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# Studies On A Novel Human Cardiospecific Transcription Factor And Its Involvement In Omi/htra2 Mediated Cell Death

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# STUDIES ON A NOVEL HUMAN CARDIOSPECIFIC TRANSCRIPTION FACTOR AND ITS INVOLVEMENT IN OMI/HTRA2 MEDIATED CELL DEATH

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## ABSTRACT

Omi/HtrA2 is a mitochondrial serine protease that is known to translocate to the cytoplasm upon induction of apoptosis and to activate caspase-dependent and caspase-independent cell death. The molecular mechanism of Omi/HtrA2's function is not clear but involves degradation of specific substrates. These substrates include cytoplasmic, mitochondrial, as well as nuclear proteins. We have isolated a new Omi/HtrA2 interactor, the THAP5 protein. THAP5 is a fifth member of a large family of transcription factors that are involved in cell proliferation, apoptosis, cell cycle control, chromosome segregation, chromatin modification and transcriptional regulation. THAP5 is an approximately 50kDa nuclear protein, with a restricted pattern of expression. Furthermore, there is no mouse or rat homolog for this protein. THAP5 mRNA is highly expressed in the human heart but some expression is also seen in the brain and skeletal muscle. The normal function of THAP5 in the heart or heart disease is unknown. THAP5 protein level is significantly reduced in the myocardial infarction (MI) area in the heart of patients with coronary artery disease (CAD). This part of the heart sustains most of the cellular damage and apoptosis. Our data clearly show that THAP5 is a specific substrate of the proapoptotic Omi/HtrA2 protease and is cleaved and removed during cell death. The molecular mechanism of THAP5's function is unclear. THAP5 can bind to a specific DNA sequence and repress transcription of a reporter gene. Our work suggests that THAP5 is a tissue specific transcriptional repressor that plays an important role in the normal function of the human heart as well as in the development of heart disease.

# ACKNOWLEDGMENTS

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# CHAPTER 1: GENERAL INTRODUCTION

Omi/HtrA2 is a serine protease and a homolog of the bacterial high temperature requirement A (HtrA/DegP) protein [1]. It was originally identified as an interactor of Mxi2, an alternatively spliced form of p38 stress activated kinase, using the yeast two-hybrid system [1]. It was also isolated independently as an interactor of Presenilin-1 (PS-1) by another group [2]. Full-length Omi is a nuclear encoded 458 amino acid protein, which is designated into the following domains; mitochondrial targeting sequence (MTS) at its amino terminus, a transmembrane segment (TM), inhibitor of apoptosis protein (IAP) binding motif (IBM), serine protease domain and a PDZ domain at the carboxyl terminus [1-4]. Omi undergoes autocatalysis at Ala133 thereby removing the amino terminal region including the MTS and TM domains and exposing the IBM (AVPS) motif. The IBM motif is very similar to Drosophila IAP inhibitor proteins Reaper, Hid and Grim and their mammalian counterpart Smac/DIABLO [4-8]. The figure below sows full length Omi/HtrA2 and its autocatalytic processing.
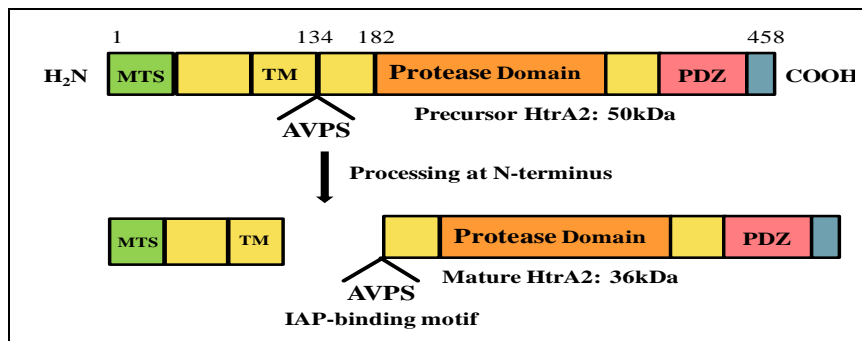


**Figure 1: Schematic Diagram of Omi/HtrA2 and its N-terminal processing**

Upon induction of apoptosis, Omi/HtrA2 gets released into the cytoplasm where it binds to IAPs and proteolytically degrades them, thereby activating the caspase cascade and apoptosis [4, 5]. In addition, it can also activate caspase independent apoptosis that involves its proteolytic

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activity [8]. Homotrimerization of Omi/HtrA2 has been shown to be essential for the protease activity in addition to the presence of the PDZ domain that plays a regulatory role in protein-protein interactions [3]. Omi/HtrA2 is predominantly present in the mitochondrial intermembrane space (IMS), but it has also been found in the endoplasmic reticulum (ER) and nucleus [1, 2, 9, 10]. The mechanism of its pro-apoptotic activity involves interaction and degradation of specific substrates that includes mitochondrial, cytoplasmic and nuclear proteins. The cytoplasmic interacting proteins of Omi/HtrA2 include Inhibitor of Apoptosis proteins (IAPs) such as XIAP, cIAP1, cIAP2, Apollon/Bruce, PED/PEA15, and receptor-interacting protein RIP1 [11-14]. The nuclear interactors include Grim-19, WARTS, p73, and Wilms' Tumor Suppressor protein (WT1) [9, 15-17]. Several of these interactions are mediated via the PDZ domain and the protease activity of Omi/HtrA2 is always necessary and required for its pro-apoptotic function. The table below summarizes some of the known substrates and interactors of Omi reported so far.

**Table 1: List of known interactors of Omi/HtrA2**

| Protein | Localization | Function | References |
|---------|--------------|----------|-----------|
| cIAP1, cIAP2, XIAP, Apollon/Bruce | Cytosol | Inhibitor of apoptotic proteins | [11, 13, 14] |
| Ped/Pea15 | Cytosol | Antiapoptotic protein | [12] |
| Rip1 | Cytosol | Apoptosis, TNF-a signaling | [13] |
| EndoG | Mitochondria | Endonuclease proapoptotic protein | [18] |
| Presenilin-1,APP, γ-secretase | Mitochondria | Precursors of b amyloid senile plaques | [21, 22, 23] |
| **Hax-1** | Mitochondria | Antiapoptotic protein | [36] |
| P73 | Nucleus | Tumor Suppressor, p53 family of proteins | [17] |
| WARTS | Nucleus | Tumor Suppressor, G1/S cell cycle block | [9] |
| Grim-19 | Nucleus/ Cytoplasm | Apoptotic protein | [16] |
| WT1 | Nucleus | Transcription factor/tumor suppressor | [15] |
| **THAP5** | Nucleus | Transcription factor/ Cell Cycle (G2/M block) | [71] |

## Function of Omi/HtrA2 in the mitochondria

It is to be noted that the pro-apoptotic function of Omi/HtrA2 comes to play when it is released into the cytoplasm from the mitochondria. However, while confined in the mitochondria Omi/HtrA2 seems to have yet another function distinct from its pro-apoptotic activity. The other function of Omi/HtrA2 in mitochondria is not yet known, but it is speculated to play a role in cell survival thereby contributing to maintenance of mitochondrial homeostasis [19]. Disturbance of

this function is linked to the development of several disorders including neurodegeneration and tumors.

A single missense mutation Ser276Cys in the protease domain of Omi/HtrA2 results in Motor Neuron Degeneration2 (Mnd2) disease in mice [19, 20]. Mnd2 homozygous mice display striking similarities with Parkinson's disease (PD) phenotype such as lack of bodily coordination, reduced motility and tremor. They also suffer from muscle wasting, neurodegeneration, significant reduction in the size of organs such as heart, kidneys, spleen and thymus and death within 40 days of birth [20]. A similar phenotype results in Omi/HtrA2 knockout mice as well [19]. The loss of Omi/HtrA2 has been shown to result in the selective loss of striatal neurons, accumulation of unfolded proteins in mitochondria, disruption of mitochondrial respiration and also increased susceptibility to apoptotic stimuli [19].

## Omi/HtrA2 and neurodegeneration

Recently, two single nucleotide polymorphisms in the Omi/HtrA2 gene that cause missense mutations (A141S and G399S) have been identified in relation to Parkinson's disease [21]. In all cases, these mutations destroy/ severely impair the proteolytic activity of the enzyme. Some studies have also implicated Omi/HtrA2 in the development of Alzheimer's disease. Originally, Omi/HtrA2 was identified as an interactor of Presenilin-1 in yeast two-hybrid screening [2]. This was followed by another study which showed that the C-terminal portion of Presenilin-1 can interact and enhance the proteolytic activity of Omi/HtrA2 [22]. More recent work has shown that Omi/HtrA2 is involved in the processing of APP [23] and can also interact with gamma-secretase [24], suggesting an important role in Alzheimer's disease as well and possibly some other neurodegenerative disorders such as Huntington's disease [25].

## Omi/HtrA2 in ischemia/reperfusion (I/R) injury

Omi/HtrA2 shows ubiquitous expression in human tissues, and the proteolytic activity of the protein is significantly upregulated upon ischemia/reperfusion (I/R) injury. Not so long ago, a specific inhibitor of Omi/HtrA2 namely ucf-101(5-[5-(2-nitrophenyl)furfuryl iodine]1,3-diphenyl-2-thiobarbituric acid was characterized [26]. This inhibitor has been used successfully in I/R studies in different organs. Using a mouse model of kidney I/R injury, it has been shown that inhibiting Omi/HtrA2 by use of ucf-101 or downregulating the protein by siRNA interference can confer significant protection to kidney cells from cisplatin or I/R mediated apoptosis [26-28]. Recent work has shown that HtrA2 gets translocated to the cytosol from the mitochondria where it promotes death of cardiomyocytes. Use of ucf-101 results in decrease in myocardial apoptosis along with reduction in infarct size, recovery of mean arterial blood pressure and improvement in contractile dysfunction ([29-31]. Not only that, work by Althaus et al also has shown that inhibiting Omi/HtrA2 prior to brain ischemia in rats significantly reduced cellular damage and conferred neuroprotection [32].

## Omi/HtrA2 in cancer

Besides Omi/HtrA2's role in neurological disorders, a lot of recent work has identified potential involvement of the protein in tumor development as well. Omi/HtrA2 has a cell-type specific varied expression levels in several tumors at different stages in comparison to normal tissues [33]. Omi/HtrA2 has been shown to be expressed in lower levels in endometrial and ovarian cancers [34], whereas shows higher expression in prostate cancer and many advanced gastric adenocarcinomas [34, 35]. Interestingly Omi/HtrA2 has also been shown to be involved in

anoikis; a form of apoptosis caused by the disruption of cell attachment and may be possibly helping in removing cancer cells [36].

All these studies put together, seem to point to the fact that Omi/HtrA2 might have an important cell survival function depending on the subcellular localization. One of the approaches used in the Zervos' laboratory to study Omi/HtrA2 function is employing the Yeast Two-hybrid System to identify interacting partner proteins. These interacting proteins could be new substrates of Omi/HtrA2 or modulators of its proteolytic activity. In one such screen two new Omi/HtrA2 interactors were isolated. One of them is Hax-1 (HS-1 associated protein X-1), a mitochondrial anti-apoptotic protein that serves as a substrate for Omi/HtrA2 [37]. Hax-1 gets cleaved in the mitochondria before Omi/HtrA2 translocates to the cytoplasm suggesting that it precedes the cleavage of IAPs that takes place in the cytoplasm [37]. Our work identified a central role for Hax-1 and Omi/HtrA2 in regulation of apoptosis in mammalian cells [37].

The other interactor we isolated is called THAP5, whose function is yet to be characterized and which constitutes the topic of interest of my dissertation. THAP5 belongs to the family of THAP proteins, which are a recently described evolutionarily conserved family of cellular factors [38]. The following chapters of the dissertation will give a detailed characterization of this novel transcription factor which is believed to be important in various activities such as transcription repression, cell cycle arrest, and DNA damage response and also implicated in Coronary Artery Disease (CAD) and several tumors including melanoma.

# CHAPTER 2: THAP5 IS A HUMAN CARDIAC SPECIFIC INHIBITOR OF CELL CYCLE THAT IS CLEAVED BY THE PRO-APOPTOTIC OMI/HTRA2 PROTEASE DURING CELL DEATH

**Introduction**

Omi/HtrA2 is a mitochondrial serine protease that has an essential role in both mitochondrial homeostasis as well as cell death [13]. Most of the studies on Omi/HtrA2 have substantially contributed to our understanding of the mechanism of its pro-apoptotic function. On the contrary very little is known about its normal function within the mitochondria. Upon induction of apoptosis, Omi/HtrA2 is released to the cytoplasm where it participates in caspase-dependent and caspase-independent cell death [4-8]. The mechanism of its pro-apoptotic activity involves degradation of specific substrates that includes mitochondrial protein HAX-1, cytoplasmic proteins XIAP, PEA/PED, and Apollon/Bruce [11, 12, 14, 37, 39] as well as, nuclear factors Grim-19, WARTS, and p73 [9, 16, 17, 40].

The protease activity of Omi/HtrA2 is always necessary and essential for its pro-apoptotic function. We have used the yeast two-hybrid system to isolate and characterize new Omi/HtrA2 interacting proteins [26]. These interacting proteins could be new substrates of Omi/HtrA2 or modulators of its proteolytic activity. Previous studies have shown that the proteolytic activity of Omi/HtrA2 can be regulated through specific protein-protein interactions mediated via its PDZ-domain [41]. In this report, we describe one such new Omi/HtrA2 interactor, the THAP5 protein. The THAP (**Th**anatos-**A**ssociated **P**rotein) family of proteins comprises a group of nuclear factors defined by the presence of an approximately 90-residue protein motif (the THAP domain) [38]. THAP domains are atypical zinc fingers with specific zinc-dependent DNA binding activity and show similarity to the site-specific DNA-binding domain of the P element

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transposase from Drosophila [38, 42]. THAP proteins are transcription factors and the limited information that exists on their function suggests that, they might be involved in gene regulation, cell cycle control, and/or apoptosis [43-45]. The figure below shows a schematic diagram of the human and drosophila THAP proteins.
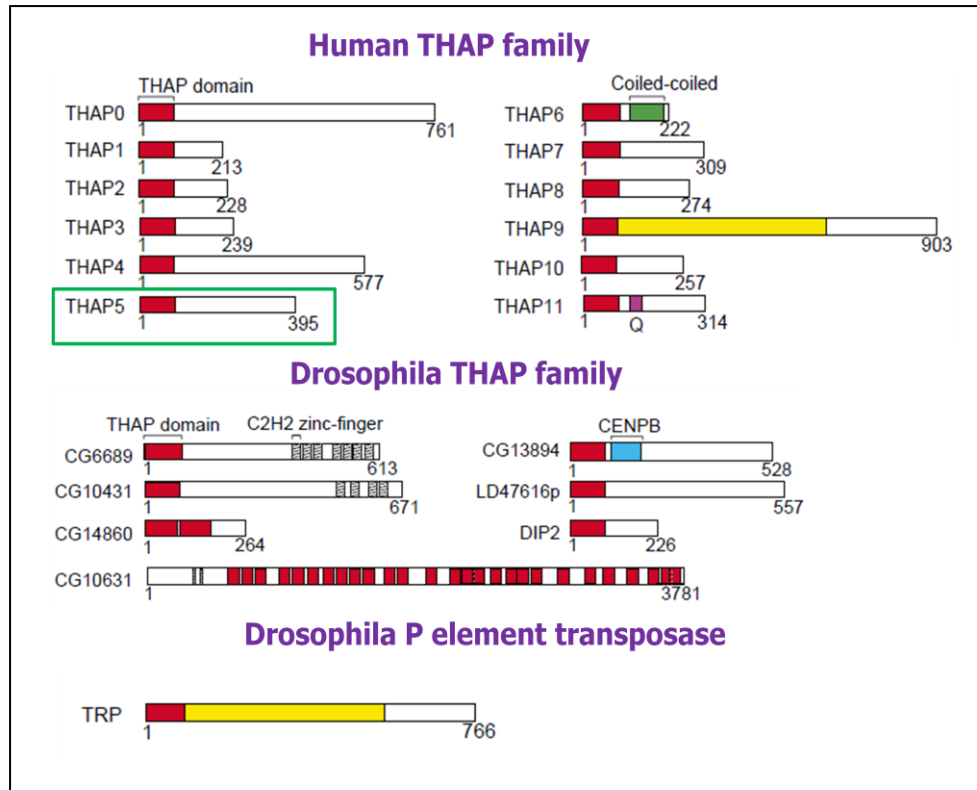


**Figure 2: Schematic Diagram of Human and Drosophila THAP proteins**

THAP5 is the fifth member in the twelve-member family of human THAP proteins and is unique since outside its THAP domain, it shares no sequence homology to any other reported protein. THAP5 interacted with Omi/HtrA2 both in yeast and mammalian cells under pro-apoptotic conditions where Omi/HtrA2 is known to be released from mitochondria to the cytoplasm. Furthermore, THAP5 could be cleaved very efficiently *in vitro* by Omi/HtrA*2* protease. Since very little is known about the function of THAP5 we performed a detailed study to characterize

its normal function and the significance of its interaction and degradation by Omi/HtrA2. We found THAP5 to be a tissue specific nuclear factor that is predominantly expressed in the human heart. Interestingly, there is no mouse or rat orthologue of THAP5; this is a characteristic of some THAP-family members since it has also been reported for four other proteins, namely THAP6, THAP8, THAP9, and THAP10 [44, 46]. The figure below shows a diagram representing the THAP proteins including the human and Drosophila THAP proteins.

The normal function of THAP5 is the regulation of cell cycle and ectopic expression of the protein caused cell cycle arrest. During cell death, THAP5 was cleaved and removed by Omi/HtrA2 in cells treated with cisplatin and $H_2O_2$, but it was not affected in cells treated with etoposide and camptothecin. Using the ucf-101 inhibitor of Omi/HtrA2, we could very effectively block THAP5 degradation and protect cells from undergoing apoptosis. The degradation of THAP5 seen during experimentally induced cell death or cell injury is a physiological event that follows cellular damage and was observed in the myocardial infarction area (MI) of the heart tissues from patients with coronary artery disease (CAD).

## Materials and Methods

*Yeast Two-Hybrid Screen.* We used the yeast two-hybrid system to screen a HeLa as well as a melanocyte cDNA library as previously described [37]. The bait used was the mature, proteolytically active form of the Omi/HtrA2 protein (aa 134-458) cloned in the pGilda (Clontech) bait vector. Several interacting proteins were identified in this screen. One of these Omi/HtrA2 interactors isolated from the melanocyte cDNA library was a partial clone of a previously uncharacterized protein called THAP5. The full-length cDNA for THAP5 encodes 395 amino acids and was isolated from a Marathon Ready human heart cDNA library

(Clontech). The specificity of THAP5 interaction with Omi/HtrA2 in yeast was tested using HtrA1, a mammalian homolog of Omi/HtrA2 that has 68% amino acid sequence similarity. The presence and stability of the recombinant proteins in yeast cells was monitored by Western blot analysis using LexA-antibodies (for baits) or HA-antibodies (for preys).

***Interaction between Omi/HtrA2 and THAP5 in mammalian cells.*** HEK293 cells were transfected in duplicates with either pEGFP-C1 empty vector (Clontech) or EGFP-THAP5 plasmid using Lipofectamine 2000 reagent (Invitrogen). EGFP-THAP5 encodes the full-length THAP5 protein fused in frame to EGFP-C1 vector. Fourteen hours later, half of the cells were treated with cisplatin (50μM) for 10 hours. Cell lysates were prepared using RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 1% NP-40, 0.25% Na-DOC) containing the protease-inhibitor cocktail (Roche). Approximately 200μg of total protein cell lysates were pre-cleared by mixing with protein G-Agarose beads (Roche) for 1 hour followed by incubation with the Omi/HtrA2 polyclonal antibody [37] for 2 hours at 4°C. Protein G-Agarose beads were then added and allowed to bind overnight at 4°C. Immunoprecipitates were collected by brief centrifugation, washed extensively with RIPA buffer, and resolved by SDS-PAGE. They were then electro-transferred onto a PVDF membrane and probed with a mouse monoclonal GFP antibody (Santa Cruz Biotechnology) followed by a secondary goat anti-mouse HRP-conjugated antibody and the immunocomplex visualized by ECL (Pierce). We also performed the reverse of this experiment by transfecting HEK293 cells with pEGFP-N1-Omi (1-458). Approximately 200μg of total protein cell lysates were pre-cleared by mixing with protein G-Agarose beads (Roche) for 1 hour and then incubated with THAP5 polyclonal antibody, followed by Western blot using the GFP antibody as described above.

***Northern blot analysis of THAP5 mRNA expression in human tissues***.  A human mRNA tissue blot (Clontech) representing twelve human tissues was probed with a radiolabeled DNA probe corresponding to THAP5 protein sequence residues 163-395.  This DNA sequence is unique and has no homology to any other known gene in the GenBank.  The blot was hybridized with the radiolabeled probe at 42°C, washed at 65°C, and subjected to autoradiography [1].  To verify that equal amount of mRNA was present on each lane, the blot was stripped and re-probed for β-actin mRNA expression.

***Degradation Assay***.  The ability of His-Omi$_{134-458}$ to cleave recombinant His-THAP5 protein *in vitro* was investigated.  The full-length cDNA for the THAP5 protein was cloned in frame in pET-28 vector (Novagen).   Bacterially expressed recombinant His-THAP5 was purified on nickel-nitrilotriacetic acid (NTA-agarose) affinity resin as described [37].  The synthesis and properties of the His-Omi$_{134-458}$ has been previously reported [37].  His-Omi$_{134-458}$ was incubated with His-THAP5 in 15μL of reaction volume in assay buffer (20mM Na$_2$HPO$_4$ pH 8, 200mM NaCl and 5% glycerol).  In some of the reactions, the ucf-101 inhibitor was used.  After various incubation times at 37°C the reactions were stopped through the addition of SDS-sample buffer.  Reaction products were analyzed by SDS-PAGE followed by Coomassie blue staining.

***Cell Culture***.  HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Hyclone), 2mM L-glutamine, 1.5g/L sodium bicarbonate, 1mM sodium pyruvate, 50 units/ml penicillin, 50μg/ml streptomycin (Invitrogen).  HeLa cells were grown in DMEM supplemented with 10% fetal calf serum, 2mM L-glutamine, 50units/ml penicillin, 50μg/ml streptomycin.

***Sub-cellular localization of the THAP5 protein.*** To investigate the sub-cellular localization of the THAP5 protein, the full-length cDNA (aa 1-395) was cloned in frame into EGFP-C1 vector (Clontech). Furthermore, DNA sequence corresponding to amino acids 1-162 or 163-395 of THAP5 protein were also amplified by PCR and cloned into EGFP-C1 vector. HeLa cells were grown on glass cover slips in 12-well plates. Approximately 60% confluent cells were transiently transfected with 1μg of the various GFP-constructs using Lipofectamine 2000 Transfection Reagent (Invitrogen). Twenty-four hours after transfection, cells were washed and fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100 and incubated with Texas red-phalloidin (Molecular Probes) to stain cytoplasm. The cover slips were then washed and placed on microscope slides using Fluoromount-G as the mounting solution. Slides were observed using a LSM510 confocal laser-scanning microscope (Zeiss). The expression and stability of the various GFP-fusion proteins was verified by Western blot analysis using GFP-specific antibodies (Santa Cruz Biotechnology).

***Expression, purification of His-tagged THAP5163-395 protein and antibody production.*** PCR was used to amplify DNA sequence corresponding to a partial carboxyl terminus THAP5 (aa 163-395) polypeptide. The PCR product was cloned in-frame in the bacterial expression vector pET-28 (Novagen). For protein expression, BL21 (DE3) (Novagen) bacteria were transformed with pET-THAP5 and single colonies were grown overnight in LB medium containing Kanamycin. The overnight culture (1ml) was used to inoculate 1L of LB medium and growth was continued at 37°C until the $OD_{600}$ was approximately 0.8. At this time, 2mM IPTG was added and the culture was placed in a shaking incubator for 4 hours at 25°C. Bacteria were harvested by centrifugation and lysed in a buffer containing 6M Urea, 0.1M

NaH$_2$PO$_4$, 20mM Tris-HCl pH 8, and a protease inhibitor cocktail (Sigma). The bacterial suspension was then sonicated and recombinant His-THAP5$_{163-395}$ protein purified using Ni-NTA His-Bind Resin (Novagen). The quality of the His-THAP5$_{163-395}$ protein was monitored by SDS-PAGE followed by Coomassie blue staining. To make the polyclonal antibody, 15mg of purified human His-THAP5$_{163-395}$ was used to immunize two rabbits. Immunization, production, and purification of the polyclonal THAP5 antibody were performed by New England Peptide Inc.

*Western blot analysis of THAP5 protein expression in various human tissues.* Since THAP5 is expressed only in human cells we used a commercially available Western blot that represents five human tissues (Calbiochem). Each lane of the blot contains 10μg of crude protein extract from the corresponding tissue. We used our THAP5 polyclonal antibody at a 1:5000 dilution. The method and conditions for the Western blot analysis have been previously described [27, 37].

*Cell Cycle Analysis.* Human embryonic kidney cells (HEK293) were seeded onto six-well plates, synchronized with serum starvation [47, 48], and then transiently transfected with EGFP-C1 empty vector (Clontech) or EGFP-THAP5 using Lipofectamine 2000 Transfection Reagent (Invitrogen). Forty or fifty hours after transfection, cells were washed and fixed in 4% paraformaldehyde (PFA). The cells were then washed, permeabilized with 0.1% Saponin and stained with 7-amino-actinomycin D (7-AAD) (5μL per 100μL of PBS) and incubated for 30 minutes at 37°C. GFP positive cells in the transfected population were measured using a FACSCalibur Flow Cytometer (BD Biosciences) [49] and analyzed using ModFitLT software.

*Cell Death Assays.* HeLa cells were treated for 12 hours with different chemicals: camptothecin (100μM), cisplatin (5μM), etoposide (20μM), H$_2$O$_2$ (0.2mM) and Staurosporine

(100µM). HeLa cells were grown in six-well plates in the appropriate medium until they reached 70-80% confluency; they were then treated with ucf-101 (20 or 30µM) for 20 minutes followed by cisplatin (5µM) or $H_2O_2$ (0.2mM) treatment for 12 hours. Cells were detached with 1X Trypsin-EDTA (Gibco), washed twice with ice-cold PBS, half of them were used for Western Blot analysis and the rest for apoptotic assay. Western Blot analysis was performed as described [27, 37]. The apoptotic assay was performed according to BD Biosciences protocol. Briefly, cells were suspended in 1X binding buffer and stained with Annexin V (apoptotic cell) and 7-AAD (necrotic cells) [50-52]. Samples were analyzed on a FACSCalibur Flow Cytometer (BD Biosciences).

*Expression of THAP5 in the heart of patients with coronary artery disease (CAD).*
Human cardiac tissues were prepared as described [53]. Briefly, human cardiac tissue samples were taken from the left ventricles of failing human hearts that were explanted in the course of heart transplantation. The study's protocol was approved by the local ethics committee, and written informed consent given by patients, according to the National Disease Research Interchange (NDRI). Hearts from patients with end-stage heart failure who were undergoing cardiac transplantation because of either DCM (Dilated Cardiomyopathy) or CAD (Coronary Artery Disease) were investigated. Furthermore, healthy donor (HD) hearts that were ultimately rejected for transplantation because of technical reasons were also included in this study as healthy tissue control. For Western blot analysis, 100 mg of tissue was ground in liquid nitrogen and homogenized with an Ultra-Turrax T8 (IKA-werke) in ice-cold lysis buffer containing 150mM NaCl, 20mM Tris-HCl pH 7.6, 1mM $CaCl_2$, 1mM $MgCl_2$, 10% Glycerol, 1% NP40 and protease and phosphatase inhibitors cocktail (Roche). Homogenate was cleared by

centrifugation for 10 min at 14,000 g. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad). Approximately 20μg of whole cell extract was resuspended in SDS-sample buffer and boiled for 3 minutes. Samples were resolved by SDS-PAGE and electro-transferred onto PVDF membranes (Pall Life Sciences) using a Semi-Dry cell Transfer Blot (Bio-Rad). 4% nonfat dry milk in TBST buffer was used to block any non-specific binding. The membrane was incubated with our THAP5 polyclonal antibody (1:5000) followed by a secondary HRP-conjugated goat anti-rabbit (Jackson ImmunoResearch) (1:15000) and visualization by ECL (Pierce).

*Statistical analysis.* All quantitative data are expressed as mean ±SD. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Post-hoc test. A value of $P<0.05$ was considered significant.

## Results

**Isolation of THAP5 as an Omi/HtrA2 interactor.** We used the yeast two-hybrid system to isolate Omi/HtrA2 interactors. We screened two different cDNA libraries derived from HeLa cells and primary human melanocytes. We used two different cDNA libraries in order to screen as many diverse proteins as possible, including any potential tissue specific interactors of Omi/HtrA2. Furthermore, one of the cDNA libraries was prepared from primary cells to avoid a potential problem often seen in cDNA libraries prepared from transformed cell lines. Cells lines such as HeLa used here often have deregulated expression of genes involved in cell growth as well as under-representation of pro-apoptotic cDNAs. The bait in this screen was the LexA-Omi$_{134-458}$ that represents the mature active form of the Omi/HtrA2 enzyme [37]. The screen was performed as described and several novel Omi/HtrA2 interacting proteins were identified [37]. One of the specific interactors from the human melanocyte cDNA library was a partial polypeptide of THAP5. THAP5 is the fifth member of a recently described twelve-member family of proteins characterized by the presence of a THAP (Thanatos-Greek for death) motif at the amino terminus of the protein [38]. Using specific primers and RACE we were able to isolate the full-length cDNA, which was then cloned back into the pGJ4-5 vector and its interaction with Omi/HtrA2 monitored. Figure 3A shows that full-length THAP5 interacts strongly with the Omi/HtrA2. Furthermore, the specificity of the interaction was tested using HtrA1, a human homologue of Omi/HtrA2 that has 68% amino acid sequence similarity. No interaction was observed in this yeast two-hybrid assay between these two proteins (Fig. 3A). The expression and stability of the different baits and preys was monitored and verified by Western blot analysis (results not shown).

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**Interaction of Omi/HtrA2 with THAP5 in mammalian cells during apoptosis.** To investigate whether Omi/HtrA2 can interact with THAP5 *in vivo*, HEK293 cells were transfected with a construct encoding GFP-THAP5. Cells were treated with cisplatin for 12 hours to induce apoptosis. After this time, Omi/HtrA2 antibodies were used to precipitate endogenous Omi/HtrA2 protein and the presence of any GFP-THAP5 protein in the complex was monitored by Western blot analysis using a GFP-specific monoclonal antibody (Santa Cruz Biotechnology). Figure 3B shows that endogenous Omi/HtrA2 interacts with GFP-THAP5 in HEK293 only in cells treated with cisplatin and undergoing apoptosis. No interaction between Omi/HtrA2 and GFP-THAP5 was observed under normal non-apoptotic conditions. We also performed the reverse experiment by transfecting HEK293 cells with GFP-Omi and using the THAP5 antibody to precipitate endogenous protein and any GFP-Omi associated with it. This experiment also clearly shows that THAP5 and GFP-Omi can associate in mammalian cells but only during apoptotic conditions (Fig. 3C)

**THAP5 is cleaved by Omi/HtrA2 protease in vitro.** To test the ability of Omi/HtrA2 protease to cleave the THAP5 protein *in vitro*, bacterially made His-THAP5 was incubated with His-Omi/HtrA2$_{134-458}$ protease for different time periods. Figure 3D shows that His-Omi/HtrA2$_{134-458}$ was able to degrade the THAP5 protein in this assay. To verify that cleavage of THAP5 was due to Omi/HtrA2 activity and not by some other contaminated bacterial protease we used its specific inhibitor ucf-101 [26]. When used in this assay, ucf-101 was able to prevent the degradation of THAP5 by Omi/HtrA2 (Fig.3D).

**Expression of THAP5: mRNA and protein.** Figure 4A is a schematic diagram of the

THAP5 protein. The nucleotide sequence predicts a protein consisting of 395 amino acids. The

THAP domain is located at its amino terminus and shows high homology to the other eleven

members of the human THAP family of proteins. Outside the THAP domain, THAP5 protein

has no similarity to any other known protein sequence that is uploaded in the GenBank. A

coiled-coil region is also predicted at its carboxyl-terminus. Since there is no mouse or rat

orthologs for THAP5, a human Northern blot representing various tissues was used to investigate

THAP5 mRNA expression. THAP5 expression is represented by a specific mRNA band of

approximately 3.2kb (Fig. 4B). THAP5 mRNA shows very high expression in the human heart

and when the blot is overexposed, some expression is also seen in the human brain and skeletal

muscle (Fig. 4B). No expression of THAP5 is detected in any of the other human tissues

represented on this blot. To verify that there is a bona-fide THAP5 protein present in these

human tissues we raised a polyclonal antibody against a recombinant His-THAP5$_{163-395}$. This

antibody was used on a Western blot containing extracts from various human tissues

(Calbiochem). Figure 4C shows that a 50kDa polypeptide is readily detectable in human heart,

brain, and skeletal muscle. A higher molecular weight band is also detected in the human brain

that corresponds to a phosphorylated form of the same THAP5 protein (unpublished data). The

relative levels of the THAP5 protein versus mRNA in the three human tissues do not correspond

and this is probably due to the poor quality (protein degradation) of the commercially available

human Western blot.

**Sub-cellular localization of the THAP5 protein.** The cDNA sequence corresponding to the full-length THAP5 protein was cloned into the GFPC1 vector. The GFP-THAP5 vector was transfected into HeLa cells and 24 hours later the sub-cellular localization of the protein was observed using a confocal microscope as previously described [27]. Figure 5A shows that, the full-length GFP-THAP5 protein is localized exclusively in the cell nucleus but is excluded from the nucleoli. There are two discernible domains in the THAP5 protein: (a) the THAP domain (90aa) present at the amino terminus that shares homology with the corresponding domain in the other eleven members of the THAP family of proteins and (b) the rest of the THAP5 protein (91-395aa) that shows no homology to any other known protein sequence in the data banks. Therefore, we cloned DNA sequence corresponding to the amino terminus of the protein including the THAP domain or the unique carboxyl-terminus of THAP5 into the GFP vector in order to investigate their potential role targeting the protein to the nucleus. Figure 5B shows that the THAP5 protein that lacks the amino terminus is now predominantly cytoplasmic, whereas the THAP domain alone localizes exclusively in the cell nucleus (Fig. 5C). Figure 5D is a Western blot to verify the expression and stability of the three different GFP fusion proteins used in this experiment.

**THAP5 is a potential regulator of cell cycle.** Previous reports suggested that some members of the THAP-family of proteins could inhibit cell cycle progression [43]. Therefore, we investigated whether ectopic expression of a GFP-THAP5 protein in human embryonic kidney (HEK293) will interfere with the cell cycle progression. Cells were synchronized and transfected with GFP-THAP5 or the empty GFP vector and 40 or 50 hours later the percentage of transfected cells at various points of the cell cycle were estimated. Figure 6 shows an

accumulation of GFP-THAP5 expressing cells at the G2/M phase compared to cells expressing GFP alone forty hours post transfection. This difference becomes more pronounced after fifty hours where over 28% of the GFP-THAP5 expressing cells were found in the G2/M phase versus 2% of cells transfected with GFP vector.

**THAP5 is degraded in cells treated with cisplatin or $H_2O_2$.** In order to investigate whether THAP5 protein is regulated during apoptosis, HeLa cells were treated with various chemicals known to induce apoptosis including: camptothecin, cisplatin, etoposide, $H_2O_2$ and staurosporine, subsequently cell death was measured using Annexin V staining and Flow Cytometry [37]. THAP5 protein level was monitored by Western blot analysis of cell extracts and it was significantly reduced in cells treated with cisplatin or $H_2O_2$ (Fig. 7). These chemicals also induced the maximum cell death (64% and 58% respectively) in this assay. In cells treated with cisplatin the phosphorylated form of THAP5 (upper band on the blot) was preferentially cleaved whereas in cells treated with $H_2O_2$ both forms of the protein were equally degraded.

**Ucf-101 inhibitor prevents THAP5 degradation.** We investigated whether the Omi/HtrA2 protease is responsible for the degradation of THAP5 observed in HeLa cells during apoptosis. For this, HeLa cells were treated with cisplatin and the percentage of cell death monitored by Annexin V staining and Flow Cytometry and the levels of THAP5 protein monitored by Western blot analysis. HeLa cells treated with 5μM cisplatin for 12 hours resulted in 42% cell death of the population (Fig. 8). The cell death in these cells coincided with a dramatic reduction in the level of THAP5 protein. We also performed the same experiment in the presence of two different concentrations of ucf-101, a specific inhibitor of the proteolytic activity of Omi/HtrA2 [26]. In this case, the percentage of apoptotic cells in the population was

reduced to 21%.  The ucf-101 inhibitor was also able to protect the degradation of the THAP5 protein implicating Omi/HtrA2 protease in this process.

**Expression of THAP5 protein in heart tissues derived from patients with coronary artery disease.**  THAP5 expression is highest in the human heart suggesting a potential role of this protein in the normal function of the cardiomyocytes.  We monitored the protein level of THAP5 in the heart of patients with various heart diseases including Coronary Artery Disease (CAD) and Dilated Cardiomyopathy (DCM).  Figure 9A shows the expression of THAP5 protein in the remote zone (CAD-RZ), border zone (CAD-BZ), and myocardial infarction area (CAD-MI) in hearts of three CAD patients.  In two of the patients, there is clear reduction in the level of THAP5 in the CAD-MI area compared to the CAD-RZ and CAD-BZ. The myocardial infarction area is the part of the heart that sustains maximum injury in CAD. Therefore, this area is expected to contain most damaged and apoptotic cells.  Figure 9B shows the expression of THAP5 protein in the heart tissue of three patients with dilated cardiomyopathy (DCM) and three healthy donors (HD).  No significant difference is seen in the level of THAP5 protein in the hearts of DCM patients or healthy donors.  The blot was also probed with GAPDH-specific antibody to verify that equal amount of protein extract was used in each lane.

## Discussion

Omi/HtrA2 is a nuclear encoded mitochondrial protease that has homology to bacterial *HtrA* chaperones [1, 54, 55]. Recent evidence suggests that Omi/HtrA2 has two distinct functions both requiring its protease activity. Omi/HtrA2 can be a pro-apoptotic protein as well as a pro-survival factor depending on its sub-cellular location. The pro-survival function of Omi/HtrA2 was based on studies on motor neuron degeneration 2 (*mnd2*) mice that carry a single mutation affecting the proteolytic activity of Omi/HtrA2 [20]. *Mnd2* homozygous animals suffer muscle wasting, neurodegeneration, and die by six weeks of age. Based on these studies, it is assumed that the primary function (physiological function) of Omi/HtrA2 in mammalian cells is to somehow maintain mitochondrial homeostasis necessary for cell survival [19, 20]. The other function of Omi/HtrA2 (pathological function) becomes operational only under conditions leading to cell death where Omi/HtrA2 is released to the cytoplasm as a pro-apoptotic protein and participates in caspase-dependent as well as caspase-independent apoptosis [4, 5, 8].

Most of the studies reported so far have provided significant new information on the mechanism of Omi/HtrA2's pro-apoptotic function but little if nothing on its pro-survival function is known. Omi/HtrA2, when released to the cytoplasm, binds and degrades several substrates including IAPs, PEA/PED, and Apollon/Bruce [11, 12, 14, 39]. Some of these proteins are clearly anti-apoptotic factors and their removal by Omi/HtrA2 can undoubtedly accelerate cell death. Others have a more obscure function and their association and cleavage by Omi/HtrA2, in the context of inducing cell death, is more difficult to explain. In the present study, we report the characterization of THAP5, the fifth member of the THAP family of proteins as a specific interactor and substrate of Omi/HtrA2 protease. THAP proteins comprise a recently described

family of cellular factors with unique structural and functional characteristics. They are defined by the presence of the THAP domain, an atypical zinc-dependent DNA-binding domain of approximately 90 amino acids.

There are 113 THAP proteins listed in the databases and in humans, there are 12 distinct proteins (THAP0-THAP11) [38]. All 12 human THAP proteins have a single THAP domain at their amino terminus. In Drosophila there are seven THAP proteins two of which contain more than one THAP domain, CG14860 has two THAP domains and the CG10631 is predicted to have as many as 27 THAP domains [38]. THAP domains are unique as DNA binding motifs due to a significant similarity with the DNA binding domain of Drosophila P element transposase. This similarity includes the size of the THAP domain, its location in the protein and the conservation in the number and residues that define the domain [38]. THAP0, also known as the death-associated protein DAP4/p52rIPK, was identified as one of the genes induced by IFN-γ-induced apoptosis and in an independent study as an interactor of PKR protein kinase [56, 57]. THAP1 was originally defined as a pro-apoptotic nuclear factor localized at the promyelocytic leukemia nuclear bodies (PML NBs) and interacting with prostate-apoptosis-response-4 (Par-4) protein [45].

Since very little is known about THAP5 we embarked on a detailed study beginning with its expression. In human tissues THAP5 mRNA is predominantly expressed in the human heart although low expression is also detected in skeletal muscle and brain. More significantly, we found no orthologs for THAP5 in mouse (*Mus musculus*) or rat (*Rattus norvegicus*). The closest orthologs were detected *in Macaca fascicularis, Bos taurus*, and *Gallus gallus*. The absence of orthologs in mouse and rat has also been reported for four other members of the THAP family,

the THAP6, THAP8, THAP9, and THAP10 proteins [46]. It was suggested that some THAP genes might have originated from a domestication event of a single copy of P element transposase that took place in the human lineage after it had diverged from the rodent lineage [42]. Alternatively, THAP gene domestication might have occurred earlier in the evolution but was later lost in the lineage leading to rodents [42].

To verify that THAP5 is a *bona fide* human protein, we raised a polyclonal antibody against the unique part of the protein (aa163-395) that does not include the THAP domain. This polyclonal antibody clearly recognized a specific band of 50 kDa, corresponding to THAP5 in human heart, skeletal muscle, and brain. In human brain a 52kDa polypeptide was also detected that could possibly correspond to a phosphorylated form of the same THAP5 protein. The THAP5 antibody did not detect any specific protein bands when tested against various mouse or rat tissue extracts (results not shown).

To investigate the sub-cellular localization of THAP5, we expressed the full-length protein fused to GFP in HeLa cells. The fusion protein, GFP-THAP5, shows exclusive nuclear localization but is excluded from the nucleoli. The amino terminus of the protein that includes the THAP5 domain is responsible for its nuclear localization. This is the part of the protein implicated in DNA binding as well as protein-protein interactions [58]. The interaction with Omi/HtrA2 is mediated by the carboxyl-terminus of THAP5 that binds the PDZ-domain of Omi/HtrA2. The carboxyl-terminus of THAP5 has the amino acid sequence EVTMI* that conforms to the consensus sequence for PDZ (Omi) binding proteins [41, 59].

Previous studies have shown two members of the same family, THAP1 and THAP11 can regulate cell proliferation [43, 60]. Furthermore, genetic studies using C. *elegans* support a role

of THAP proteins in cell cycle regulation. These studies demonstrated a genetic interaction between LIN-35/Rb, which is the C. *elegans* retinoblastoma homologue and four C. *elegans* THAP proteins, LIN-36, LIN-15B, LIN-15A, and HIM-17. LIN-36 and LIN-15B are known inhibitors of G1/S cell cycle transition [44, 61-65]. Also, in zebrafish the ortholog of the cell cycle transcription factor E2F6 has a THAP domain and functions as a repressor of E2-F-dependent transcription during S phase [66]. Based on these reports we decided to investigate if THAP5 plays any role in the regulation of cell cycle.

For these studies, we ectopically expressed GFP-THAP5 in a human embryonic kidney (HEK293). This cell line expresses endogenous THAP5 protein and can be transfected with high efficiency. The results from these experiments clearly show, GFP-THAP5 had a dramatic effect blocking the progression of transfected cells through the cell cycle progression and causing high accumulation of cells at the G2/M phase. The mechanism by which THAP5 inhibits cell cycle is not known and it most likely will involve the direct regulation of cell cycle genes. THAP5 has an atypical zinc finger domain at its amino terminus that can bind to specific DNA sequences on different promoters. A recent study showed that another member of the same family, THAP11, could inhibit cell cycle by binding to the promoter of c-Myc and down-regulating its expression [60].

To investigate the role of the THAP5-Omi/HtrA2 interaction during apoptosis, HeLa cells that normally express THAP5 were treated with various chemicals to induce cell death. Under these conditions, Omi/HtrA2 is released from mitochondria and becomes a pro-apoptotic protein. In these experiments, the THAP5 protein was cleaved in cells treated with cisplatin or $H_2O_2$ and the degree of THAP5 degradation increased proportionally with the rate of apoptosis in the cell

population. No significant cleavage of THAP5 was seen in cells treated with Etoposide and Camptothecin but these chemicals also caused minimal cell death in this protocol and cell line used for these experiments. We used a specific inhibitor to show that Omi/HtrA2 is responsible for THAP5 degradation. This inhibitor, ucf-101, was developed in our laboratory and has been extensively studied in various systems of cell death [26, 31, 37]. When the ucf-101 inhibitor was used, it clearly blocked THAP5 cleavage and protected cells from apoptosis.

THAP5 is hyper-expressed in the human heart but there are no rodent orthologs, therefore limiting the type of studies that can be done to investigate its potential involvement in the development or progression of heart disease. We obtained small tissue samples from patients with several human heart diseases including coronary artery disease (CAD) and dilated cardiomyopathy (DCM). In CAD patients, we were able to detect a decrease in the THAP5 protein level in the myocardial infarction (MI) area when compared to remote zone (RZ) or border zone (BZ) areas of the same heart. These results, though preliminary, suggest that during pathological conditions resulting in myocardial cell death, such as MI, the THAP5 protein decreases significantly in areas of the heart that sustained maximum cell damage. There was no difference in THAP5 protein levels in DCM patients compared to normal (donor) heart. Previous studies have shown that Omi/HtrA2 is a key player in myocardial ischemia/reperfusion injury [29]. Furthermore, inhibition of the proteolytic activity of Omi/HtrA2 using the ucf-101 inhibitor improved post-ischemic myocardial function and reduction of the myocardial infarct size [29-31].

THAP5 is the first human, heart-specific substrate of Omi/HtrA2 identified thus far. Under normal, physiological conditions THAP5 functions as an inhibitor of cell cycle. This dual

function of THAP5 supports previous studies that have shown the existence of a cross talk between cell cycle control and apoptosis in cardiomyocytes. For example, over-expression of the cell cycle inhibitor p57$^{kip2}$ in cardiomyocytes was shown to attenuate ischemia-reperfusion injury in the mouse heart [67]. In another study, cyclin A/cdk2 activation was involved in hypoxia-induced apoptosis in cardiomyocytes [68]. The hyper-expression of THAP5 in human heart and its regulation during cell death by Omi/HtrA2 suggests this pathway plays a significant in the normal function of the heart. Furthermore, THAP5 might be involved in the development and progression of heart disease especially in coronary artery disease.
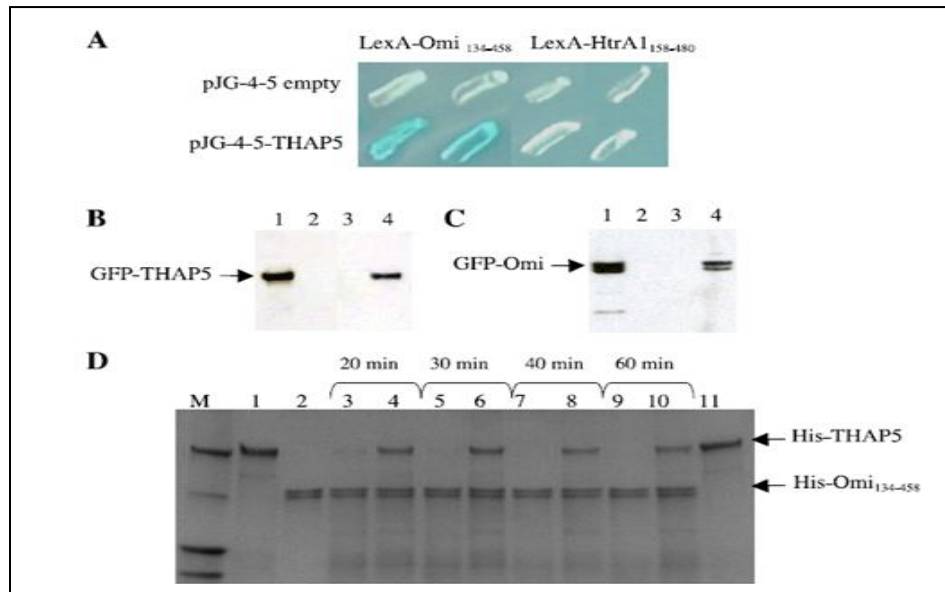
**Figure 3: THAP5 is an interactor and substrate of Omi/HtrA2 protease.**

A, Yeast colonies were transformed with plasmids encoding the indicated baits and full length THAP5 cloned into the prey vector. The specificity of THAP5 and Omi/HtrA2 interaction was verified using a closely related homologue, the HtrA1 protein. Blue color results from a positive protein-protein interaction. B, HEK293 cells were transfected with EGFP, EGFP-THAP5 or GFP-Omi. Twelve hours after transfection one plate was treated with Cisplatin to induce apoptosis and one plate was used as control. A polyclonal Omi/HtrA2 antibody was used for immunoprecipitation. The immunoprecipitated complex was resolved on SDS-PAGE, transferred and the presence of GFP-THAP5 fusion protein detected using a specific anti-GFP antibody. Lane 1 shows crude lysates of GFP-THAP5 transfected cells. Lane 2 shows Co-IP lysates obtained from cells transfected with GFP empty vector. Lane 3 shows Co-IP lysates obtained from cells transfected with GFP-THAP5 control cells and lane 4 shows Co-IP lysates obtained from cells transfected GFP-THAP5 and then treated with cisplatin. C, Shows the reverse experiment described in Fig 1B. HEK293 cells were transfected with GFP-Omi and THAP5 antiserum was used in the Co-IP and anti-GFP on the Western blot to detect the GFP-Omi. Lane 1 shows total cell lysates of GFP-Omi transfected cells. Lane 2 shows Co-IP lysates obtained from cells transfected with GFP empty vector. Lane 3 shows Co-IP lysates obtained from cells transfected with GFP-Omi control cells and lane 4 shows Co-IP lysates obtained from cells transfected GFP-Omi and then treated with cisplatin. In both Figures 1B and 1C THAP5 was co-precipitated with Omi/HtrA2 but only in cells where apoptosis was induced. D, Purified His-THAP5 was incubated with His-Omi$_{134-458}$ at 37°C for the indicated time periods. For some assays, Omi/HtrA2 was pre-incubated with ucf-101 inhibitor 10 min prior to addition of His-THAP5. The reactions were resolved on SDS-PAGE and the gel stained with Coomassie Blue. Lane 1, His-THAP5 control (400ng); lane 2, His- Omi$_{134-458}$ control (400ng), lanes 3, 5, 7 and 9, His-Omi+His-THAP5 at different time points; lane 4, 6, 8, and 10, His-Omi + His-THAP5+ ucf-101 (50μM); lane 11, His-THAP5 + ucf-101 control
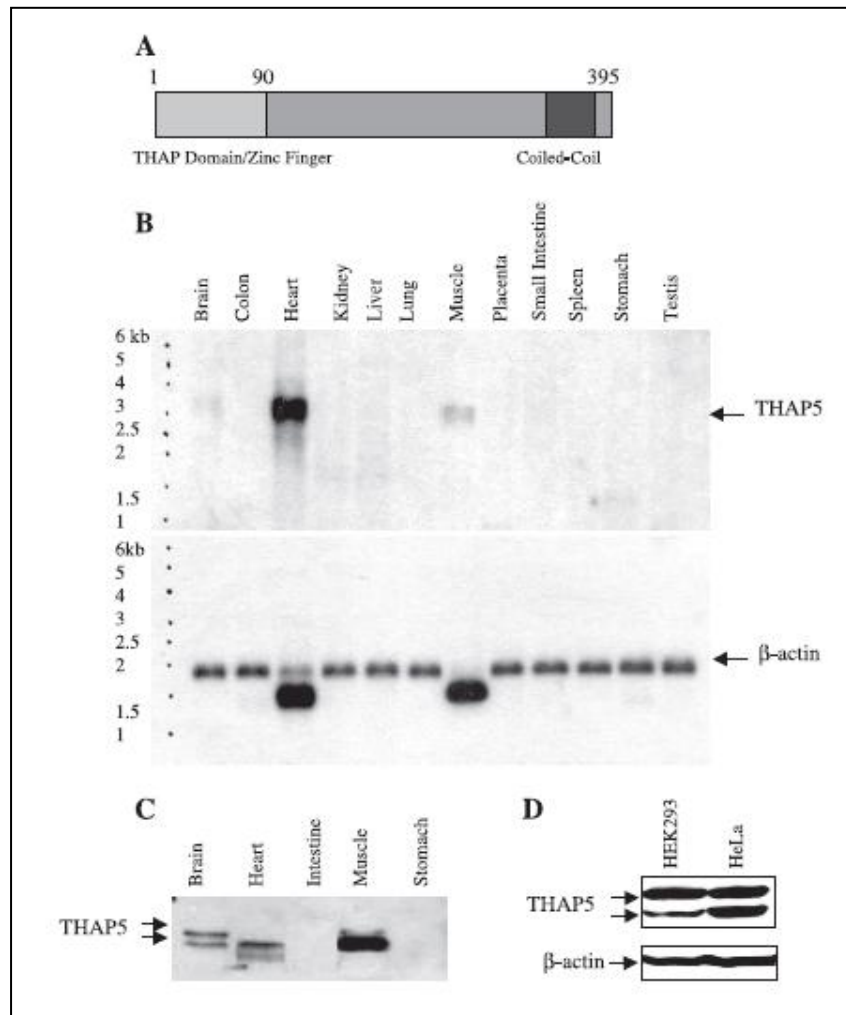
**Figure 4. Expression of THAP5: mRNA and protein in human tissues.**

A, Schematic representation of the THAP5 protein encoded by 395 amino acids. The light gray box represents the 90aa THAP domain, which is characteristic of all THAP proteins. B, Northern blot analysis of THAP5 expression in human tissues. A commercially available Northern blot (Clontech) containing 2μg/lane poly (A) mRNA from various adult human tissues was probed with $^{32}$P-THAP5 (539-1342) cDNA. A single transcript was detected of about 3.2 kb. The blot clearly shows high expression of THAP5 in the human heart, some expression was also seen in skeletal muscle and brain. β-actin probe was used to verify that equal amounts of mRNA were present in each lane. C, Western blot analysis of THAP5 protein in multiple human tissue; A commercial western blot (INSTA-blot Calbiochem) containing 15μg/lane of total protein from adult human tissues was incubated with rabbit-polyclonal anti-THAP5 antibody followed by a secondary antibody, HRP-conjugated goat anti-rabbit and chemiluminescence detection.
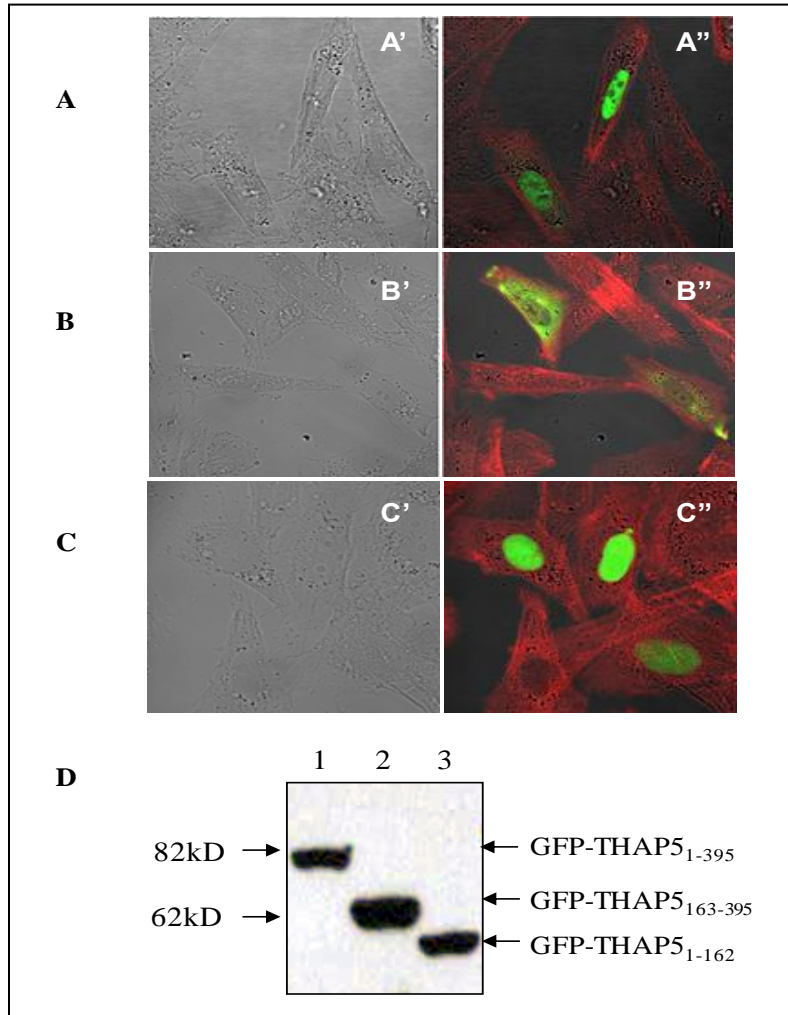
**Figure 5. Sub-cellular localization of the THAP5 protein in mammalian cells.**

A, Confocal image of HeLa cells transfected with GFP-THAP5$_{1-395}$ shows exclusive nuclear localization (green in panel A"). B, HeLa cells transfected with GFP-THAP5$_{163-395}$ show cytoplasmic localization (green in panel B"). C, HeLa cells transfected with GFP-THAP5$_{1-162}$ show exclusive nuclear localization (green in panel C") of this protein. The cells were also stained with Texas red-phalloidin (Molecular Probes) that stain actin filaments (red). Nomarski/DIC images of the same cells are shown in panel A', B' and C' respectively. D, The stability of EGFP-fusion proteins was monitored by Western blot analysis using an anti-GFP antibody. Equal amounts of whole-cell lysates, obtained 24 hrs post-transfection were subjected to SDS-PAGE followed by Western blot analysis using anti-GFP monoclonal antibody as described in Materials and Methods. Lane 1, lane 2 and 3 represent lysates obtained after transfection of HeLa cells with GFP- THAP5$_{1-395}$, GFP-THAP5$_{163-395}$, and GFP- THAP5$_{1-162}$ respectively.
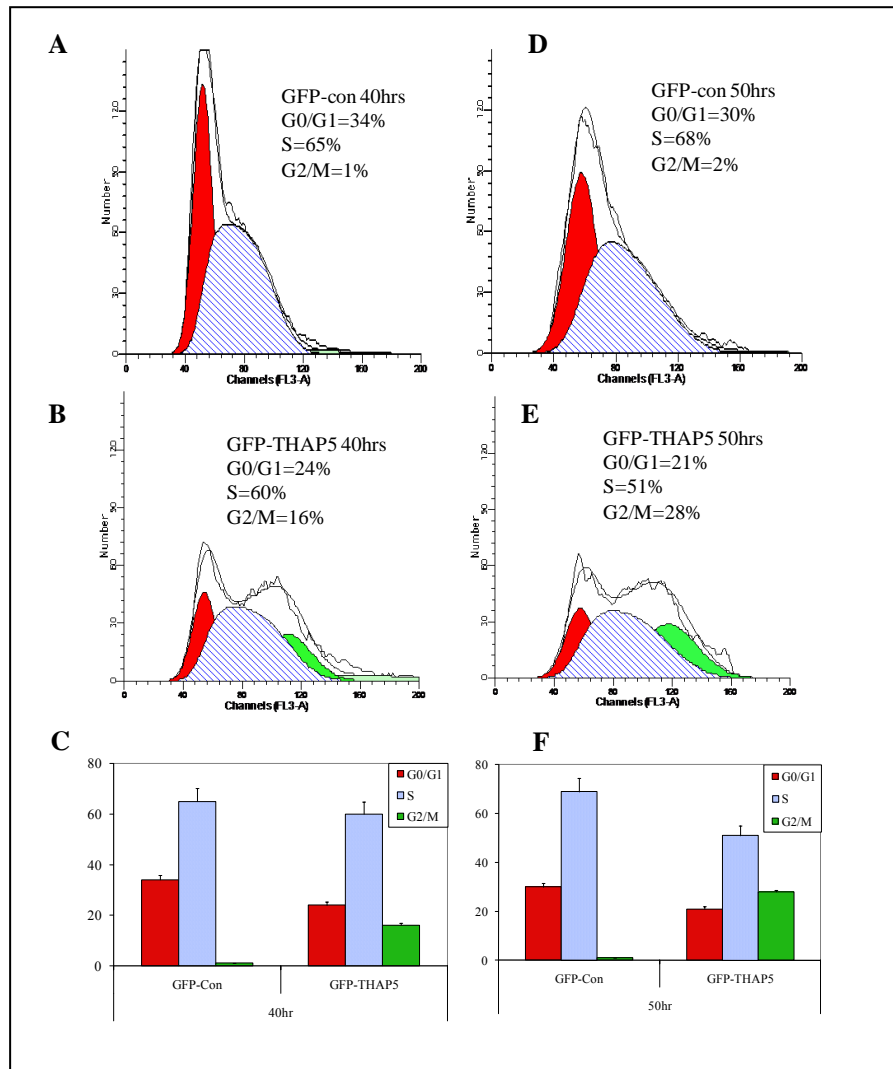
**Figure 6. Over-expression of GFP-THAP5 causes accumulation of cells in G2/M phase.**

HEK293 cells were transiently transfected with GFP vector alone or GFP-THAP5. 40hrs and 50hrs post transfection cells were stained with 7-AAD and the DNA content was analyzed using ModFitLT software. Panel C represents the percentages of GFP positive cells at different phases of cell cycle 40 hrs after transfection and panel F represents 50hrs. A, Histogram results from representative experiments in cells transfected with GFP vector at 40 and D, 50 hours. B, Cells transfected with GFP-THAP5 40 and E, 50 hours show significant increase in the percentage of cells in G2/M phase. Data are means ±SD of four independent experiments.
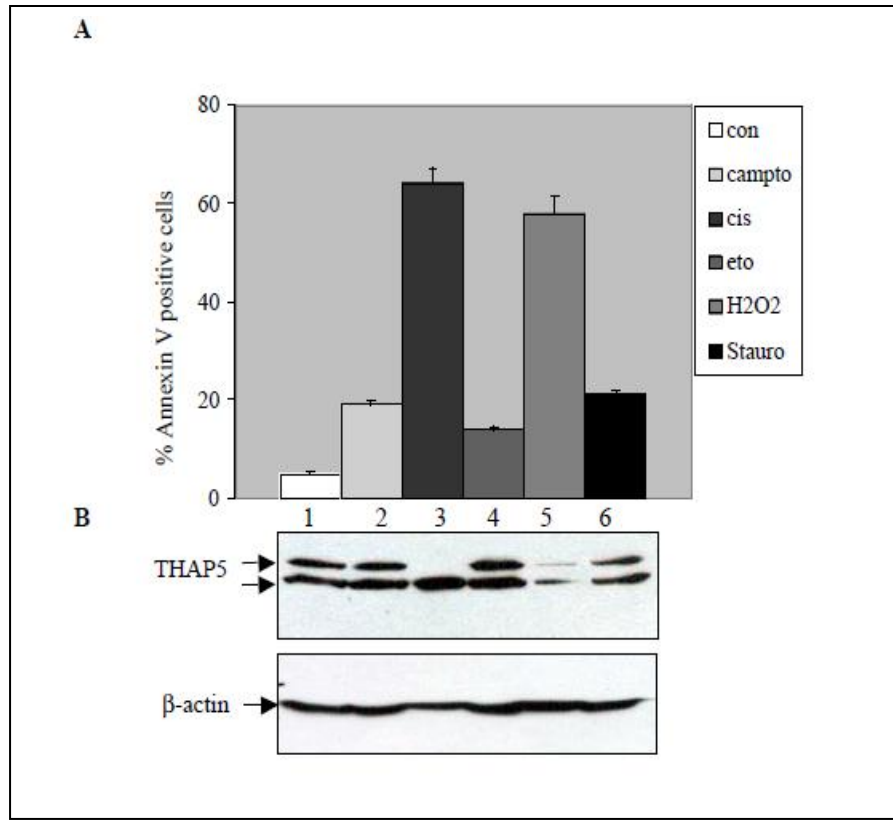
**Figure 7. THAP5 protein level is regulated during apoptosis.**

Total cell lysates were prepared from HeLa cells after induction of apoptosis using different chemicals (Camptothecin, Cisplatin, Etoposide, H₂O₂ and Staurosporine). A, Cell death was monitored in the treated cell populations by Annexin V and 7-AAD staining and analyzed by Flow cytometry. B, Cells extract were prepared followed by Western blot analysis of the same samples. Lane 1 shows lysates from control, untreated cells, lanes 2, 3, 4 show cell extracts from HeLa treated with the chemical indicated above. β−actin antibody was used to verify that equal amounts of protein were present in each lane.
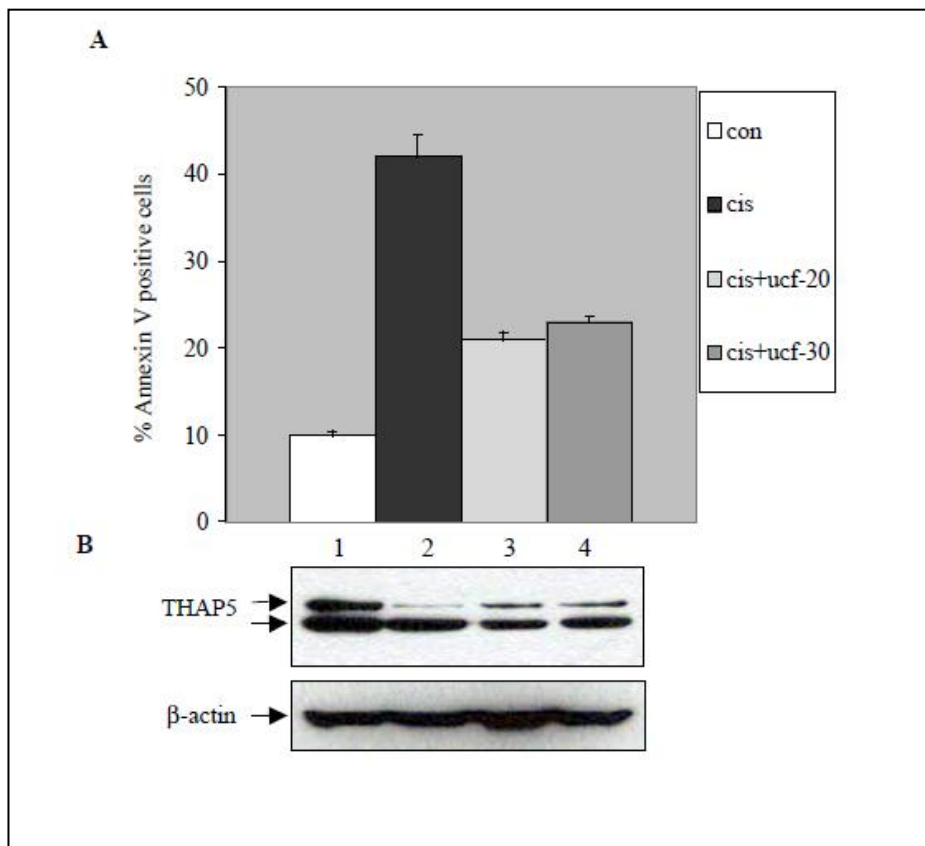
**Figure 8. A specific inhibitor of Omi/HtrA2 blocks THAP5 cleavage and protects HeLa cells from apoptosis.**

A, HeLa cells were treated with 20 or 30μM of ucf-101 and apoptosis was induced with 5μM cisplatin for 12 hours. Apoptosis was monitored using Annexin V and 7-AAD staining and analyzed by Flow cytometry. B, Extracts were prepared from the same cell population and analyzed by SDS-PAGE and Western blot analysis using THAP5 antibody. Cisplatin treatment caused a dramatic reduction in THAP5 protein level (lane 2). This corresponds with increased apoptosis in the cell population. When HeLa cells were treated with ucf-101 followed by cisplatin, the inhibitor substantially protected THAP5 proteins and the percentage of apoptotic cells was significantly reduced (lanes 3 and 4). β−actin antibody was used to verify that equal amounts of protein were present in each lane.
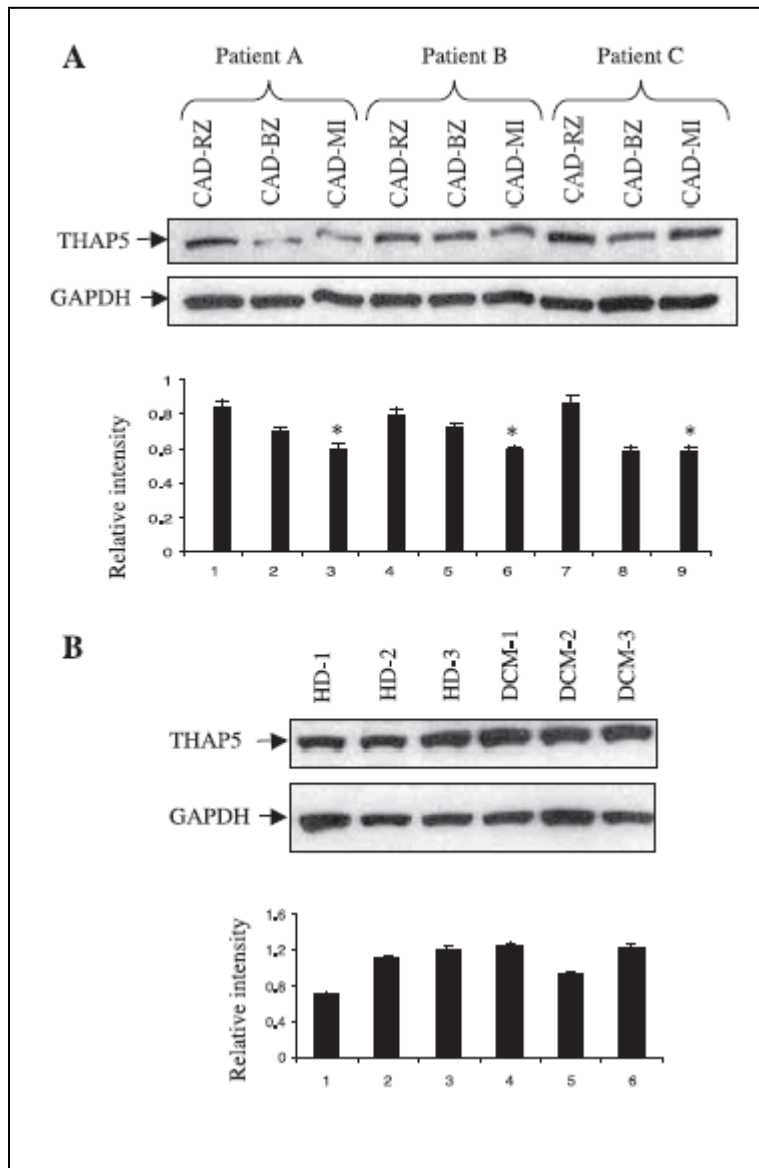
**Figure 9. THAP5 protein levels in heart tissues from patients with coronary artery disease (CAD).**

A, Heart tissue extracts from three patients with coronary artery disease (CAD) were used in a Western blot to monitor the expression of THAP5 protein. Extracts were prepared from three areas on each heart corresponding to the remote zone (CAD-RZ), border zone (CAD-BZ), and the infarction (CAD-MI) area. B, Human heart tissue lysates from three healthy donors (HD 1 to 3) and three DCM patients (DCM 1 to 3) were probed for THAP5 expression.

## CHAPTER 3: THAP5 IS A DNA BINDING PROTEIN AND A TRANSCRIPTIONAL REPRESSOR WITH A ROLE IN MELANOMA CELL DEATH

**Introduction**

THAP proteins are zinc dependent DNA binding proteins involved in several functions such as cell proliferation, apoptosis, cell cycle, chromosome segregation, chromatin modification and transcriptional regulation [38]. THAP stands for Thanatos Associated Protein (derived from Thanatos which means death in Greek) [38]. These proteins are defined by the presence of an approximately 90 amino acid THAP domain at their amino terminus. THAP domains are evolutionarily conserved motifs with specific amino acid residues that are necessary for the DNA binding activity of the THAP proteins [38, 42, 44]. Twelve human THAP proteins (THAP0-11) have been identified but most of the studies have been focused on THAP0 (DAP4), THAP1, THAP7 and THAP11 [43, 44, 56-58, 60]. Very little, if anything, is known about the function of the other members of the THAP family proteins.

The first THAP protein to be studied was THAP0/ DAP4 (death associated protein-4) [56, 57]. THAP0 has been shown to enhance apoptosis caused by the overexpression of MST-1 protein [69]. THAP1 has been shown to be a nuclear protein associated with promyelocytic leukemia nuclear bodies and TNF-α induced apoptosis [45]. It can also act as a regulator of cell cycle (G1/S phase) by regulating the expression of the RRM11 gene, a pRb/E2F target protein [43]. THAP7 is a chromatin associated transcriptional repressor protein that recruits histone deacetylase 3 (HDAC3) and nuclear hormone receptor corepressors (NCoR) [58, 70]. THAP11 has been shown to suppress cell growth by transcriptional downregulation of c-Myc [60]. The

mouse homolog of THAP11, called Ronin, was identified as an essential factor for embryogenesis and embryonic stem (ES) cell pluripotency [71].

In our previous work, we identified the THAP5 protein as an interactor and substrate of the mitochondrial serine protease Omi/HtrA2 [72] (The Am Physiol Soc, used with permission). THAP5 is a nuclear protein with a restricted pattern of expression and is present predominantly in the human heart. Furthermore, there is no mouse or rat homolog for THAP5. The closest orthologs have been detected in monkey, cow and chicken. The absence of mouse/rat homolog has also been noted in some other THAP proteins, namely THAP6, THAP8, THAP9 and THAP10 [46]. Our studies showed that THAP5 is an inhibitor of cell cycle and is cleaved by the pro-apoptotic protease Omi/HtrA2 during cell death [72]. The levels of THAP5 protein are significantly reduced in the myocardial infarction area of human heart tissues obtained from patients suffering from coronary artery disease (CAD) suggesting a potential role of this protein in the development and/or progression of human heart disease [72].

In this work, we extend our studies on the mechanism of the normal function of THAP5 and its potential role in cell death. For this, we used human melanoma cells since THAP5 was originally isolated from a human melanocyte cDNA library [72]. In addition, although THAP5 is mostly expressed in human heart, human heart tissues are hard to obtain and there is no human heart cell line. Nothing is known about the biological role of THAP5 in melanocytes and melanoma cells. We found, upon UV or cisplatin treatment of melanoma cells, THAP5 protein is induced considerably. The induction of THAP5 mirrors the degree of apoptotic cell death in the melanoma population. Overexpression of THAP5 sensitizes melanoma cells to UV-induced apoptosis. To further investigate the mechanism of THAP5 function we used an *in vitro* binding

selection and identified an 11-nucleotide consensus DNA-binding sequence specifically recognized by THAP5 protein. In addition to its DNA binding ability, THAP5 was able to repress the transcription of a reporter gene in a heterologous system. In summary, our work defines THAP5 as a DNA binding protein that can potentially repress the transcription of target genes involved in UV or cisplatin-induced damage and cell death of melanoma cells.

## Materials and methods

*Cell culture.* Melanoma (MEL-2, MEL-18, MMG1, SMYM, 397mel, 624mel, 888mel, 928mel, SKmel23, RPM-ML, PM-WK, and MM-LH), stomach cancer (MNK7 and MNK28), lung cancer (A549), Ovarian Cancer (ES2), normal monkey kidney (cos7) cell lines were grown in Life Technologies RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). All cell lines were cultured at 37 ˚C in a humidified atmosphere containing 5% $CO_2$, as previously described [73]. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Hyclone), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). MeWo and SK-Mel-28 cell lines were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum, 50U/ml penicillin, and 50μg/ml streptomycin. Peripheral blood lymphocytes (PBL) from healthy normal donors were used as controls. Paraffin-embedded archival tissue (PEAT) specimens were obtained from melanoma patients who underwent surgical resection at Shinshu University Hospital (Nagano, Japan) between 1999 and 2009. All of these specimens were initially assessed by surgical pathologists at Shinshu University Hospital and diagnosed as melanoma.

*Quantitative Real-time (qRT)-PCR assay.* Total RNA was extracted from cell lines and PBL specimens using Tri-Reagent (Molecular Research Center Inc). The THAP5 primer sequences used were: 5'- GAAAGGTGCACGCAAAGTTAAT-3' (forward); 5'-CAGGAGTAAAATGGTCACTACATAGAA-3' (reverse). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sequences were: 5'- CCATGTTCGTCATGGGTGT -3' (forward); 5'- CCAGGGGTGCTAAGCAGTT -3' (reverse). For cell lines and PBL specimens, reverse transcription (RT) reactions were performed using Moloney murine leukemia virus RT (Promega) with oligo-dT primer. The qRT-PCR assay was performed with the LightCycler System and Universal Probe Library Set, Human (Roche Applied Science) using 250 ng of total RNA. Specific plasmid controls of THAP5 and GAPDH were synthesized as described previously [73], and standard curves for each gene were generated with a threshold cycle of six serial dilutions of plasmid templates (106–101 copies). THAP5 expression was calculated as a ratio of THAP5 /GAPDH copy numbers.

*Immunohistochemistry.* Immunohistochemistry was performed on formalin-fixed, paraffin embedded sections from primary and metastatic melanoma tissues. After deparaffinization, endogenous peroxidase was quenched with peroxidase block (Fisher Scientific). For antigen retrieval, the sections were boiled using microwave for 20 minutes in 10 mmol/L citrate buffer (pH 6.0), and exposed to blocking solution (Protein Block Serum-Free, DakoCytomation). The sections were incubated with polyclonal anti-THAP5 antibody for 60 minutes. For immunohistochemical detection, HRP-labeled anti-rabbit immunoglobulin antibodies (DAKO EnVision+ System, HRP, DakoCytomation) was used (AEC + Substrate-Chromogen,

DakoCytomation). Sections were counterstained via Gill's hematoxylin (Fisher Scientific), and then mounted [74].

***Sub-cellular localization of THAP5 protein.*** To investigate the subcellular localization of the THAP5 protein, the full-length cDNA (aa 1-395) was cloned in frame into EGFP-C1 vector (Clontech). MeWo cells were grown on glass cover slips in 12-well plates. Approximately 60% confluent cells were transiently transfected with 1 µg of the GFP-THAP5 plasmid using Lipofectamine 2000 Transfection Reagent (Invitrogen). Twenty-four hours after transfection, cells were washed and fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100. The cover slips were then washed and placed on microscope slides using mounting solution with DAPI to stain the nucleus. Slides were observed using a LSM510 Confocal laser-scanning microscope (Zeiss).

***Western blot analysis of THAP5 protein expression.*** Cell lysates were prepared using RIPA buffer (150 mM NaCl, 50 mM TrisHCl, pH 7.5, 1% Nonidet P-40, 0.25% deoxycholic acid sodium salt) containing the protease-inhibitor cocktail (Roche) and resolved by SDS-PAGE. They were then electro-transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with polyclonal THAP5 antibody at a 1: 5000 dilution followed by secondary goat anti-rabbit horseradish peroxidase conjugated antibody [72]. The immunocomplex was visualized using enhanced chemiluminescence (Pierce).

***Cell death assays.*** MeWo cells were grown in six-well plates in the appropriate medium until they reached 90% confluency followed by treatment for 10h with increasing concentrations of cisplatin (1mM, 5mM, 10mM and 15mM respectively). For UV-irradiation, cells were treated for 10h with increasing doses of UVC (25 mj/cm$^2$, 30 mj/cm$^2$, 35 mj/cm$^2$, 40 mj/cm$^2$ and 50

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mj/cm$^2$ respectively) using a Spectroline UV crosslinker (254nm) [75, 76]. Cells were detached with 1x Trypsin-EDTA (Gibco) and washed twice with ice-cold PBS. One-half of them were used for Western blot analysis, and the rest for apoptotic assay. Western Blot analysis was performed as described. The apoptotic assay was performed according to BD Biosciences protocol. Briefly, cells were suspended in 1x binding buffer and stained with Annexin V (apoptotic cell) and 7-amino-actinomycin D (7-AAD for necrotic cells). Samples were analyzed on a FACS Calibur Flow Cytometer (BD Biosciences) [37]. For the other apoptotic assay, MeWo cells were transfected with GFPC1 (control) and GFPC-THAP5 plasmids. Thirty six hours after transfection, cells were treated with 50mj/cm$^2$ of UVC. 10 hours following UVC treatment the cells were stained with Annexin V and 7-AAD. Approximately ten thousand GFP positive cells were counted the percentage of apoptotic cells estimated [72].

*Electrophoretic Mobility Shift Assay (EMSA).* THAP5 DNA-binding assays were performed as described previously [77, 78]. Briefly, nuclear extracts were prepared from cultured MeWo and NIH3T3 cells in hypertonic buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 20 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM Na$_4$P$_2$O$_7$, 1 mM DTT, 0.5 mM PMSF, 0.1 mM aprotinin, 1 mM leupeptin, 1 mM antipain). Extracts containing 3 μg of total protein were incubated with a double-stranded $^{32}$P-radiolabeled WT oligonucleotide probe (5'-TGCCTGGTGCAAGTAACT) and mutant probe (5'-TGCCTGGTACACGTAACT). Protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography.

*Cyclic Amplification and Selection of Targets (CASTing).* The CASTing procedure described by Wright et al was modified to select for the THAP5 binding site [79, 80]. A

randomized DNA template, 5'-TGG GCA CTA TTT ATA TCA A-N25- AAT GTC GTT GGT GGC CC-3' (where N25 is a 25-base sequence of randomly inserted nucleotides) was synthesized and amplified with the primers 5'-ACCGCAAGCTTGGGCACTATTTATATCAAC-3' and 5'-GGTCTAGAGGGC CACCAACGACAT T-3' in a PCR reaction. PCR cycling conditions used were: $94^{o}C$ for 2 minutes, 30 cycles of $94^{o}C$ (20 sec), $50^{o}C$ (20 sec) and $72^{o}C$ (20 sec), with a final extension at $72^{o}C$ (10 min). Approximately 6μl of MeWo cell extract was incubated with the PCR product for 30 minutes in 100 μl of binding buffer (20 mM Tris, pH 7.5; 100 mM NaCl; 0.05% NP40; 0.5 mM EDTA; 100 μg/ml BSA; 50 μg/ml Poly dI-dC; 5μg/μl single stranded salmon sperm DNA) at RT. This was followed by addition of THAP5 specific antibody and Protein G-agarose beads. After 30 minute incubation, the mixture was washed five times in 100 μl of NT2 buffer (20 mM Tris, pH 7.5; 100 mM NaCl; 0.05% NP40) [71]. Beads were resuspended in 100 μl of H2O, and THAP5 bound DNA was purified by phenol/chloroform extraction and ethanol precipitation and then resuspended in 10 μl of H2O. Two microliters of DNA isolated from the first round of THAP5 binding were used in a second round of PCR amplification and purification using the same conditions. After seven rounds of PCR amplification (with reduced number of PCR cycles for the next round of CASTing), the oligonucleotides enriched for THAP5 DNA binding site were into the pGEM-T Easy vector (Promega). Twenty five randomly selected clones were sequenced and analyzed using the Multiple EM for Motif Elicitation (MEME) software (meme.sdsc.edu).

***Bioinformatics Analysis.*** The software tool MEME was applied to the 25 sequences generated from CASTing. The first motif that was found was much more significant than other

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reported motifs, with p-value at least 400 times smaller. By using the consensus of this motif, GTGNAANNAAC, the upstream 1kb region plus 5' UTR of cell cycle genes that are reportedly involved in G2/M transition were scanned according to methods described in [81]. The repeats in these sequences were removed by Repeat Masker tool (http://www.repeatmasker.org/). A total of 20 G2/M cell cycle genes were used as input and by allowing one nucleotide mismatch, 15 genes were found to contain at least one instance of to the consensus.

*Reporter Gene Studies.* 293T cells were seeded into 48-well plates to be 50-70% confluent the next day. Cells were transfected with Gal4 TK-Luc reporter and pRL-SV40 Renilla luciferase (Promega), Gal4 DNA-binding protein (DBD) alone, Gal4 DBD-THAP5, or Gal4 DBD-THAP7 in increasing concentrations (0ng, 5ng, 25ng) using Lipofectamine 2000 (Invitrogen). PCDNA3 was used to maintain the total concentration of transfected DNA [70]. Cells were lysed after 48 h and luciferase activity was measured according to the manufacturer's instructions (Promega).

*Mutation Analysis.* Three exon regions corresponding to THAP5 were amplified by PCR from cDNA obtained from several melanoma cell lines, primary melanocytes and metastatic melanoma samples. The following specific primers were used for Exon1 (677 bp): Forward 5'-GAGTCGACAGACGAGGCGG -3', Reverse 5'- CATTCCGGGCCTCAGTTTCC -3'; Exon2 (386 bp): Forward 5'- CCTCTTGTAGTACTTACTTGAG -3', Reverse 5'-CTCTCCCACCTGATCACCTG -3'; and Exon3 (1200 bp): Forward 5'-GCTTGGCCAGTGTACTACTC -3', Reverse 5'- CTCTCCAGCCCAACTCTCTG -3'. Following amplification, the PCR products were sequenced and analyzed by ClustalW alignment software.

*Statistical analysis.* All quantitative data are expressed as mean ±SD. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Post-hoc test. A value of $P<0.05$ was considered significant.

## Results

**Expression of THAP5 in melanoma cells/tissues.** THAP5 is highly expressed in the human heart, however the lack of availability of human cardiomyocyte cell lines restricts the experiments we can perform to characterize the protein. This prompted us to use a different system to study the function of THAP5. We originally isolated THAP5 from a melanocyte cDNA library. Before we proceeded with the use of melanoma cells as a model system, we performed RT-PCR analysis on several cell lines including melanoma and some other cancer cells along with normal kidney cells taken as control. We found that THAP5 is highly expressed in some of the melanoma cell lines and not expressed in normal kidney cells (Figure 10A). We obtained human primary and metastatic melanoma tissues and used THAP5 antibody to probe these tissues for THAP5 expression. We found that THAP5 shows high levels of staining in both primary and metastatic melanoma (Figure 10B).

**Sub-cellular localization of the THAP5 protein.** We have previously shown that THAP5 is localized in the nucleus in Hela cells [72]. To verify that THAP5 has a similar subcellular location in melanoma, we cloned the cDNA sequence corresponding to the full-length THAP5 protein into the GFPC1 vector. The GFP-THAP5 vector was transfected into MeWo cells and 24 hours later the subcellular localization of the protein was observed using a Confocal microscope as previously described [72]. Figure 11 shows that, the full-length GFP-THAP5 protein is exclusively localized in the cell nucleus but is excluded from the nucleoli.

**THAP5 protein is induced in melanoma cells upon UV treatment.** In order to investigate whether THAP5 protein is regulated during apoptosis, MeWo cells were treated with increasing doses of UVC and subsequently cell death was measured by Annexin V staining and Flow Cytometry [72]. THAP5 protein level was monitored by Western blot analysis of cell extracts, following UVC treatment and was found to be significantly induced in cells in a dose dependent manner (Fig. 12 A, B). THAP5 induction corresponded with increased apoptosis, both under conditions of increasing doses of UV and increasing time points. (Fig. 12 C, D). Similar result was obtained using another melanoma cell line Sk-Mel28.

**THAP5 protein level dramatically increases in MeWo cells following cisplatin treatment.** Cisplatin is another chemical that is known to activate DNA damage pathways. In order to investigate whether cisplatin causes a change in levels of THAP5, MeWo cells were treated with increasing doses of cisplatin and cell death was measured by Annexin V staining and Flow Cytometry [72]. THAP5 protein levels were monitored by Western blot analysis of cell extracts and found to be significantly increased in cells in treated with increasing doses of cisplatin. This induction also correlated with a corresponding increase in percentage of apoptotic cells (Figure 13). Similar results were obtained using another melanoma cell line SK-Mel28.

**THAP5 sensitizes cells to UV induced cell death.** To test whether THAP5 is proapoptotic or cytoprotective, MeWo cells were transfected with GFPC1 (control) and GFPC-THAP5 plasmids. Thirty six hours after transfection, cells were treated with increasing doses of UVC. 10 hours following UVC treatment the cells were stained with Annexin V and 7-AAD. Among the GFP-positive cells, the percentage of apoptotic cells was estimated. We found that in cells overexpressing GFP-THAP5, there was increased cell death compared to those expressing

GFP alone (Figure 14). This clearly shows that THAP5 sensitizes MeWo cells to UVC induced apoptosis [37, 72].

**THAP5 can repress transcription of a reporter gene.** THAP proteins are reportedly nuclear transcription factors with the ability to directly regulate expression of target genes. In fact, transcriptional regulation activity has been shown before in THAP1, THAP7 and THAP11 [43, 58, 60, 70]. Therefore, we expected THAP5 to be such a transcription regulator based on amino acid sequence similarity with the rest of proteins of the THAP-family. Since we do not know the target genes that might be regulated by THAP5, we used a transcription repression system that targets the Gal4-THAP5 to a reporter gene containing four Gal4 DNA binding sites. Gal4-THAP5 was able to repress the transcription of Gal4 reporter gene (Figure 15). As a positive control for these experiments we used Gal4-THAP7 previously shown to be a potent repressor of transcription when used in the same system [70]. Gal4-THAP5 repressor activity was not as effective as the THAP7 suggesting these two proteins might have distinctly different functions.

**Mutation Analysis of THAP5 gene.** THAP5 is known to arrest cells at G2/M phase of the cell cycle [72]. However, it is also highly expressed in several cancer cell lines and tissues in very high levels. We wanted to test if the THAP5 gene is mutated. So using specific primers, we amplified the three different exons of THAP5 from cDNA obtained from several obtained from several melanoma cell lines, primary melanocytes and metastatic melanoma tissues and sequenced them. However, we found no mutations in THAP5. This suggests that THAP5 is highly expressed in melanoma cell lines but not degraded and this could account for some unique

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function in cancer cells. Figure 16 shows a schematic diagram representing the three exons of THAP5 gene.

**Characterization of THAP5 binding site.** THAP5 has an atypical zinc finger domain (THAP domain) at its amino terminus which has been shown to be a sequence-specific DNA-binding domain in THAP1 and mouse homolog of THAP11 (Ronin) [44, 71]. Based on this information, we expected THAP5 to bind to a specific DNA sequence. To determine whether the THAP domain of THAP5 possesses sequence-specific DNA-binding activity and identify a consensus target-binding site, we used a modified PCR-based approach, CASTing (Cyclic Amplification and Selection of Targets) [79, 80]. We synthesized an approximately 100 bp random oligonucleotide using specific primers. This oligonucleotide pool was mixed with MeWo cell extract to permit the formation of THAP5/DNA complexes and then immunoprecipitated with Protein-G agarose beads and anti-THAP5 polyclonal antibody. The protein-DNA complexes were washed and then re-amplified. This process was repeated six times to enrich for oligonucleotides bound to THAP5 and after the seventh round, the PCR product was cloned into suitable vector (described in methods) and sequenced. Figure 17 A shows a consensus DNA binding site specific to THAP5.

**DNA-binding site specificity of THAP5.** To confirm the binding of THAP5 to the consensus DNA binding site, we performed electrophoretic mobility shift assays (EMSA) [77, 78]. We used labeled oligonucleotides containing the THAP5 consensus sequence (WT 5'-TGCCTGGTGCAAGTAACT), resolved the protein-DNA complexes by non-denaturing polyacrylamide gel electrophoresis (PAGE) and detected the bands by autoradiography. Figure 17B shows specific binding of THAP5 to the consensus sequence in MeWo extracts. Use of a

THAP5 specific antibody significantly reduced the binding of the complex. In addition, we do not see any binding of THAP5 in NIH3T3 mouse extracts (which lack THAP5 protein), showing that the DNA binding site is highly specific to THAP5. We also used 100 molar excess of cold probe (WT and mutant) and found that the binding of THAP5 was abolished completely. We mutated the consensus DNA binding site by modifying the invariable G and A nucleotides at positions 3 and 6, and found that the binding of THAP5 to the mutant probe was significantly diminished (Fig 17C).

**Identification of THAP5 binding site in promoter of G2/M genes.** In previous studies, THAP1, THAP11 and the mouse homolog of THAP11 (Ronin) were shown to bind a specific DNA sequence and regulate transcription of specific genes RRM1, c-Myc and HCF-1 respectively [44, 60, 71]. We know THAP5 is present exclusively in the cell nucleus and ectopic expression of THAP5 induces G2/M arrest in transfected cells [72]. Therefore, if THAP5 is indeed a transcription factor, one of its functions will be direct regulation of genes involved in cell-cycle. Based on this information and using the consensus THAP5 binding motif, GTGNAANNAAC, nucleotides spanning the 1kb region upstream of the promoters including 5' UTR of known cell cycle genes involved in G2/M transition [81] were scanned using specific software described in the methods section. A total of 20 G2/M genes were used as input and by allowing one nucleotide mismatch, 15 promoters were found to contain at least one instance of the consensus (shown in figure 16) (described in methods).

**Discussion**

THAP proteins are characterized by the presence of a THAP domain at the N-terminus with specific conserved residues and these include, a C2CH zinc finger motif, three other residues

(P26, W36, F58) and a C-terminal AVPTIF box (Ala-Val-Pro-Thr-Ile-Phe), which have been shown to be essential for the DNA binding activity of these proteins [38]. Abolishing any of these key residues eliminates DNA binding activity [44, 82]. In this study, we have expanded our studies on THAP5, one of the twelve human THAP proteins. For this purpose, we used melanoma cells because we originally isolated THAP5 as an interactor of Omi/HtrA2 using a melanocyte library and it is also expressed in melanocytes /melanoma.

We performed RT-PCR analysis of several cancer cell lines and found THAP5 to be highly expressed in several melanoma cell lines but not in normal cells. Moreover primary and metastatic melanoma tissues also show high levels of expression of THAP5. This raises a question as to why a protein which is involved in cell cycle arrest would be highly expressed in cancer cells. This could be the result of some mutation in THAP5 gene in these cells. To test this, we performed mutation analysis of several of the melanoma cell lines and metastatic melanomas using PCR amplification of the three exons encoding for THAP5 gene followed by sequencing of the exons. However, we found no mutations in the THAP5 gene. This suggests that THAP5 plays is highly expressed but not targeted for degradation. The stabilization of THAP5 could account for it function in melanoma cells and other cancers. We have previously shown that THAP5 contains a nuclear localization signal able to translocate the protein to the nucleus of HeLa cells [72]. To verify that THAP5 is also nuclear in melanoma cells we transfected MeWo cells with GFP-THAP5 fusion protein. These experiments clearly showed GFP-THAP is exclusively nuclear.

Some of the THAP proteins have been implied in functions such as apoptosis and cell cycle control. THAP0/DAP4 was originally identified while screening a Hela library for IFN-γ

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induced apoptosis genes [57]. It is speculated that THAP0/DAP4 may promote MST1-induced apoptosis by enabling the colocalization of MST-1 protein with p53 [69]. Also, THAP1 has been shown to be a proapoptotic protein associated with promyelocytic leukemia nuclear bodies (PML NBs), which interacts with Par-4 and induces serum withdrawal and TNFα-induced apoptosis [45]. Par-4 is a transcriptional regulator which is upregulated in prostate cancer cells undergoing apoptosis [83, 84]. Additionally, overexpression of Par-4 in neurons, prostate cancer and melanoma cells also sensitizes these cell types to apoptosis [43]. To investigate THAP5's role during apoptosis in melanoma, we treated MeWo cells with UVC. We used UVC to induce apoptosis because it is the most relevant physiological stimulus for these cells and it is also known to be responsible for the development melanoma cancers. In MeWo cells, upon treatment with UVC, we found that THAP5 is considerably induced and this increase correlated with the degree of apoptosis in the cell population. Similar results were obtained using a melanoma cell line, SK-Mel28.

The induction of THAP5 in melanoma cells treated with UV could have a pro-apoptotic or cytoprotective function. To test this we transfected MeWo cells with GFP-THAP5. We irradiated these cells with increasing doses of UVC and found that GFP-THAP5 over-expressing cells were more sensitive to UVC radiation compared to cells expressing GFPC vector alone. This suggests that THAP5 has a proapoptotic function in melanoma cells treated with UV.

We investigated whether UV alone can induce THAP5 or other cytotoxic chemicals known to cause DNA damage can have a similar effect. For these experiments, MeWo cells were treated with cisplatin. Cisplatin forms adduct with DNA and the resulting inter and intra strand crosslinks disrupt the structure of the DNA thereby interfering with replication and transcription

processes [85]. This alteration in the structure is recognized by the cellular proteins to repair cisplatin-induced DNA damage [85]. THAP5 protein was also induced when melanoma cells were treated with cisplatin in a similar manner as cells treated with UV. These results suggest THAP5 is probably involved in a previously unknown pathway that gets activated by DNA damage leading to cell death.

THAP proteins constitute the second largest family of zinc finger proteins after the C2CH family [82]. The few members that have been studied were shown to have DNA binding activity and to recognize specific target sequences. THAP1 binds to an 11 nucleotide motif and Ronin (mouse homolog of THAP11) recognizes a slightly longer and different 15 nucleotide sequence [44, 71]. These findings as well as recent structural analysis has speculated that each THAP protein may bind to a unique DNA sequence and therefore perform a distinct function in cells where it is expressed [44, 71, 86]. We employed a modified protocol of the Cyclic Amplification and Selection of Targets (CASTing) assay and identified an 11 nucleotide consensus binding sequence recognized by THAP5 [79, 80]. To verify that THAP5 can bind to this sequence in cells we performed electrophoretic mobility shift assays (EMSA). We confirmed the specificity of binding by using THAP5 specific antibody which reduced the binding significantly. Not only does the probe bind to THAP5 specifically but also shows no binding in mouse nuclear extracts (NIH3T3) which lack THAP5. We mutated the two nucleotides which were invariable in all sequences ($G_3$ and $A_6$) and found that the binding of THAP5 to the sequence is considerably reduced.

It has been proposed that some members of the THAP family of proteins (THAP7 and HIM-17) play a role in chromatin regulation because of their interaction with chromatin-modifying

proteins [58, 70]. THAP7 has been shown to associate with histone tails, HDACs and NCoRs [58, 70] and act as a transcriptional repressor. HIM-17 is involved in recruitment of the methyltransferase activity to histone H3 [87, 88]. Ronin interacts with HCF-1, an important transcriptional regulator [71].

Since there's no information on the potential gene targets of THAP5, we first performed a transfected reporter gene assay. For this assay, we used THAP5 fused to a Gal4 DNA binding domain and cotransfected it with a vector containing the firefly luciferase gene under the control of Gal4 binding sides and a control Renilla luciferase reporter. We measured the luciferase activity relative to the Renilla luciferase values. As a control for this experiment we used Gal4-THAP7 which has already been shown to be a transcription repressor [58, 70]. Gal4-THAP5 shows repressor activity but it was not as high as the Gal4-THAP7. Although at this point, we have only demonstrated that THAP5 can function as a repressor in a heterologous system, we still do not know if it can repress genes in cells and under what conditions this function comes into play.

Some of the THAP proteins have been shown to exert their function by acting on specific gene targets. THAP1 has been shown to act on RRM1 gene, a pRb/E2F cell cycle target [43]. THAP11 represses c-Myc and negatively downregulates growth of cells [60]. Since we already know the specific DNA sequence that THAP5 binds to, we decided to look for this binding site in promoters of genes which are involved in G2/M transition. The reason we did this was because we have previously shown that THAP5 is involved in G2/M cell cycle arrest [72]. We took a set of 20 known genes involved in G2/M cell cycle arrest. Using the consensus binding site for THAP5 we scanned the promoter region of these genes and found the binding site in

several of the genes. Of these genes, we are particularly interested in the sites that showed up only in human, rhesus and dog because THAP5 is not expressed in mouse/rats.

Currently we are looking at a set of genes and using their promoter regions containing potential THAP5 binding sites in luciferase assays to test if THAP5 regulates their transcription. It'll be interesting to identify potential targets of THAP5 because it can provide a lot of information on THAP5's functions in cells. As a starting point, it might be worthwhile to look into HCF-1, a large protein involved in transcriptional control. Previously, Ronin (mouse homolog of THAP11) has been shown to associate with a large HCF-1 protein containing complex, in which even THAP7 was pulled down [71]. Recently another group also showed that THAP1 and THAP3 can interact with HCF-1 and activate transcription [89]. They also showed that most of the THAP proteins have a HCF-1 binding motif called HBM through which they interact with the amino-terminal kelch domain of HCF-1. The HBMs are always located upstream of the coiled-coil domains and found at similar locations in the orthologs of THAP proteins in other species such as zebrafish and xenopus [89]. In fact, in a yeast two hybrid screen, they showed that most of the THAP proteins including THAP5 can interact with HCF-1 protein. Therefore, it has been proposed that interaction of THAP proteins with HCF-1 may be important for both transcriptional activation and repression, depending on the THAP protein involved, the cellular context and/or the cell cycle status [89].
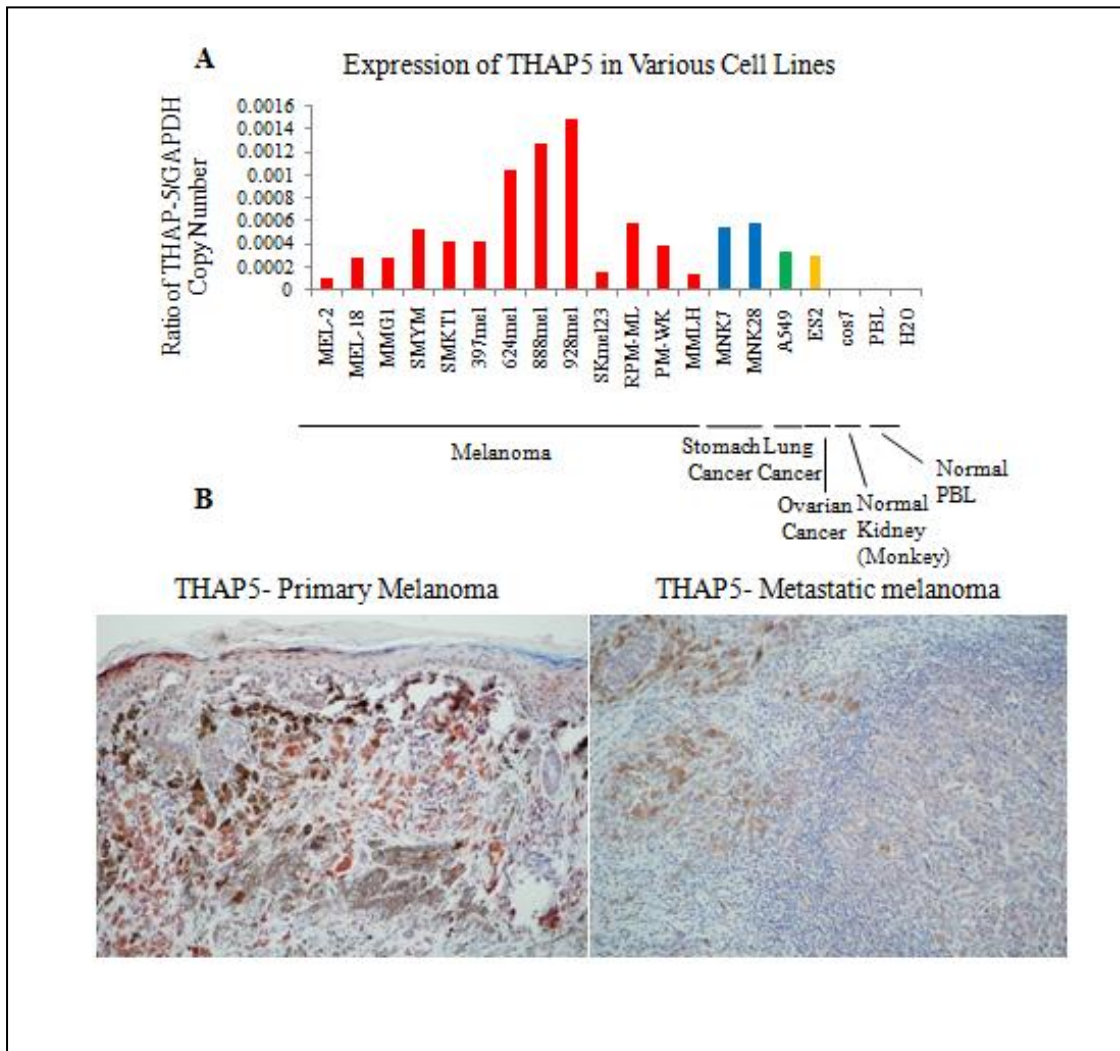
**Figure 10. Expression of THAP5 in melanoma cells and tissues.**

A, qRT-PCR analysis of various cell lines showing THAP5 expression. qRT-PCR was performed on several cell lines including melanoma and some other cancer cell lines with normal kidney cells as control. THAP5 is highly expressed in some melanoma cell lines. B, Immunohistochemistry staining of primary and metastatic melanoma tissues showing high levels of THAP5.
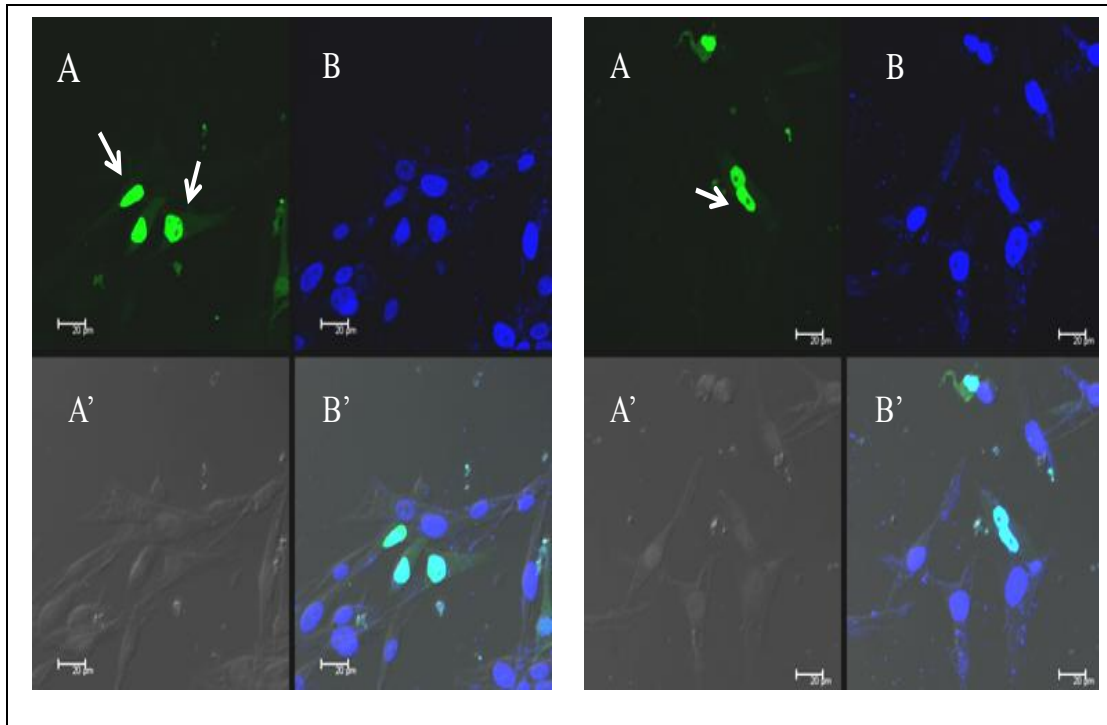
**Figure 11. Subcellular localization of the THAP5 protein in melanoma (Mewo) cells.**

*C*onfocal images of Mewo cells transfected with GFP-THAP5$_{1-395}$ shows exclusive nuclear localization (green in A). Nomarski/DIC images of the same cells are shown in A'. Panel B shows cells with DAPI staining for the nucleus and B' shows merged image of Panels A, B and A'.
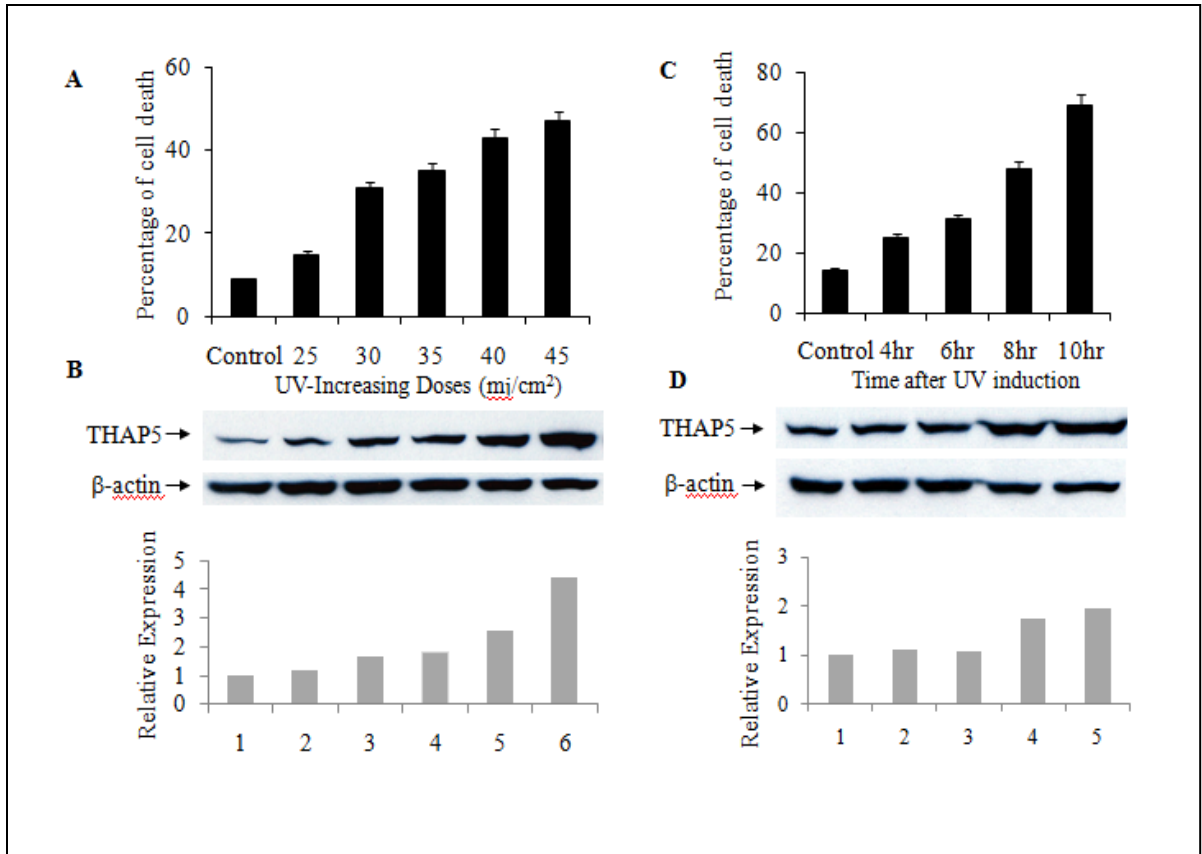
**Figure 12. THAP5 protein is induced in MeWo cells upon UV treatment.**

A, Cell death was monitored in the treated cell populations, by Annexin V and 7-AAD staining and analyzed by flow cytometry; B, Cell extracts of the treated samples were prepared, followed by Western blot analysis of the same. *Lane 1* shows lysate from control cells; *lanes 2*, *3*, *4, 5 and 6* show extracts from MeWo cells treated with increasing doses of UVC; $25mj/cm^2$, $30mj/cm^2$, $35\ mj/cm^2$, $40mj/cm^2$, $50\ mj/cm^2$ respectively. THAP5 protein level increases with time. *C*, Cell death was monitored in the UVC treated cell populations, by Annexin V and 7-AAD staining and analyzed by flow cytometry. *D*, cell extracts were prepared followed by Western blot analysis of the treated samples. *Lane 1* shows lysates from control cells; *lanes 2, 3, 4, 5 and 6* show extracts from cells treated with $50\ mj/cm^2$ of UVC after 4, 6, 8 and 10 hrs respectively. β-Actin antibody was used to verify that equal amounts of protein were present in each lane. Data are means ± SD of four independent experiments.
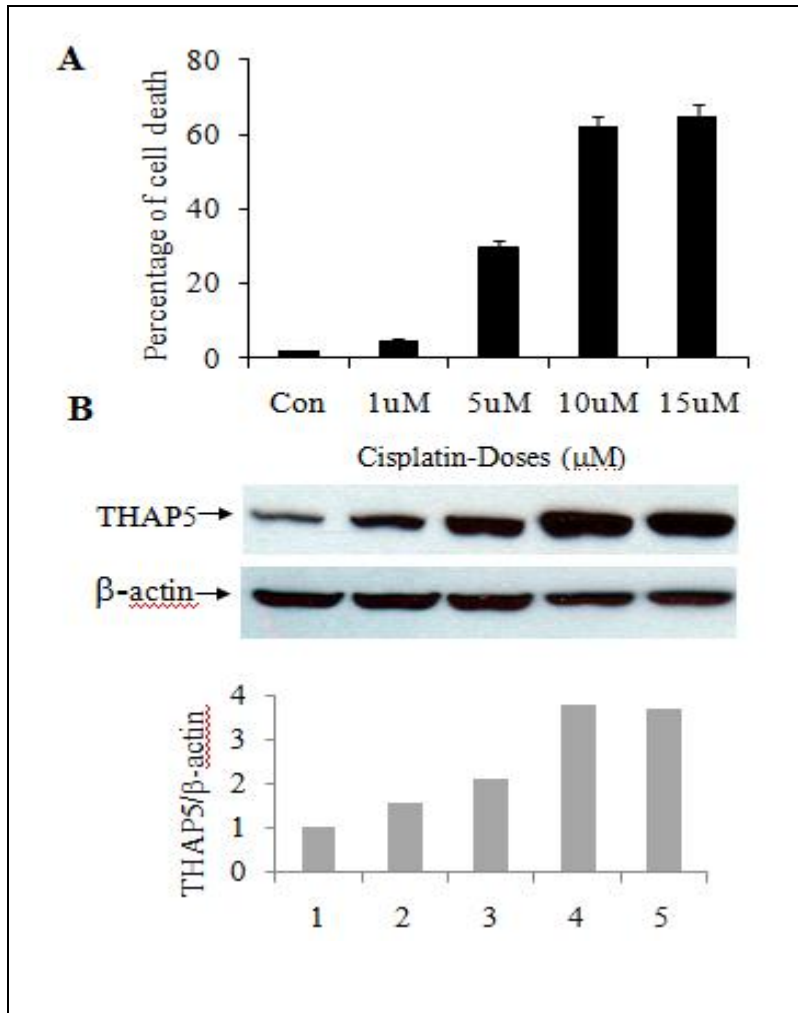
**Figure 13. THAP5 protein levels increase following cisplatin treatment.**

MeWo cells were treated with increasing doses of cisplatin to induce apoptosis. A, Cell death was monitored in the treated cell populations, by Annexin V and 7-AAD staining and analyzed by flow cytometry. B, Cell extracts were prepared followed by Western blot analysis of the treated samples. *Lane 1* shows lysates from control cells; *lanes 2*, *3*, *4, and 5* show cells treated with 1mM, 5mM, 10mM and 15mM cisplatin for 10 hours respectively. β-actin antibody was used to verify that equal amounts of protein were present in each lane. Data are means ± SD of four independent experiments.
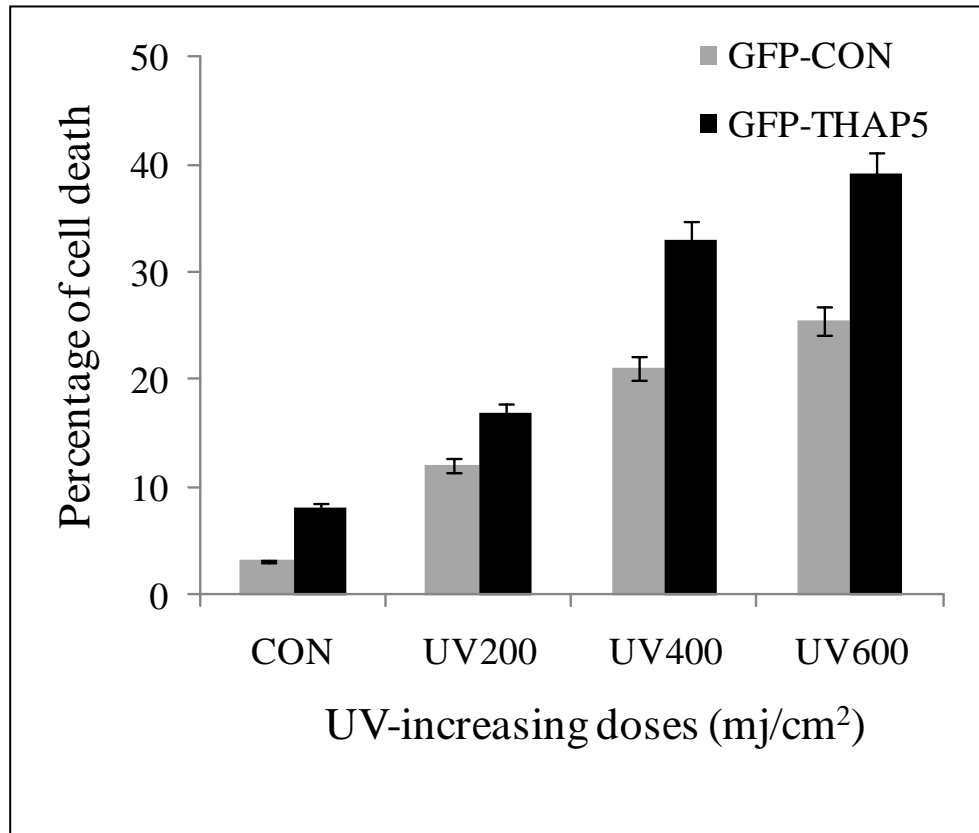
**Figure 14. Overexpression of THAP5 sensitizes cells to UV induced apoptosis.**

MeWo cells were transfected with GFPC vector and GFPC-THAP5 plasmids. 36 hours following transfection, cells were treated with increasing doses of UVC. Cell death was monitored in the treated cell populations, by Annexin V and 7-AAD staining and GFP positive cells were counted analyzed by flow cytometry. Data are mean ± SD of 3 different experiments.
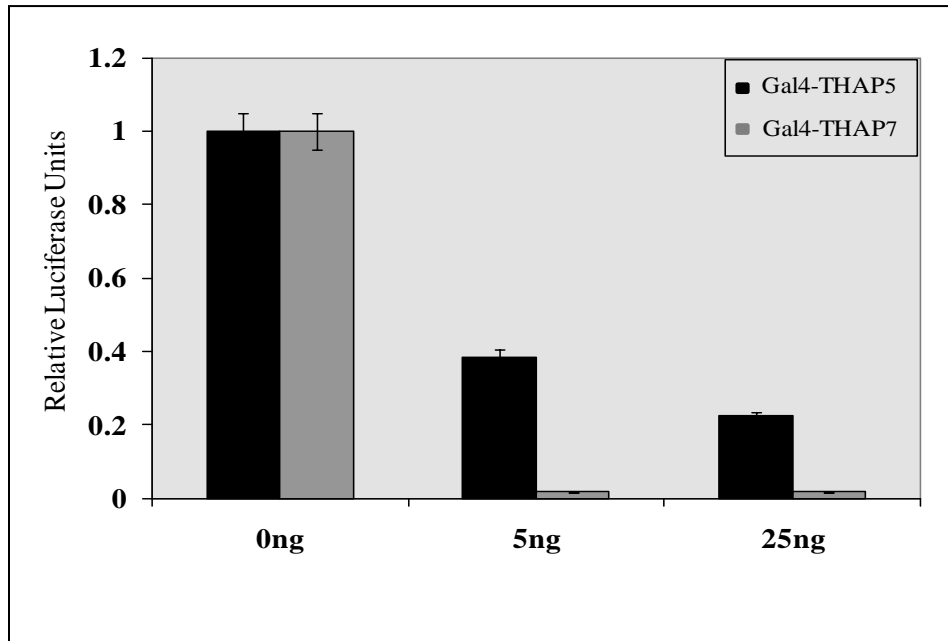
**Figure 15. THAP5 represses transcription of a reporter gene.**

Gal4DBD-Thap5 and Gal4DBD-Thap7 were transfected separately in increasing amounts (0ng, 5ng and 25ng respectively) into HEK-293T cells along with Gal4 MH100TK-luc reporter and a control Renilla luciferase reporter (pRL-SV40 Luc). Luciferase assays were performed 48 hrs post transfection. Values are reported relative to Renilla Luciferase reporter. Data are means ± SD of four independent experiments.
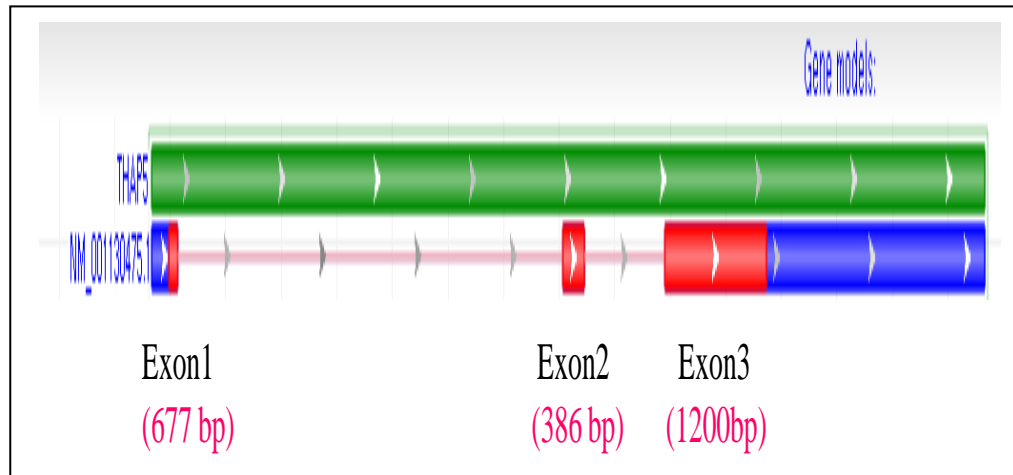
**Figure 16.  Schematic representation of the THAP5 gene.**

Full-length THAP5 gene contains 3 exons regions.  Exon1 is 677 base pairs (bp), Exon2 is 386 bp and Exon 3 is 1200 bp long.  The three exon regions depicted in the figure were amplified by PCR from cDNA obtained from several melanoma cell lines, primary melanocytes and metastatic melanoma tissues.  Specific primers were used for amplification of the three exons followed by sequencing and analysis of the PCR products for mutations.
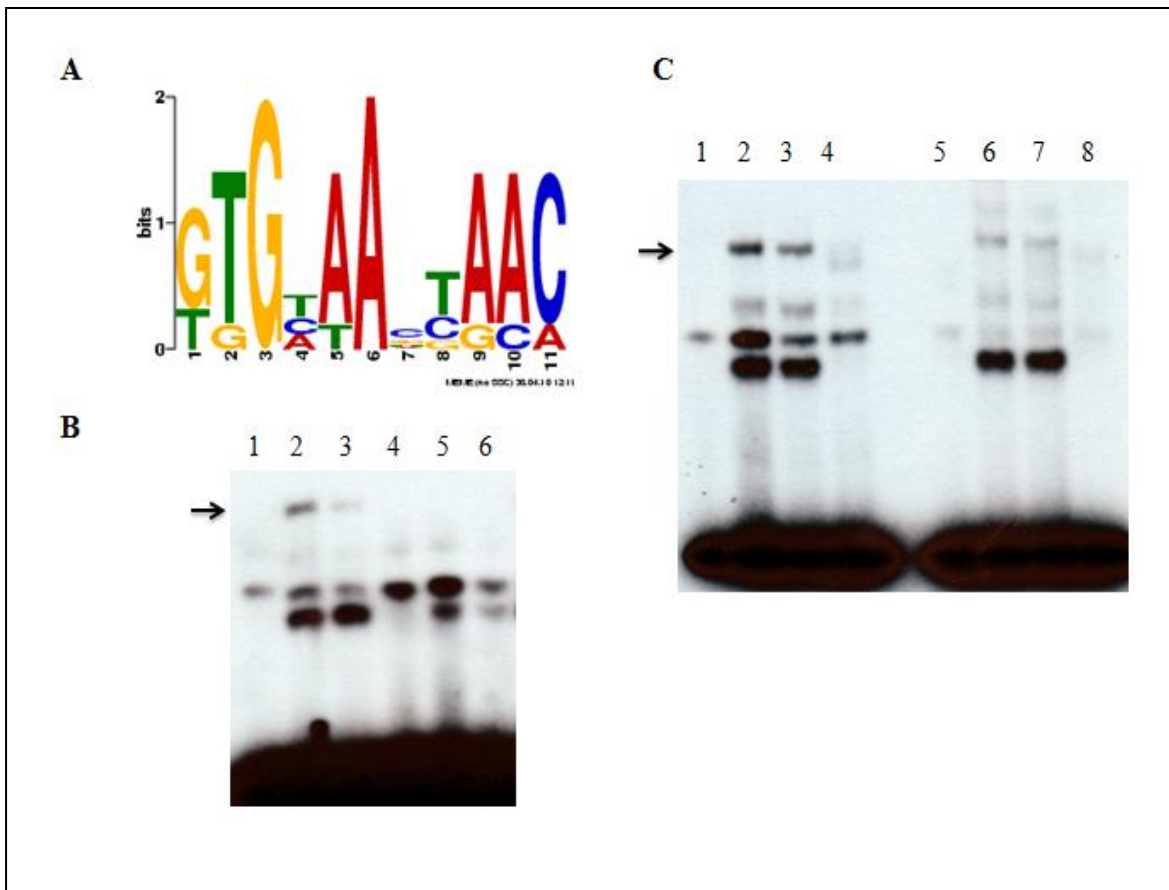
**Figure 17. Identification of a THAP5 consensus DNA-binding site.**

A, The 11-nucleotide THAP5 DNA binding site. The oligonucleotide sequences recovered after 7 rounds of selection were analyzed by the motif-discovery program MEME. B, Electrophoretic Mobility Shift Assay showing THAP5 binding to consensus sequence. Lane 1, control (no extract); lane 2, MeWo nuclear extract; lane 3, MeWo extract with anti-THAP5 antibody; lane 4, NIH 3T3 extract; lane 5, MeWo extract plus 100 molar excess of cold THAP5 specific probe (WT 5'-TGCCTGGTGCAAGTAACT ) and lane 6, MeWo extract plus 100 molar excess of mutant probe (Mut 5'-TGCCTGGTACACGTAACT), incubated with $^{32}$P labeled wild type probe. The protein-DNA complexes were analyzed by electrophoretic mobility shift assay (EMSA) as described in methods. C, Specificity of THAP5 Binding. First four lanes show THAP5 binding to the labeled WT probe. Lane 1, control (no extract); lane 2, MeWo nuclear extract; lane 3, MeWo extract with anti-THAP5 antibody; lane 4, NIH 3T3 extract. Lanes 5-6 show reduced binding of THAP5 to the labeled Mutant probe. Lane 5, control (no extract); lane 6, MeWo nuclear extract; lane 7, MeWo extract with anti-THAP5 antibody; lane 8, NIH 3T3 extract.

| Human Gene | Motif | Species |
|---|---|---|
| CCNB1 | GTGCCACGAAC | Human, Mouse, Chimp |
| CDNK3 | GAGTAAGAAAC | Human, Mouse, Chimp, Dog |
| GTF2H1 | CTGGAAACAAC | Human, Mouse, Chimp, Dog |
| MNAT1 | TTGAAAAGAAC | Human, Mouse, Chimp, Dog |
| SERTAD1 | GTGTAAGAAGC | Human, Dog, Rhesus |
| ANAPC4 | CTGCAACTACC | Human, Mouse, Rhesus |
| BCCIP | GCGGAAGAAAA | Human, Mouse, Rhesus, Dog |
| CCNG1 | TTTTAAGCAAC | Human, Mouse, Rhesus, Dog |
| CDK5R1 | CTGACAGGAAC | Human, Mouse, Rhesus, Dog |
| DDX11 | GTGAAAAAAAC | Human, Rhesus, Dog |
| DIRAS3 | GTGCACGTATC | Human, Rhesus |
| DNM2 | GAGCAACGAAG | Human, Rhesus, Mouse, Dog |
| HERC5 | GCTTAACAAAC | Human, Elephant |
| KPNA2 | ATGCAATTAAA | Human, Rhesus, Dog |

**Figure 18. Identification of potential promoters containing THAP5 binding site.**

Using the consensus THAP5 binding motif, GTGNAANNAAC, nucleotides spanning the 1kb region upstream of the promoters including 5' UTR of known cell cycle genes involved in G2/M transition were scanned using the software listed in Materials and Methods. A total of 20 G2/M genes were scanned and by allowing one nucleotide mismatch, 15 genes were found to contain at least one instance of the consensus (shown in table).

# CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

Omi/HtrA2 is an extensively studied serine protease. Its role as a proapoptotic protease is very well established however, its normal function in cells is not known [3, 5, 6, 11]. Most of the recent studies seem to point out to a protective function for Omi/HtrA2 [19]. Some groups believe it may be involved in the maintenance of cellular equilibrium. In order to define the functions of a protein it is important to find out what other proteins it interacts with. The interacting/partner protein sheds light on the possible pathways/ functions of the protein of interest. The yeast two-hybrid system is a popular method used to isolate new protein interactors. We employed this method to discover new partners of Omi/HtrA2. Among the approximately 30 interactors obtained, the one of most interest to us was THAP5. There was no published information on this protein. The only known information on THAP5 was it belongs to a large family of transcription factors called THAP proteins [45]. All the proteins in this family have zinc dependent DNA binding activity and are involved in various important cellular functions [45].

Through my dissertation we were able to obtain key information on the function of this novel THAP5 protein. THAP5 is a 395 amino acid protein which localizes in the nucleus. It encodes for an approximately 50kDa protein. THAP5 mRNA is highly expressed in the human heart and some expression is seen in the brain and skeletal muscle. It interacts with Omi/HtrA2 in yeast and in mammalian cells. However, in mammalian cells, THAP5 interacts with Omi/HtrA2 only under conditions of apoptosis. When cells are subjected to apoptotic stimuli, Omi/HtrA2 is released from the mitochondria and this creates a physiological meeting point for these two proteins. Furthermore, the interaction of Omi/HtrA2 with proteins from different cellular

compartments has been well documented before. We have been able to show that THAP5 can serve as a substrate for Omi/HtrA2 both in vitro and in mammalian cells. The degradation of THAP5 can be prevented by the use of ucf-101, a specific inhibitor of Omi/HtrA2. However, it is not known what the function of this degradation of THAP5 means [72].

To explore the normal function of THAP5, we overexpressed the protein in cells and found that it induced cell cycle arrest at G2/M phase. This could possibly explain the high levels of expression of THAP5 in the human heart which represents a terminally differentiated organ with very small percentage of dividing cells. This function of THAP5 is also in agreement with some other proteins of the THAP family. The most significant finding is the deregulation of THAP5 in heart tissues from Coronary Artery Disease (CAD) patients. This occurs in the myocardial infarction area (MI), where maximum damage and loss of cardiomyocytes happens. Although the actual function of THAP5 in the human heart is unknown at this point, its deregulation coupled with the high expression levels in the human heart suggests that the protein plays a very important role in the development/ progression in heart disease [72]. The absence of mouse/rat homolog for THAP5 made studies with the proteins very difficult. Besides, there are no human cardiomyocyte cell lines available. Therefore, we decided to use melanoma cells to study the mechanism of THAP5 function, where we know THAP5 is expressed.

We found that THAP5 has a proapoptotic function in melanoma cells. THAP5 is not only induced upon UV and cisplatin mediated apoptosis, its overexpression also sensitizes melanoma cells to these apoptotic stimuli. Through our results, we believe that THAP5 could be involved in a very important DNA damage/repair pathway. This function of THAP5, we believe is independent of the Omi/HtrA2 pathway. We have also identified a DNA binding site which is

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specifically recognized by THAP5. Although at this moment, we do not know the genes that THAP5 regulates, we have narrowed down a list of G2/M phase genes that potentially contain THAP5 binding site in the promoter region. Identifying the gene targets of THAP5 will provide great insight into its function as a transcription factor. Furthermore, THAP5 can repress the transcription of a reporter gene adding to the set of transcriptional repressors belonging to the THAP family of proteins. In summary, our work identifies a novel cardiospecific transcription factor with an important role in heart disease and melanoma apoptosis.

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