well plate. Next day, cells were transfected with $siRNA_{human\text{-}fortilin/TCTP}$, $siRNA_{MCL1}$, or $siRNA_{Luciferase}$ for 48 h; challenged the cells with 1 mM 5-FU for 24 h; and, then, determined their viability using trypan blue assay. In the presence of native human-fortilin/TCTP, the higher the intracellular levels of MCL1 were, the lower the susceptibility of cells to 5-FU-induced cell death was (Figure 35, no MCL1 (lane 4) *versus* native MCL1 (lane 5)

versus native plus overexpressed MCL1 (lane 6), 60.1 ± 2.1% *versus* 40.5 ± 1.7% *versus* 29.0 ± 0.4%, $p < 0.01$ by ANOVA). The MCL1 protected U2OS cells from 5-FU-induced cytotoxicity in a dose-dependent fashion in the presence of native human-fortilin/TCTP.

Furthermore, an experiment was conducted to determine whether this dose-dependence would be hold in the absence of human-fortilin/TCTP in the cells by treating $U2OS_{MCL1\text{-}6}$ and $U2OS_{Empty\text{-}1}$ cells with $siRNA_{human\text{-}fortilin/TCTP}$ to silence fortilin/TCTP. In some cases, both MCL1 and fortilin were simultaneously knocked down by cotransfection of $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{MCL1}$ as optimized in Result 1.3 and shown in Figure 27. As shown in Figure 35, the introduction of $siRNA_{human\text{-}fortilin}$/TCTP into the cells caused the intracellular level of human-fortilin/TCTP to fall down to an undetectable level (Figure 35, Western blot analysis, human-fortilin/TCTP, lanes 1-3). In case of double knock down, Western blot analysis showed no significant signals of either MCL1 or human-fortilin/TCTP proteins (Figure 35, Western blot analysis, MCL1 and human-fortilin/TCTP, lane 1). The increasing intracellular concentration of MCL1 in these cells was associated with increasing resistance to 5-FU-induced cell death in the absence of human-fortilin/TCTP (Figure 33, no MCL1 (lane 1) *versus* native MCL1 (lane 2) *versus* native plus overexpressed MCL1 (lane 3), 74.6 ± 3.3% *versus* 60.2 ± 7.4% *versus* 32.9 ± 0.9%, $p < 0.01$ by ANOVA). MCL1 was equally capable of protecting cells from 5-FU-induced cytotoxicity in both the presence and absence of human-fortilin/TCTP and did it in a dose-dependent manner (Figure 35, lanes 4-6 *versus* lanes 1-3). Together, these data suggested that MCL1 does not require the presence of human-fortilin/TCTP to protect U2OS cells from 5-FU-induced cell death.

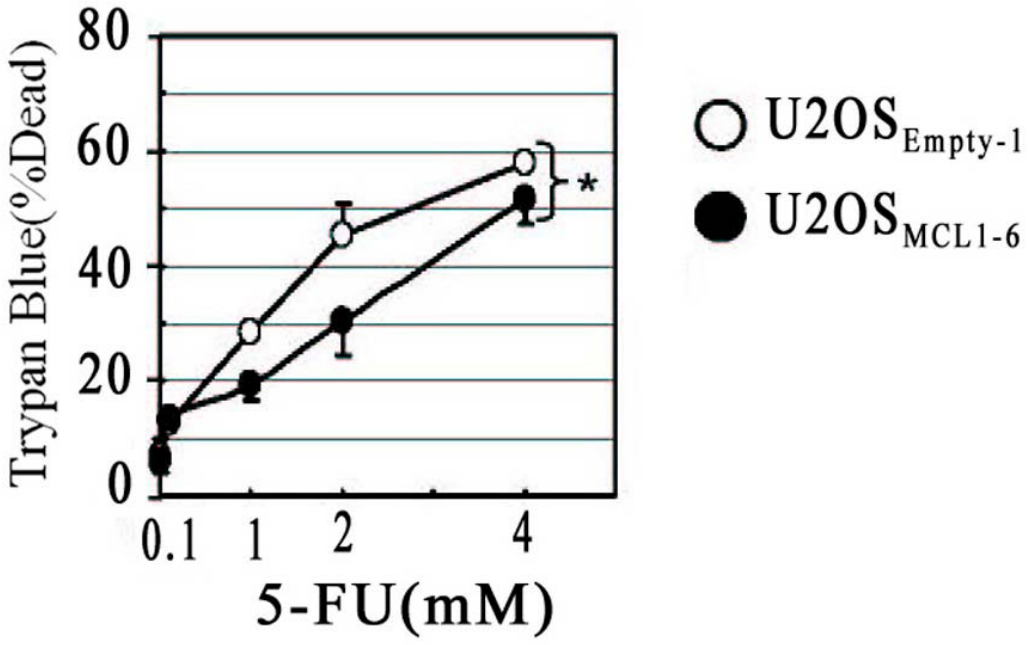


Figure 34. Protection from 5-FU-induced cytotoxicity in U2OS cells overexpressing MCL1.

MCL1 protects cells from undergoing cell death in the absence of its protein partner, human-fortilin/TCTP. Cells were challenged with 0-4 mM 5-FU and subjected to trypan blue assay. Overexpression of MCL1 was associated with significantly higher survival in U2OS cells. Trypan blue assays were performed in triplicate. *, $p < 0.05$ by ANOVA, comparing $U2OS_{Empty-1}$ and $U2OS_{MCL-6}$ cells.

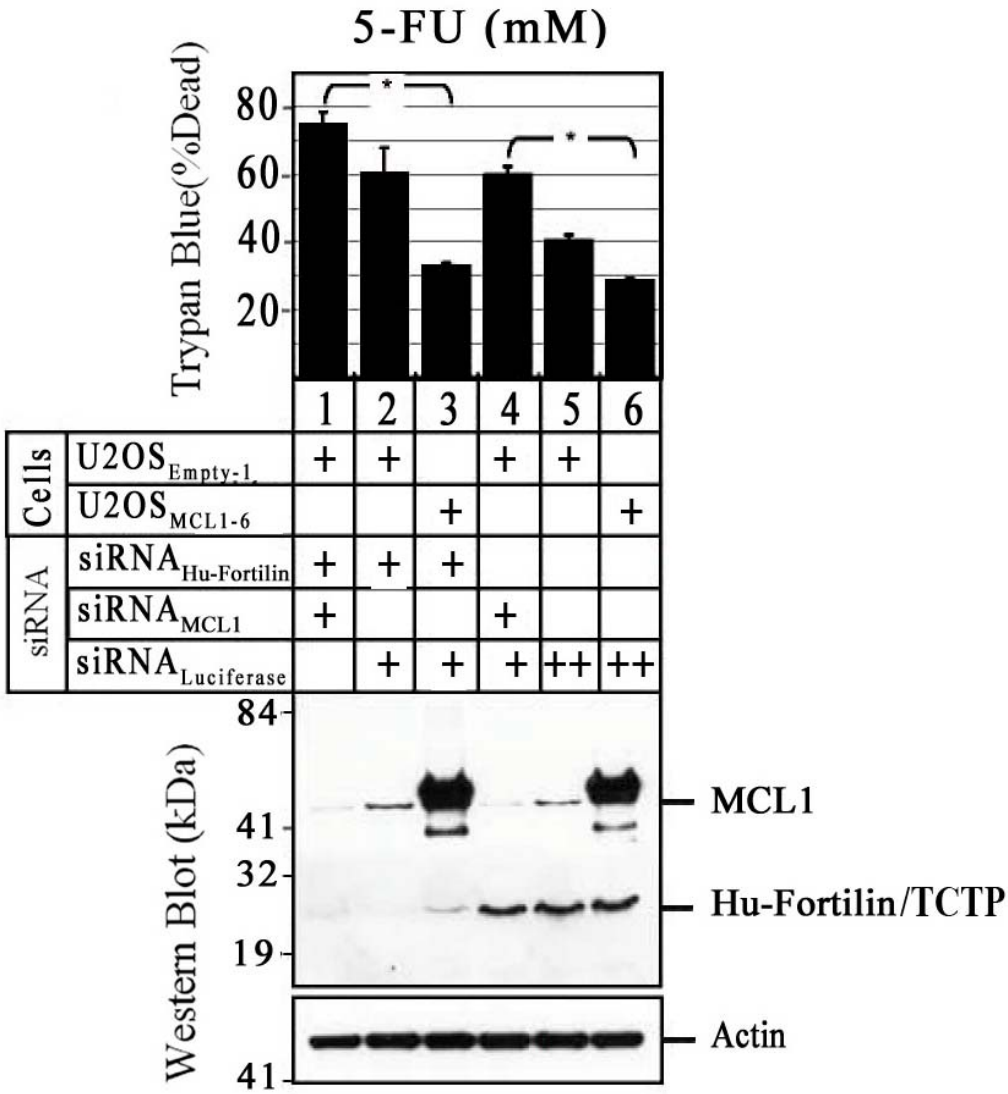


Figure 35. Dose-dependent protection of 5-FU-challenged U2OS cells by MCL1 in the absence of human-fortilin/TCTP.

MCL1 protects cells from undergoing cell death in the absence of its protein partner, human-fortilin/TCTP. $U2OS_{Empty-1}$ or $U2OS_{MCL1-6}$ cell were transfected with $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{MCL1}$ (lane 1), $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{Luciferase}$ (lanes 2 and 3), $siRNA_{MCL1}$ and $siRNA_{Luciferase}$ (lane 4), or $siRNA_{Luciferase}$ alone (lanes 5 and 6). Western blot analysis showed that both $U2OS_{Empty-1}$ and $U2OS_{MCL1-6}$ cells

expressed no human-fortilin/TCTP after $siRNA_{human\text{-}fortilin/TCTP}$ treatment (lanes 1-3). Double transfection of $siRNA_{MCL1}$ and $siRNA_{human\text{-}fortilin/TCTP}$ resulted in the reduction of intracellular MCL1 and human-fortilin/TCTP to undetectable levels (lane 1). In the presence of human-fortilin/TCTP (lanes 4-6), higher intracellular MCL1 levels were associated with lower cytotoxicity as assessed by trypan blue assay. In the absence of human-fortilin/TCTP (lanes 1-3), higher intracellular MCL1 levels were still associated with lower cytotoxicity as assessed by trypan blue assay. Experiments were performed in triplicate. *, $p < 0.05$ by ANOVA.

6. In polyclonal cell populations, human-fortilin/TCTP prevents cells from undergoing cell death in the absence of its protein partner MCL1.

To make certain that the independent cytoprotective effects of protein partners human-fortilin/TCTP represented in Figure 33 did not originate from the selection process associated with the establishment of monoclonal cell populations, polyclonal populations of U2OS cells that stably expressed human-fortilin/TCTP ($U2OS_{human\text{-}fortilin/TCTP\text{-}poly}$) were generated. Western blot analyses showed that $U2OS_{human\text{-}fortilin/TCTP\text{-}poly}$ cells robustly overexpressed human-fortilin/TCTP (Figure 36A).

8×10^4 $U2OS_{human\text{-}fortilin/TCTP\text{-}poly}$ cells were seeded onto each well of 24-well plate. Next day, cells were transfected with 25 nM $siRNA_{MCL1}$ and with varying amounts of $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{Luciferase}$ (for 24 h), which were added to keep the total amount of siRNAs constant; challenged the cells with 1 mM 5-FU for 24 h; and then determined their viability using the trypan blue assay. With 25 nM $siRNA_{MCL1}$, there was no detectable MCL1 in the cells (Figure 36B). In this situation, increasing the amount of $siRNA_{human\text{-}fortilin/TCTP}$ caused the intracellular human-fortilin/TCTP concentration to drastically decrease. The reduction of intracellular human-fortilin/TCTP was associated with the increase in susceptibility of the cells to 5-FU-induced cell death ($p < 0.0001$ by ANOVA). In summary, human-fortilin/TCTP protected U2OS cells from 5-FU-induced cytotoxicity in a dose-dependent manner in the absence of MCL1. These data again suggest that human-fortilin/TCTP does not require the presence of MCL1 to protect U2OS cells from 5-FU-induced cell death.

The data derived from monoclonal cell population was entirely consistent with that derived from polyclonal cell population. Together, these data strongly

suggest that antiapoptotic protein, human-fortilin/TCTP, independently protect cells from 5-FU-induced cytotoxicity.

7. In polyclonal cell populations, MCL1 prevents cells from undergoing cell death in the absence of its protein partner human-fortilin/TCTP.

To make certain that the independent cytoprotective effects of protein partners MCL1 represented in Figure 35 did not originate from the selection process associated with the establishment of monoclonal cell populations, polyclonal populations of U2OS cells that stably expressed MCL1 ($U2OS_{MCL1\text{-}poly}$) were generated. Western blot analyses showed that $U2OS_{MCL1\text{-}poly}$ cells robustly overexpressed MCL1, respectively (Figure 37A).

8×10^4 $U2OS_{MCL1\text{-}poly}$ cells were seeded onto each well of 24-well plate. Next day, cells were transfected with 25 nM $siRNA_{human\text{-}fortilin/TCTP}$ and with varying amounts of $siRNA_{MCL1}$ and $siRNA_{Luciferase}$ for 24 h, challenged the cells with 1 mM 5-FU for 24 h, and then determined their viability using the trypan blue assay. With 25 nM $siRNA_{human\text{-}fotilin}$/TCTP, there was no detectable human-fortilin/TCTP in the cells (Figure 37B). In this situation, increasing the amount of $siRNA_{MCL1}$ caused the intracellular MCL1 concentration to drastically decrease. The reduction of intracellular MCL1 was associated with the increase in susceptibility of the cells to 5-FU-induced cell death ($p < 0.0001$ by ANOVA). In summary, MCL1 protected U2OS cells from 5-FU-induced cytotoxicity in a dose-dependent manner in the absence of human-fortilin/TCTP. These data again suggest that MCL1 does not require the presence of human-fortilin/TCTP to protect U2OS cells from 5-FU-induced cell death.

The data derived from monoclonal cell population was entirely consistent with that derived from polyclonal cell population. Together, these data strongly suggest that antiapoptotic protein, MCL1, independently protect cells from 5-FU-induced cytotoxicity.

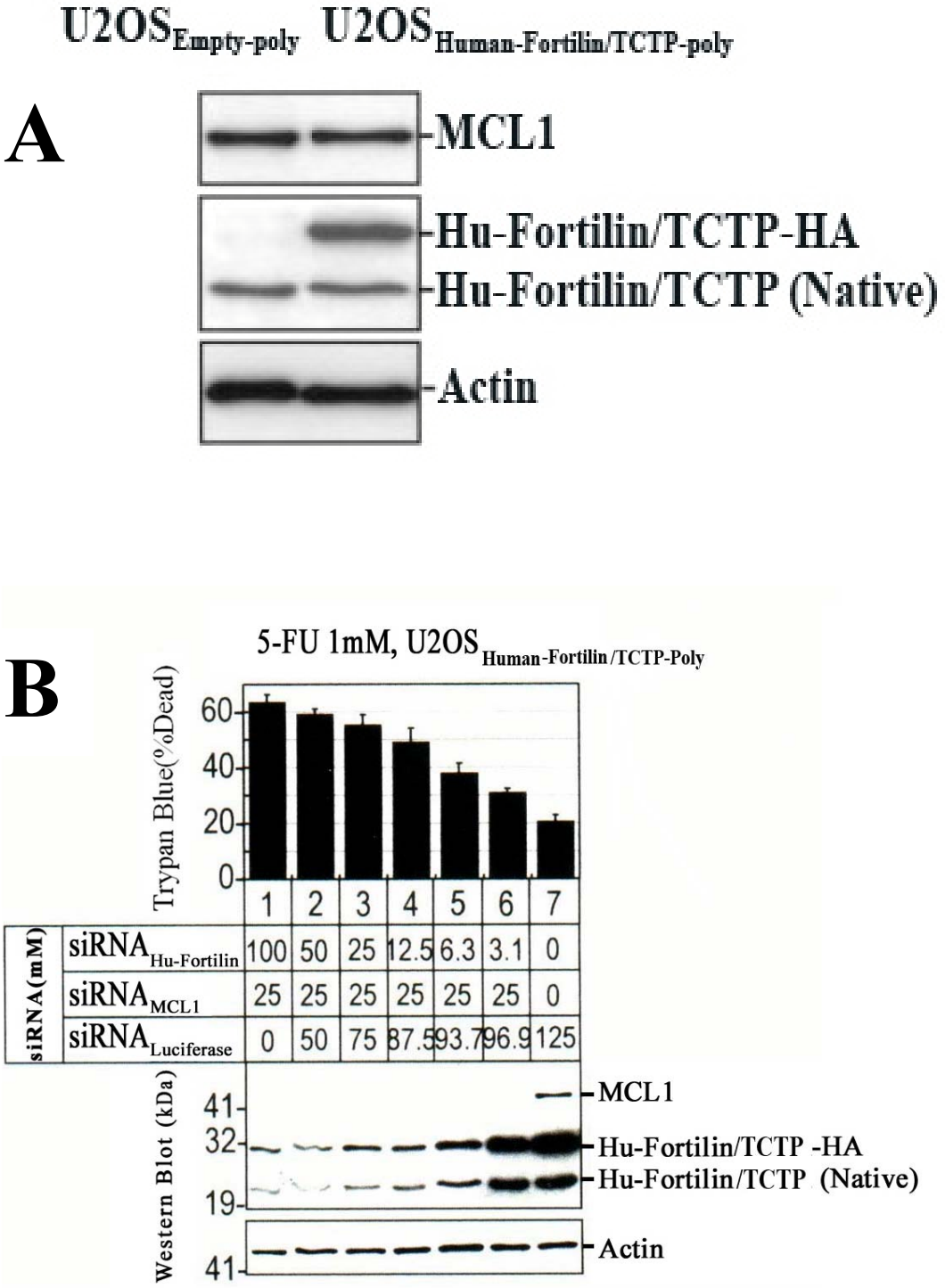
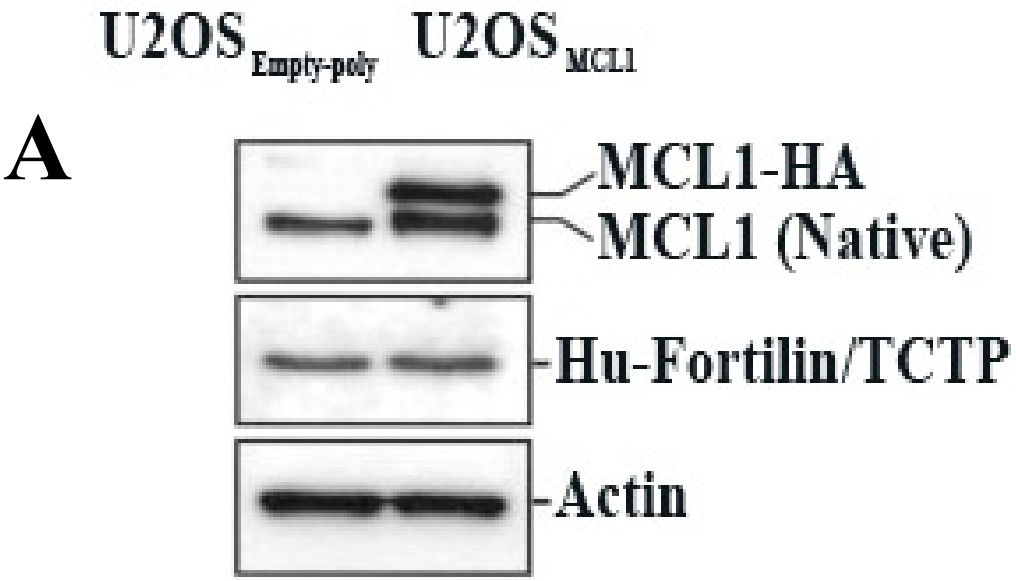


Figure 36. Human-fortilin/TCTP protects cells from undergoing cell death in the absence of its protein partner, MCL1, as shown in studies using polyclonal cell population.

A, characterization of a polyclonal population of U2OS cells stably expressing human-fortilin/TCTP ($U2OS_{human\text{-}fortilin/TCTP\text{-}poly}$). Western blot analyses using anti-MCL1, anti-human-fortilin/TCTP, and anti-actin antibodies showed that $U2OS_{human\text{-}fortilin/TCTP\text{-}poly}$ cells contained a significantly higher concentration of human-fortilin/TCTP but expressed the same amount of MCL1. *Human-fortilin/TCTP-HA*, human-fortilin/TCTP encoded which $pcDNA4_{human\text{-}fortilin/TCTP\text{-}HA}$. ***B***, Dose Dependent protection of 5-FU-challenged U2OS cells by human-fortilin/TCTP in the absence of MCL1. $U2OS_{human\text{-}forilin/TCTP\text{-}poly}$ cells were transfected with 25 nM $siRNA_{MCL1}$ to create a MCL1-free environment and with various amounts of $siRNA_{human\text{-}fortilin/TCTP}$ and $siRNA_{Luciferase}$ to evaluate the dose-dependent nature of human-fortilin/TCTP's cytoprotection. Western blot analyses showed that $siRNA_{MCL1}$-treated $U2OS_{human\text{-}fortilin/TCTP\text{-}poly}$ cells expressed no detectable MCL1. In the absence of MCL1, higher intracellular human-fortilin/TCTP levels were associated with lower cytotoxicity as assessed by trypan blue assay. These data were entirely consistent with those obtained using monoclonal populations of U2OS cells (Figures 32 and 33). Difference in cell death was significant at $p < 0.0001$ by ANOVA. Experiments were performed in triplicate.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U2OS MCL1
A
MCL1-HA
MCL1 (Native)
Hu-Fortilin/TCTP
Actin

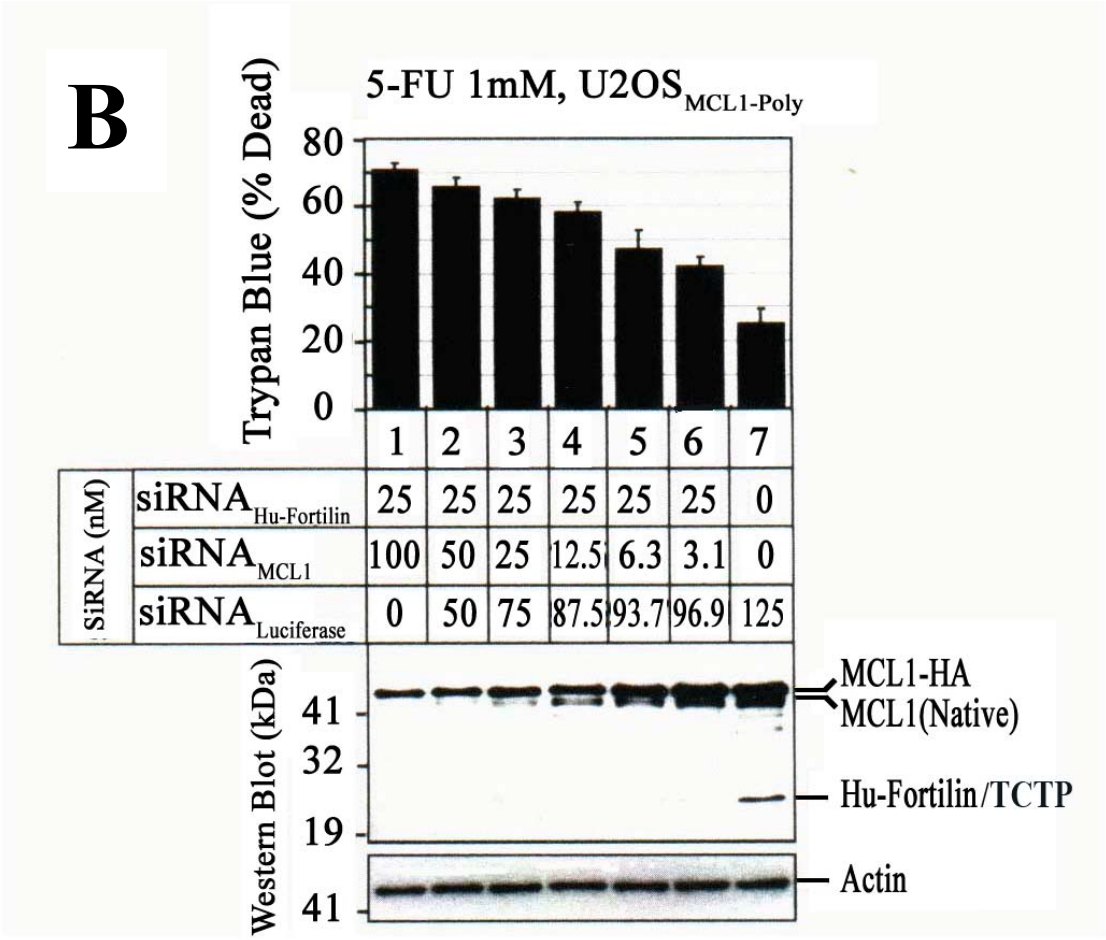
B
5-FU 1mM, U2OS MCL1-Poly
Trypan Blue (% Dead)
80
60
40
20
0
1 2 3 4 5 6 7
SiRNA (nM)
siRNA Hu-Fortilin 25 25 25 25 25 25 0
siRNA MCL1 100 50 25 12.5 6.3 3.1 0
siRNA Luciferase 0 50 75 87.5 93.7 96.9 125
Western Blot (kDa)
41
32
19
41
MCL1-HA
MCL1(Native)
Hu-Fortilin/TCTP
Actin

Figure 37. MCL1 protects cells from undergoing cell death in the absence of its protein partner, human-fortilin/TCTP, as shown in studies using polyclonal cell population.

A, characterization of a polyclonal population of U2OS cells stably expressing MCL1 ($U2OS_{MCL1\text{-}poly}$). Western blot analyses using anti-MCL1, anti-human-fortilin/TCTP, and anti-actin antibodies showed that $U2OS_{MCL1\text{-}poly}$ cells contained a significantly higher concentration of MCL1, but expressed the same amount of human-fortilin/TCTP. MCL1-HA, MCL1 encoded for by $pcDNA4_{MCL1\text{-}HA}$. ***B***, Dose Dependent protection of 5-FU-challenged U2OS cells by MCL1 in the absence of human-fortilin/TCTP. $U2OS_{MCL1\text{-}poly}$ cells were transfected with 25 nM $siRNA_{human\text{-}fortilin/TCTP}$ to create a human-fortilin/TCTP-free environment and with various amounts of $siRNA_{MCL1}$ and $siRNA_{Luciferase}$ to evaluate the presence of the dose-dependent nature of MCL1's cytoprotection. Western blot analyses showed that $siRNA_{human\text{-}fortilin/TCTP}$-treated $U2OS_{MCL1\text{-}poly}$ cells expressed no detectable human-fortilin/TCTP. In the absence of human-fortilin/TCTP, higher intracellular MCL1 levels were associated with lower cytotoxicity as assessed by trypan blue assay. These data were entirely consistent with those obtained using monoclonal populations of U2OS cells (Figures 34 and 35). Difference in cell death was significant at $p < 0.0001$ by ANOVA. Experiments were performed in triplicate.