4. METERIALS AND METHODS

I. Materials

1. Bacterial strains

Escherichia coli XL1-Blue strain: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^{r})] was purchased from Stratagene (USA).

Escherichia coli TOP10F' strain: [F'{*lac*I^{q}, Tn*10*(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC), Ø80*lacZ*ΔM15Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R})] *end*A1 *nup*G was purchased from Invitrogen (USA).

2. Animals

Adult *Penaeus monodon* 12-16 g in weight were collected from shrimp farm in Songkhla and Satun provinces, Thailand. The animals were maintained in 60 liter aquarium tanks with salinity between 20-25 ppt at room temperature for two days before starting the experiment. During cultivation, shrimp were fed commercial pellets and fresh squid.

3. WSSV viral stock

WSSV stock for experimental infections was prepared according to the method described by Supamattaya et al. (1998). Infected tissues (mesodermal, eyestalk, gill, filament and heart) were isolated from WSSV-infected *P. monodon* with prominent white spot and were ground into powder in the presence of liquid nitrogen

and resuspended in K199 medium. The ratio of tissue to K199 medium was 1:5. After centrifugation at 3000 rpm for 10 min at 4^{o}C, the supernatant was filtered through a 0.45 μm sterile membrane. The stock solution of virus was kept in K199 medium at -80^{o}C and thawed at 4^{o}C before use.

4. Primers

The nucleotide primers for RT-PCR, as shown in Table1, were purchased from Life Technologies, USA.

5. Plasmid Vector

- pGEM® - Teasy Vector was purchased from Promega (USA).
- pcDNA4/HisMax C Vector was purchased from Invitrogen (USA).

6. Cell line

The U2OS cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acid, 1% L-glutamine, and 1% antibiotics (penicillin/steptomycin) in the presence of 5% CO_2.

7. siRNA

The siRNAs, as shown in Table 2, were purchased from Dharmacon Research, Inc., USA.

8. Chemicals

All chemicals and solvents (analytical grade) used in this thesis were purchased from Life Technologies, USA; Fisher Biotechnology, USA; Sigma, USA; PIERCE, USA; Amersham Biosciences, UK; QIAGEN, Germany; and BIO-RAD, USA.

9. Enzymes

All enzymes (T4 DNA ligase) and other modifying enzymes (*Bam*HI, *Eco*RI, *Sal*I, *Xho*I) were purchased from New England Biolabs, USA; and Invitrogen, USA.

10. Antibodies

All antibodies used in this thesis were purchased from Santa Cruz Biotechnology, Inc., USA; Covance, USA; Jackson ImmunoResearcg Laboratories, USA; Molecular probes, USA; Sigma, USA; and Roche Molecular Biochemicals, USA.

Table 5. The sequences of the optimal primer used for reverse transcription PCR analysis (RT-PCR).

Primer	PCR fragment size (bp)	Sequence	Tm (ºC)
5'-ß-Actin	500	5'-CAGATCATGTTYGAGACCTTC-3'	46
3'-ß-Actin	500	5'-GATGTCCACGTCRCACTTCAT-3'	48
5'-Pm-fortilin/TCTP	500	5'-CTCACCAGTCGAGAATTTAGCGACG-3'	54
3'-Pm-fortilin/TCTP	500	5'-CTGGCCGCTCTTCTCCATCAATGTC-3'	56

Table 6. The target genes and sequences of siRNA.

Target gene	siRNA
Human-fortilin/TCTP	5’-AGATGTTCTCCGACATCTA-3’
	5’-CGAAGGTACCGAAAGCACA-3’
	5’-GGGAGATCGCGGACGGGTT-3’
	5’-GGTACCGAAAGCACAGTAA-3’
MCL1	5’-AAACGGGACTGGCTAGTTA-3’
	5’-TCACAGACGTTCTCGTAAG-3’
	5’-CGAGTGATGATCCATGTTT-3’
	5’-GGGACTGGCTAGTTAAACA-3’
Luciferase	5’-CGTACGCGGAATACTTCGA-3’

II. Methods

1. Expression of Pm-fortilin/TCTP in WSSV-infected shrimp

1.1 WSSV experimental infection

Shrimp were divided into two groups; WSSV infected group and mortality group. Both groups were inoculated intramuscularly with 100 µl of a $1:10^6$ dilution of the WSSV stock solution ($LD_{50} = 4.5x10^6$). After injection, haemolymph of WSSV infected group was withdrawn using a 26 ga/l inch needle fitted onto a 1.0 cc insulin syringe. For the mortality experiment, moribund shrimp were monitored for 2 days and haemolymph was withdrawn at 1.5 days post injection, and the total RNA was extracted.

1.2 Isolation of total RNA from haemolymph of *P. monodon*

Total RNA was prepared from haemolymph using Trizol LS reagent (GIBCO BRL). The detailed protocol used is as follow; after withdrawing the haemolymph from shrimp, 200 µl of haemolymph was vigorously mixed in 200 µl of Trizol LS reagent and vortex. The homogenized samples were incubated for 5 min at 30ºC to permit the complete dissociation of nucleoprotein complexes and then 0.1 ml of chloroform was added. The sample tube was vigorously shaken by hand for 15 sec and incubated for 2-3 min at room temperature. The sample was centrifuged at 12,000 rpm for 15 min at 4ºC. Subsequently, the aqueous phase was removed and put into a fresh tube and mixed with 0.25 ml of isopropyl alcohol to precipitate RNA. The sample was incubated at 30ºC for 20 min and, then, centrifuged at 12,000 rpm for 15 min at 4ºC for RNA precipitation. The pellet was washed with 0.5 ml of 75% ethanol followed by centrifugation at 7,500 rpm for 5 min at 4ºC. The RNA pellet was dried in

a vacuum, resuspended in 50 µl of RNase free water and incubated at 60ºC for 10 min in order to increase its solubility. The RNA solution was stored at -70ºC. The quantity of the RNA was determined by spectrophotometry at 260 nm and analyzed by formaldehyde agarose gel electrophoresis (Appendix A, 1).

1.3 Reverse transcription (RT)-PCR analysis

RT-PCR was used to confirm the induction and differential expression of the selected sequences. The primers (5'- and 3'-) were designed from the select sequences and synthesized by Life Technologies as shown in Material, Table 5. β-actin primer was used to amplify a PCR product 500 bp in the RT-PCR experiments as an internal control of gene expression to insure that the RT-PCR reaction tube for each sample contains the same amount of total RNA and that the RNA is intact. Total RNA (1 µl) was used as template in RT-PCR reaction mixture according to the manufacturer's instruction (QIAGEN OneStep RT-PCR, QIAGEN). The PCR reaction was performed in a total volume of 50 µl containing 1 µg of total RNA isolated from WSSV uninfected and infected samples, 1x QIAGEN One step RT-PCR buffer, 0.4 mM dNTP, 0.6 µM of each primer and 2 µl of QIAGEN One step RT-PCR enzyme mix. The thermal profile for RT-PCR condition was 50ºC for 30 min followed by an initial PCR activation step at 95ºC for 15 min. This was followed by 30 cycles of 94ºC for 1 min, specific annealing temperature for 1 min and 72ºC for 1 min. After amplification, 5 µl of the RT-PCR products were separated by 1.8 % agarose gel electrophoresis (Appendix A, 2) and revealed by ethidium bromide staining under UV-light. The amount of RNA in each sample was standardized by amplifying the ß-actin transcripts. The identifications of PCR products were confirmed by constructing

into pGEM-TEasy (Promega) (Appendix A, 3) and transformed into *E. coli* TOP10F' (Appendix A, 4). Plasmids containing the inserts were purified with the QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5) and sequenced by using the ABI Prism BigDye Terminator Cycle Sequencing kit with visualization of results on an ABI Prism 377 DNA Sequencer (Appendix A, 6).

2. siRNA system and apoptosis assay

2.1 Cells, cell lines and culture conditions

The U2OS cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acid, 1% L-glutamine and 1% antibiotics (penicillin/steptomycin) in the presence of 5% CO_2.

2.2 siRNA system (Elbashir et al., 2001a; 2001b)

The siRNAs against luciferase, human-fortilin/TCTP, and MCL1, were synthesized at Dharmacon Research, Inc. (Lafayette, CO). The siRNA against human-fortilin/TCTP ($siRNA_{human\text{-}fortilin/TCTP}$) consisted of the mixture of 4 siRNA duplexes targeting 4 different regions of human-fortilin/TCTP mRNA (Table 6). Similarly, the siRNA against MCL1 ($siRNA_{MCL1}$) consisted of the mixture of 4 siRNA duplexes targeting 4 different regions of MCL1 mRNA (Table 2). The siRNA against luciferase, a nonmammalian protein from *Photinus pyralis* (American firefly), was used as control. Luciferase target sequence (cDNA) as shown in Table 2; sense siRNA, 5'-CGUACGCGGAAUACUUCGAdTdT-3' and antisense siRNA: 5'-

UCGAAGUAUUCCGCGUACGdTdT-3'. All procedures were performed under RNAse free environment, using RNase-free water, eppendorf tubes, and pipette tips (Ambion). The transfection cells with siRNA-duplexes were performed using TransIT-TKO Transfection reagent (Mirus corp.), at the final concentration of 1.1%. The serum free medium (F-12K) was mixed with TransIT-TKO Transfection reagent and siRNA. The mixture was incubated for 10 min at room temperature and then mixed with DMEM medium. The old medium was removed from cells and then the mixture was added into plate. In order to minimize cytotoxicity from the reagent, cells were washed once with PBS and the media changed at 6 h after the transfection.

2.3 Cell harvesting (Li et al., 2001; Zhang et al., 2002)

Cells were harvested by trypsinization, washed twice with 5 ml of cold PBS, and centrifuged at 1,000 rpm for 5 min. To prevent protein degradation, the cell pellet was immediately transferred into liquid nitrogen, and then the frozen pellet was resuspended in lysis buffer (lysis buffer or RIPA buffer: 150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) NP-40, 0.25 % (w/v) Sodium deoxycholate, 1 mM EDTA). The cell mixture was vigorously vortex and subsequently incubated on ice for 10 min followed by centrifugation for 10 min at 4°C to collect the protein lysate. The supernatant was determined the protein concentration by Bradford's method (Appendix A, 7) and, then, treated at 45°C for 1 h in 4% SDS gel loading buffer (40% (v/v) glycerol, 200 mM Tris-HCl (pH 6.8), 400 mM DTT, 8% (w/v) SDS, 8% (v/v) 2-mercapto-ethanol, 0.4% (w/v) Bromophenol blue). The genomic DNA in the lysate was sheared by passing the lysate through a 27-gauge needle five times. The concentration of protein was determined by Bradford's method (Appendix A, 7). The samples were then

subjected to 12% SDS-PAGE (Appendix A, 8) and Western Blot Analysis, using anti-human-fortilin/TCTP (Dr.Fujise's lab), anti-MCL1 (Santa Cruz Biotechnology, Inc), and anti-actin (Roche Molecular Biochemicals) antibodies (Method 2.5).

2.4 Transfering protein to nitrocellulose membrane

After the protein samples were separated on 12% SDS-PAGE, the gel was soaked in water for 5 min. The nitrocellulose membrane and 3M filter paper (four pieces) were cut in the same size as that of the gel and soaked in blotting buffer (100 mM glycine, 50 mM Tris, 20% methanol). The two pieces of 3M filter paper were placed in Mini Trans-Blot sandwiches (Bio-Rad), and followed by polyacrylamide gel, nitrocellulose membrane and two pieces of wet 3M filter paper. The electroblotting was performed at a constant current, 35 mA overnight.

2.5 Western blot analysis of cell lysates (Li et al., 2001; Fujise et al., 2000)

The protein samples were transferred onto a nitrocellulose membrane. The membrane was blocked by blocking buffer (5% (w/v) skim milk in TTBS (0.5% (v/v) Tween® 20, 154 mM NaCl, 40 mM Tris-HCl, 48 mM Tris-base)) for 1 h and washed three times for 5 min each time with 1% (w/v) skim milk in TTBS buffer. The membrane was, then, incubated in 1° antibody (diluted with 5% skim milk in TTBS) for 1 h. The blot was washed three times for 5 min each time with 1% (w/v) skim milk in TTBS buffer. The membrane was incubated in 2° antibody (diluted with 5% skim milk in TTBS) for 1 h and, then, washed three times for 10 min each time with TTBS buffer. Bound antibodies were detected by using in an equal volume of Supersignal West Pico stable/peroxide solution with Supersignal West Pico luminol/enhancer

solution for 1 min (SuperSignal West Pico Chemiluminescent Substrate, PIRCE). The membrane was probed by using anti-human-fortilin/TCTP (Dr.Fujise's lab), anti-MCL1 (Santa Cruz Biotechnology, Inc) and anti-actin (Roche Molecular Biochemicals) antibodies. The excess detection reagent was drained off and the membrane was wrapped in Saranwrap. Then the membrane was placed in the film cassette containing autoradiography film (X-ray film from Kodak, USA), and exposed for 1 min. The X-ray film was developed using developing machine (Konica, USA).

2.6 Trypan Blue Assay

To examine anti-apoptotic activity of human-fortilin/TCTP in cell death, U2OS cells and U2OS overexpressing cells were exposed to various cell death stimuli; 0 μM - 10 μM of campothecin, 0 mM - 1 mM cisplatin, 0 μM - 100 μM of doxorubicin, 0 mM - 2 mM of etoposide, 0 mM - 2 mM of 5-fluorouracil, 0 mM - 1 mM of H_2O_2, 0 mM - 2 mM of hydroxyurea, 0 μM - 1 μM of staurosporine, 0 mM - 1 mM of thapsigargin, 0 nM - 100 nM of TNF-α + 10 μM of cycloheximide and incubated for 24 h. All reagents were purchased from Sigma. The mortality of cells was, then, determined as a percentage of trypan blue-stained cells in total cells.

Cytotoxicity was assessed by trypan blue exclusion assay as previously described by Lissy et al. (2000). Cells were harvested after the addition of death stimuli reagents, by a brief trypsinization. Cells were then transferred into 1.5 ml microcentrifuge tube and centrifuged at 3,000 rpm for 7 min. Both floating and attached cells were subjected to the assay. After centrifugation, the pellet was resuspended with 100 μl of new medium and 100 μl of 0.4% of trypan blue stained

solution (GIBCO). At least 150 cells were counted per treatment after being stained by trypan blue at the final concentration of 0.2%. Assays were performed in triplicate.

3. Generation of cells stably overexpressing human-fortilin/TCTP, Pm-fortilin/TCTP and MCL1.

3.1 Construction of recombinant plasmid

3.1.1 Construction of human-fortilin/TCTP or MCL1 gene into pcDNA4/HisMax C vector

To express human-fortilin/TCTP or MCL1 protein in U2OS cells, human-fortilin/TCTP-HA or MCL1-HA was subcloned from pcDNA6B into pcDNA4/HisMax C (Invitrogen) by using restriction enzymes, *Bam*HI and *Eco*RV (Appendix A, 9). The human-fortilin/TCTP and MCL1 cDNAs were fused with the nucleotide sequence encoding for influenza virus hemaglutinin (HA)-tagged at their 3' termini. After digestion had been completed, the digested products were analyzed by agarose gel electrophoresis (Appendix A, 10) and purified by QIAquick Gel Extraction kit (QIAGEN) (Appendix A, 11).

3.1.2 Construction of Pm-fortilin/TCTP gene into pcDNA4/HisMax C vector

To express Pm-fortilin/TCTP protein in U2OS cells, Pm-fortilin/TCTP was subcloned from pQE40 into pcDNA4/HisMax C by using restriction enzymes, *Bam*HI, Sal*I* and *Xho*I (Appendix A, 9). After digestion, the digested products were analyzed by agarose gel electrophoresis (Appendix A, 10) and purified by QIAquick Gel Extraction kit (QIAGEN) (Appendix A, 11).

3.2 DNA ligation into pcDNA4/HisMax C

After digestion and purification, purified DNA fragments and pcDNA4/HisMax C vector were ligated by T4 DNA ligase (Invitrogen) under the following condition; 4 µl of 5x Ligase buffer, T4 DNA ligase, 1 µl of digested pcDNA4/HisMax C, 1 unit of T4 DNA ligase and 13 µl of digested DNA fragment. The reaction mixture was incubated at 16 °C for 1 h. Then, the ligation mixture was precipitated by using seeDNA^{TM} Co-precipitant kit (Amersham Biosciences) (Appendix A, 12) and transformed into *E. coli* XL1-blue by electroporation method (Appendix A, 13). Plasmids containing the inserts were purified with the QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5.2 and 14) and sequenced by using the ABI Prism BigDye Terminator Cycle Sequencing kit with visualization of results on an ABI Prism 377 DNA Sequencer (Appendix A, 15).

3.3 Generation of cells monoclonal overexpressing human-fortilin/TCTP or Pm-fortilin/TCTP or MCL1

To generate U2OS cells stably over-expressing either human-fortilin/TCTP or Pm-TCTP/fortilin or MCL1, one million cells of U2OS were culture in 5 dishes of 10 cm dish. For control, U2OS cells were transfected with empty pcDNA4. U2OS cells were transfected using FuGENE6 reagent (Roche Molecular Biochemicals). The reaction mixtures contained 160 µl of DMEM without serum, 30 µl of FuGENE6 and 10 µg of pcDNA4-His-Max (pcDNA4), a mammalian expression vector containing a Zeocin selection marker (Invitrogen, Carlsbad, CA), containing the cDNA encoding

human-fortilin/TCTP (pcDNA4-His-Max$_{human\text{-}fortilin/TCTP\text{-}HA}$) or Pm-fortilin/TCTP (pcDNA4-His-Max$_{Pm\text{-}fortilin/TCTP}$) or MCL1 (pcDNA4-His-Max$_{MCL1\text{-}HA}$) or pcDNA4-His-Max$_{Empty}$, and incubated at room temperature for 20 min. After that, the mixtures were dropped wide to U2OS cell and cells were cultured for 24 h. The medium was changed and the cells were continued cultivation for 24 h. For the generation of monoclonal populations of cells, transfected cells were clonally selected for at least 3 weeks by using 400 µg/ml of Zeocin (Invitrogen) and characterized by Western blot analyses using commercial anti-MCL1 antibodies (Santa Cruz Biotechnology), anti-human-fortilin/TCTP antibodies raised in Dr.Fujise's lab and anti-Pm-fortilin/TCTP (Anun, 2004) (Method 2.5). The resulting cell lines were named U2OS$_{empty}$, U2OS$_{human\text{-}fortilin/TCTP}$, U2OS$_{Pm\text{-}fortilin}$/TCTP, and U2OS$_{MCL1}$, respectively.

3.4 Generation of cells polyclonal overexpressing human-fortilin/TCTP or MCL1

To generate polyclonal cell populations (Method 3.3), cells transfected with pcDNA4$_{human\text{-}fortilin/TCTP\text{-}HA}$ or pcDNA4$_{MCL1\text{-}HA}$ or pcDNA4-His-Max$_{Empty}$ were collectively selected in a 10 cm tissue culture dish for 4 weeks with 1500, 800 and 800 µg/ml Zeocin, respectively, and characterized by Western blot analyses using commercial anti-MCL1 antibodies (Santa Cruz Biotechnology) and anti-human-fortilin/TCTP antibodies raised in Dr.Fujise's lab (Method 2.5). The resulting lines were named U2OS$_{human\text{-}fortilin/TCTP\text{-}poly}$, U2OS$_{MCL1\text{-}poly}$, and U2OS$_{Empty\text{-}poly}$, respectively.

3.5 Indirect immunofluorescence and fluorescence microscopy.

For the intracellular localization of human-fortilin/TCTP and MCL1 in the presence or absence of MCL1 and human-fortilin/TCTP, respectively, U2OS cells were seed into 4 well chamber slide, transiently transfected with pcDNA4 containing the cDNA of human-fortilin/TCTP-HA ($pcDNA4_{huamn\text{-}fortilin/TCTP\text{-}HA}$) or $pcDNA4_{MCL1\text{-}HA}$, then treated with $siRNA_{human\text{-}fortilin/TCTP}$, $siRNA_{MCL1}$, or $siRNA_{luciferase}$, (Method 2) and subjected to immunocytochemical staining 48 h later. The medium was removed from slide and cells were washed with 500 µl of 1X PBS. Cells were fixed with 4% paraformaldehyde in PBS and incubated for 30 min. Cells were washed 3 times with 1X PBS and incubated with cold acetone:methanol (1:1) for 10 min at -20ºC. Fixed cells were washed 3 times with PBST (1X PBS with 0.1% Tween®20) and block with 10% normal goat serum in PBST for 30 min. Cells were rinsed with PBST and incubated with mouse anti-HA antibody (Covance) and rabbit anti-human-fortilin/TCTP or anti-MCL1 (Santa Cruz Biotechnology) antibodies for 1 h. Then, cells were washed 4 times with PBST, block with 10% normal goat serum in PBST for 30 min and rinsed with PBST. Bound antibodies were detected by Alexa fluor 488 goat anti-mouse antibody IgG antibody (Molecular Probes) and goat anti-rabbit antibody conjugated to rhodamine Red X (Jackson ImmunoResearch Laboratories) for 30 min and washed with PBS. The nuclear was stained by 4',6-Diamidino-2-phenylindole (DAPI) and then cells were washed 3 times with distilled water. The mounting medium was applied on the cell surface and followed by cover-slide. The stained cells were dried overnight at room temperature and analyzed on a Zeiss Axioskop fluorescence microscope, using a 40x objective and appropriate filter sets (Carl Zeiss Ltd., Germany) as described by Li, 2001. Images were captured using the

Spot RT SE6 Slider Camera (Diagnostic Instrument, Inc., Sterling Heights, USA) and Image Pro Plus software system (Media Cybernetics, Inc., Carlsbad, USA).

3.6 Subcellular fractionation procedure.

U2OS cells (1.5 x 10^6) were seeded onto three 10 cm-dishes. Next day, the cells were transfected with $siRNA_{luciferase}$, $siRNA_{human\text{-}fortilin/TCTP}$, or $siRNA_{MCL1}$ (Method 2.2). Forty-eight hours after the siRNA transfection, cells were harvested by trypsinization (Method 2.3) and cytosolic and nuclear fractions were separated using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce), according to the manufacture's instructions and previously described by Thompson (2003). The ice cold Cytoplasmic Extraction Reagent I (CER I) (100 µl/1 million cells) was added to cell pellet to resuspend and then 11 µl of ice cold Cytoplasmic Extraction Reagent I (CER II) was added. The suspension was vortex on the highest setting for 5 sec, incubated on ice for 1 min following by centrifugation at maximum speed for 5 min. The supernatant was transferred to clean pre-chilled microcentrifuge tube. This solution was a Cytosolic fraction. The pellet was washed 2 times with 1xPBS following by centrifugation at maximum speed for 5 min. The ice cold Nuclear Extraction Reagent (NER) (100 µl/ 1 million cells) was added to cells pellet following by vortex on the highest setting for 30 sec and incubated on ice for 40 min. The solution was centrifuged at maximum speed for 10 min. The supernatant was transferred to clean pre-chilled microcentrifuge tube. This solution was a Nuclear fraction. Protein concentrations were determined by Bradford's methods (Bio-Rad) (Appendix A, 7). Exactly 10 µg of total proteins were resolved by 12% SDS-PAGE

(Appendix A, 8) and subjected to Western blot analyses (Method 2.5). The successful purification of nuclear and cytosolic fractions was confirmed by anti-histone (clone AE-5; Santa Cruz Biotechnology) and anti-tubulin (Sigma) antibodies.