# The Translationally Controlled Tumor Protein (TCTP) associates to and destabilizes the Circadian Factor Period2 (Per2)

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# Translationally Controlled Tumor Protein (TCTP) associates to and destabilizes the Circadian Factor Period2 (Per2)

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(ABSTRACT)

Period 2 (Per2) is a core circadian factor responsible for its own negative regulation. It operates in the circadian clock, which affects multiple biological functions such as metabolic rate, hormone release, and core body temperature. The Per2 protein functions directly with factors in other biological functions such as tumor suppression, immune system, and metabolism. In many cases, the Per2 deficiency caused by disrupted expression is sufficient to create severe abnormalities in many of the mentioned functions. The sequence contains several domains and motifs in Per2 that are traditionally involved in protein interactions which suggests that Per2 serving a regulatory role by effecting downstream biological roles dependent on Per2 stability.

In this work, we perform a two-hybrid screening assay using the C-terminal region of human Per2 and identified an extensive number of interactors. Utilizing a genetic ontology program, we assorted the list of clones into groups of proteins that are biologically relevant or operated in similar function. Through this program, we validated the two-hybrid screening by the clusters of biological function already attributed to hPer2 and identified new putative biological functions. We use the new putative interactors to gain further insight on the regulatory roles that hPer2 performs, in conjunction with operating as a core factor in circadian rhythmicity.

We also show that Translationally Controlled Tumor Protein (TCTP) is capable of binding to hPer2 and is a novel interaction. When a sufficient amount of TCTP (1:1 molar stoichiometric ratio) is present in a system, a cleavage of hPer2 is observed *in vitro*. This cleavage occurs in reactions independent of ATP, ubiquitin, and the proteasome. The data points towards a method of cleavage similar to that of the archael lon-*tk* (*Thermococcus kodakaraensis*) that preferentially cleaved unstructured substrates in ATP-independent reactions.

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**Attributions**

The individuals who contributed to the work presented here and the nature of their contributions are listed here.

**Chapter 3: Results**

**Prof. Carla V. Finkielstein** – Ph.D. (Department of Biological Sciences, Virginia Tech) is the Committee Chair and provided co-direction of this project.

**Chapter 4: Discussion**

**Prof. Carla V. Finkielstein** – Ph.D. (Department of Biological Sciences, Virginia Tech) is the Committee Chair and provided co-direction of this project.

**Chapter 6: Collaborations**

**Prof. Carla V. Finkielstein** – Ph.D. (Department of Biological Sciences, Virginia Tech) acted as the director of the project "A novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor Period 2".

**Jianhua Yang** – Ph.D. (Department of Biological Sciences, Virginia Tech) contributed as first author to the publication "A novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor Period 2".

**Andrew Lucas** – B.S./M.S. (Department of Biological Sciences and Department of Chemistry, Virginia Tech) contributed to the data presented in the publication "A novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor Period 2".

**Carlo S. Santos** – M.S. (Department of Biological Sciences, Virginia Tech) contributed to the data presented in the publication "A novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor Period 2."

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**Abbreviations**

| | |
|---|---|
| ASPT | Advanced sleep-phase type |
| Bcl-2 | B-cell lymphoma 2 |
| Bcl-xL | B-cell lymphoma- extra large |
| Bmal1 | brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 |
| CHO | Chinese hamster ovaries |
| CKIε | Casein kinase I ε |
| CRY | Cryptochrome |
| DSPS | Delayed sleep-phase syndrome |
| Dss4 | Dominant suppressor of Sec4 |
| eEF1α | eukaryotic elongation factor 1 α |
| G protein | GTP-binding protein |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| hPer2 | human Period 2 |
| HRF | Histamine releasing factor |
| KO | Knock-out |
| MCL-1 | Myeloid cell leukemia 1 |
| Mss4 | Mammalian suppressor of Sec4 |
| PAS | Per2 – Arnt – Sim |
| Plk | Polo-like kinase |
| RAR | Retinoic acid receptor |
| ROR | RAR-related orphan receptor |
| SCN | Suprachiasmatic nucleus |
| siRNA | silencing RNA |
| TCTP | Translationally controlled tumor protein |

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**Chapter 1: Background and significance**



Chapter 3: Results

## Chapter 1: Background and significance

Circadian rhythms are the manifestation of a 24 h oscillatory cycle upon which physiological and behavioral functions are dependent on. For example, mammals undergo activity during the day that requires specific genes for increased metabolism, especially during feeding times and at night these genes are not required. This is done in order to maximize energy efficiency in the body. Circadian rhythm is an essential mechanism by which temporal gene control is maintained. In humans, the master circadian clock is located in the suprachiasmatic nucleus (SCN) in the anterior region of the hypothalamus. After the initial input by light entrainment, many of the cells in the body are capable of maintaining the circadian rhythm [1] even if they were to be separated from the body, representing autonomous cellular circadian activity.

### The Circadian Clock Mechanism

The mammalian circadian clock consists of components oscillating expression according to positive and negative feedback loops [2]. The two primary components of this mechanism are the genes *clock* and *Bmal1* respectively. These two genes encode transcription factors CLOCK and BMAL1, which upon dimerization through the PER-ARNT-SIM (PAS) domain, regulate the transcription of *per* and *cry* genes [2]. Transcription is activated when the CLOCK:BMAL1 complex binds to the E-box element within the promoter region of *per* and *cry* genes. Once *per* and *cry* mRNAs have been translated, the heterodimer translocates to the nucleus. It is inside this compartment that PER:CRY acts as a negative regulator of CLOCK:BMAL1-mediated transcription [2], and thus down-regulates *per* and *cry* expression. Casein Kinase I epsilon (CKIε) acts on residue 662 in hPer2 and it is responsible for the phosphorylation-dependent degradation of PER. In order to sustain the circadian mechanism, a feedback loop is necessary. Rev-Erbα is a nuclear receptor that can act as a transcriptional repressor and has an E-box in its promoter [3]. The transcription of the *Rev-erbα* gene is positively regulated by CLOCK:BMAL1 leading to Rev-Erbα accumulation and specific inhibition of *bmal1* transcription [3]. The transcriptional activation of *bmal1* is under debate with the most widely accepted theory using RAR orphan receptors (ROR) but experiments show that this is a redundant system and is not required for *bmal1* rhythmicity [3]. BMAL1 acts as the rate limiting protein in the CLOCK:BMAL1 heterodimerization complex[2] with PER:CRY acting as positive regulator. (Fig 1.1)

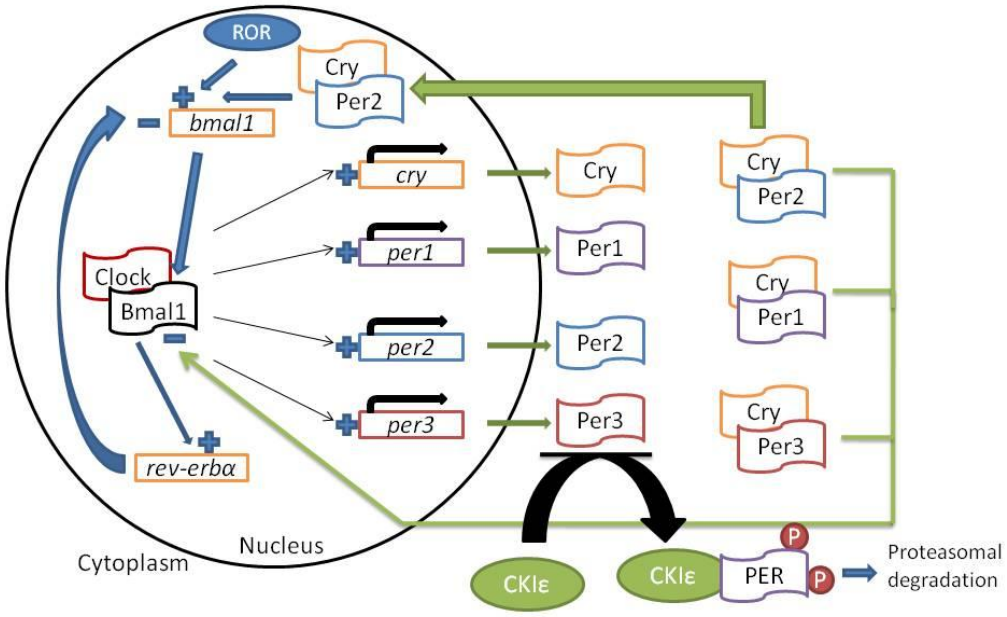


**Figure 1.1:** The Circadian clock. The circadian clock is comprises oscillations in expression. The presence of heterodimer Per/Cry in the nucleus along with ROR activates the expression of *bmal1*. Clock is constitutively expressed and therefore once Bmal1is present, the Clock/Bmal1 heterodimer binds to downstream E-boxes that activate transcription and expression of *cry* and *per* genes. These proteins are exported into the nucleus where Per is degraded at basal levels until it accumulates in order to heterodimerize and form a complex with Cry. The complex translocates to the nucleus to negatively regulate its own expression.

**Period 2**

Per2 contains many structural domains (Figure 1.2) that are potential sites of interaction. The PAS domain is in the N-terminus region and contains two repeats (PAS A and PAS B). The PAS domain is responsible for various protein-protein interactions and is also capable of ligand-binding such as heme[4] that implies Per2 involvement in sensing redox conditions in the cell. The L*XX*LL motifs are conserved in coactivators that are involved with nuclear receptors. This motif is found on both the N-terminal and C-terminal regions and although no such activity for Per2 has been reported thus far[5], it suggests alternative regulatory roles for the circadian factor. There are several nuclear export signals and one nuclear localization signal throughout Per2 and deletion of the NLS causes accumulation of Per2 in the cytoplasm [5]. Despite this, there has been evidence indicating CRY is capable of localizing Per2 to a certain degree inside the nucleus through forming the heterodimer complex[6]. The coiled coil domain is located at the C-terminal region of Per2 and is capable of forming dimers through its amino acid residue repeat pattern that forms a helix and dimerizes with the helix of another protein through hydrophobic

surface interactions[7]. There is a proline-rich sequence that hypothetically causes Per2 to be less structured which may allow Per2 to serve as a scaffold protein which could utilize the coiled coil and PAS domains to bring proteins in proximity[5].

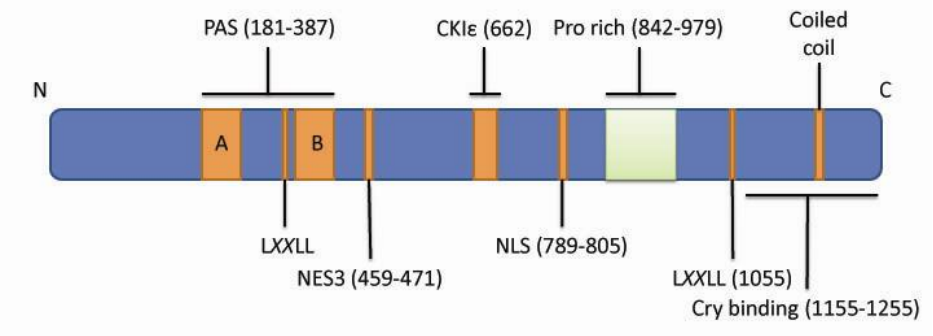


**Figure 1.2:** Per2 domains. Localization signals include the nuclear export signals (NES) and nuclear localization signal (NLS). The protein interaction domains and motifs are the helix-loop-helix motif (HLH), Per2-Arnt-Sim (PAS A and B subdomains), L*XX*LL motifs (*X* represents any amino acid), coiled coil motif, and the CKIε binding region where it becomes phosphorylated.

Per2 localizes in both the cytoplasm and nucleus of the cell. As described above, CRY is capable of promoting nuclear entry of Per2 but is not solely responsible, suggesting a cooperative relationship between NLS and CRY[5]. The interaction between Per2 and CRY is also important in preventing ubiquitination and prolonging the half-life of Per2[7]. *In vitro* data suggest other ligands, such as heme, can prevent the Per2/CRY complex from forming which could subsequently lead to further ubiquitination of Per2 as well[8].

There are multiple post-translational modifications of Per2 which alter its stability or localization[9]. There are multiple phosphorylation sites identified in Per2 but one in particular, S662, has been studied extensively for loss of CKIε binding and hypophosphorylation. The lack of phosphorylation at S662G favored destabilization and increased degradation whereas mutating the same residue to glutamic acid to mimic constitutive phosphorylation led to accumulation of Per2[10]. The results implicated that phosphorylation at S662 and downstream phosphorylation sites stabilize Per2 whereas phosphorylation at the other sites in lieu of S662 favored degradation. Polyubiquitination of Per2 by its E3 ligase, β-TrCP, leads to proteasome-mediated degradation and only recognizes Per2 once bound to CKIε[11]. Sirt1 is a deacetylase that targets histones and several transcriptional regulatory proteins involved in metabolism. Acetylation of hPer2 serves to stabilize and increase the half-life [12], evident in *sirt1* KO mice that display an abnormal accumulation of hPer2. The expression of Sirt1 significantly reduces the accumulation of hPer2.

There are three Period homologs in the mammalian circadian clock. Despite the interest in the functionality of all three, there are relatively insignificant phenotypes caused by *per1* or *per3* deficient

mice [6] in terms of the combined effects of both circadian rhythmicity and period. In the *per1*-deficient mice, the oscillations of *per2* are unaltered whereas the *per2*-mutated mice showed decreased expression of *per1* and *per2* indicating that *per2* is an upstream effector of *per1* [6]. Like *per2*-deficient mice, disruption of the *per3* gene results in a phenotype with a shortened circadian period. However, *per3*-disrupted mice maintains rhythmicity in darkness [13] suggesting that Per3 functionality has some effect on the length of the circadian period but has no significant role in maintaining circadian rhythmicity.

In mice with disrupted *per2* expression, metabolic processes are also disrupted and lose cyclical expression. *Per2* mutant mice display reduced muscle strength and endurance along with implications of increased dependence on anaerobic metabolism[14] shown by up-regulation of such genes as beta enolase or triose phosphate isomerase. Mice deficient in *per2* also have a characteristic aberrant interferon-γ (IFN-γ) mRNA cycling [15] and decreased IFN-γ in serum. Although the reduced IFN-γ production is not directly attributable to *per2* deficiency, it is a correlation that carries weight considering IFN-γ is responsible for anti-tumor functions (Figure 1.3).

Mice deficient in *per2* have shown increased tumor development and reduced apoptotic behavior after exposure to γ radiation [16]. Homozygous mice for the deletion mutation in Per2 PAS domain (*mPer2*$^{m/m}$), showed a neoplastic phenotype [6]. In addition, core circadian genes were induced in wild-type but not in *mPer2*$^{m/m}$ mice in response to γ-radiation suggesting that some clock controlled genes are involved in tumor suppression. To evaluate the apoptotic function of Per2 in the thymus of animals with *mPer2*$^{m/m}$ background, the thymus of the mutant mice and wild-type mice were taken after 18 hours. Most of the thymocytes in wild-type mice had undergone apoptosis whereas the majority of the mutant mice thymocytes were still intact and this effect correlates with the levels of cytochrome c present in the cytosol of wild-type compared with mutant animals [6]. This evidence suggests mutations in the PAS domain that disrupt heterodimerization of mPer2 causes cells that would normally become apoptotic to persist and reproduce which is similar in concept to how tumor cells function; cells with many mutations that continue to persist and reproduce.

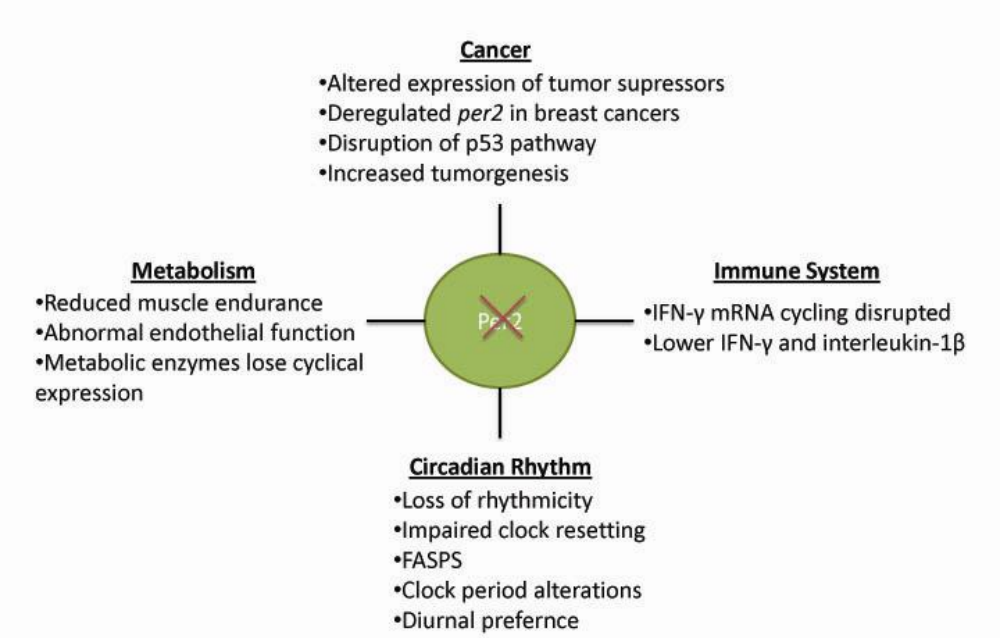


**Figure 1.3:** Per2 aberration disrupts multiple biological functions. As a core circadian factor, it comes as no surprise that the aberration of Per2 disrupts rhythmicity and behavioral traits that are controlled by circadian rhythm. The immune system is disrupted when the IFN-γ mRNA cycles deviate and IFN-γ levels drop. Dysfunctional Per2 causes increased tumorgenesis and altered expression of downstream tumor suppressors. Metabolic functions are altered in *per2* deficient or KO mice.

**The Translationally Controlled Tumor Protein (TCTP)**

The *tpt1* gene was one of the genes found up-regulated in Ehrlich ascites cells compared to normal cells[10] and was thus named Translationally Controlled Tumor Protein (TCTP). Several other groups also discovered TCTP and each gave distinct names such as fortillin due to its tubulin-binding abilities [17] or histamine-releasing factor (HRF) due to its ability to induce histamine release upon its secretion from the cell [18]. The TCTP has been implicated in over a dozen pathways and functions (for review, see [10] and references within). For example, the protein is involved in anti-apoptosis [19] and microtubule stabilization [20]. The relevance of its overexpression in cancerous cells is pertinent and not coincidental especially since down-regulating expression of TCTP can actually cause reversion of colon, lung, leukemia, and melanoma cancer cell lines [21].

Human TCTP is a small protein of 172 amino acids [10] that is conserved in over 30 different species ranging from yeast to higher mammals [10]. The conservation between human and mouse TCTP is over 95% in sequence alone. Its structure consists of one major alpha helix connected to a second alpha helix by a loop structure, multiple beta sheets and a larger unstructured loop [10]. There are two evolutionarily conserved regions in TCTP, TCTP1 (amino acids 46-55) and TCTP2 (125-148) [10] but there has been no functionality assigned to these regions. The TCTP is structurally homologous to

guanine nucleotide free chaperones Mss4 (Mammalian suppressor of Sec4) and Dss4 (Dominant suppressor of Sec4, the yeast homologue of Mss4)[22]. Guanine exchange factors such as Mss4 serve to remove the GDP and bind GTP to the G protein in order to activate it and serve as a positive regulator [14]. Mss4 and TCTP share structural homology in the Rab-binding site (Rab is a specific family of G proteins that are peripheral membrane proteins regulating trafficking of vesicles), yet TCTP has shown to instead act as a guanine nucleotide dissociation inhibitor on translational elongation factor eEF1a [14]. This prevents the GDP from being hydrolyzed and therefore serves as a negative regulator by preventing activation [14]. It was proposed that the deregulation of the eEF1a causes cells to become susceptible to malignant tumor development[23].

TCTP also plays an important role in cell cycle progression [12]. The protein associates to microtubules and stabilize them during metaphase [12]. TCTP is only removed from microtubules after phosphorylation by the polo-like kinase (Plk) and it has been discussed that Plk-dependent phosphorylation of TCTP would be needed for the cell cycle to progress from metaphase to anaphase [12]. Failure to phosphorylate this protein leads to cell death or multinucleated cells [12]. Thus, TCTP is also capable of regulating cell cycle progression by regulating the metaphase to anaphase transition.

TCTP is involved in the antiapoptotic pathway via stabilization of proteins responsible for cell survival such as MCL1, myeloid cell leukemia 1, and Bcl-xL, (both proteins belong to the Bcl-2 family) [24]. Bcl-xL acts as an antiapoptotic protein by stabilizing the mitochondrial membrane and preventing the release of cytochrome c [25]. TCTP's involvement with the immune system is apparent since it is important in T-cell survival and TCTP and Bcl-xL were both up-regulated during T-cell activation [17]. TCTP also aids in antiapoptotic behavior by preventing Bax dimerization and binding to the mitochondrial membrane [11].

RNA-dependent protein kinase (PKR) is a proapoptotic protein that is a downstream effector of p53 in the apoptotic signal cascade [26]. p53 activation resulted in a downregulation of TCTP which mirrored the effect during $Ca^{++}$ stress conditions [27]. It was proposed that in order to lead the cell through apoptosis, expression of the proapoptotic factor PKR would need to repress the expression of TCTP.

In cancerous cells, the levels of TCTP are up-regulated [13] and it has not yet been determined if the up-regulation is a result of the transformation or affects the transformation itself. Tumor reversion is a process that results in the suppression of the malignant phenotype and therefore changing the tumor cell back to a normal cell called a revertant [13]. The factors involved are being studied but many models exist for differentiating revertants and the malignant cells such as utilizing H1 parvovirus which selects for revertants or specialized media that highlight differential culturing between revertants and malignant tumor cells[28]. In all of these revertants, a correlation of TCTP being down-regulated [13] was

identified. Whereas knockdown of TCTP by antisense cDNA lead to massive apoptosis in U937 cells (leukemic monocyte lymphoma cells), there was a reorganization of the ductal and acinar structures as well as a reorganization of the cytoskeleton in breast carcinoma cells [13].

Due to the multiplicity of its function (Figure 1.4), TCTP is being considered a housekeeping protein involved in the maintenance of several pathways and protein functions. The ubiquitous nature in its expression and compartmentalization, not to mention its conservancy in sequence, support this hypothesis.

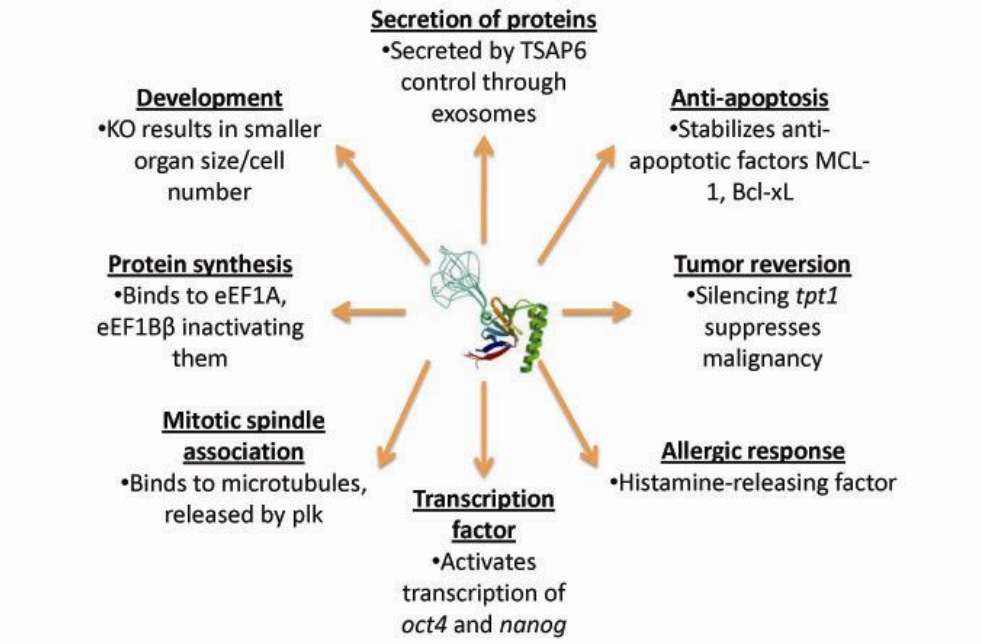


**Figure 1.4:** TCTP is involved in multiple pathways throughout the cell. TCTP's role as a housekeeping protein is strongly supported when studying the diversity of pathways it is involved in. The TCTP is implicated in DNA transcription (*oct4*, *nanog*), protein synthesis (suppressing eEF1A activity), protein stability (MCL-1, Bcl-xL), cell cycle (microtubule association and phosphorylation by plk), extracellular secretion (controlled by TSAP6), cell number and organ size, allergic responses, and suppressing malignant tumor phenotypes by its knockdown.

**Ubiquitin-mediated degradation**

Many components involved in the ubiquitin-mediated degradation pathway are constitutively expressed which is important since many cellular functions depend on consistent or cyclical degradation of proteins. In certain situations however, the activity of the pathway is up-regulated in conditions such as fasting which would increase the degradation of skeletal muscle proteins. Ubiquitin is a 76 amino acid protein that is highly conserved and covalently binds to the target protein through a carboxyl group on C-terminus.

The ubiquitin pathway consists of several core components that vary in specificity and function (Figure 1.5). The E1 (enzyme 1) is an ubiquitin activating enzyme that conjugates ubiquitin through a

thioester bond between a C-terminal carboxyl group and a cysteine residue in an ATP-dependent reaction. The E1 then transfers the ubiquitin molecule to E2 (enzyme 2) through a *trans*-esterification process from the cysteine in E1 to the active site in E2. The E3 (enzyme 3) is the ubiquitin ligase which controls the specificity of the pathway by binding to target substrate. The E2 is brought within proximity of the substrate by E3 and transfers the ubiquitin onto a lysine residue of the target substrate. The E3 ligase beta-Transducin repeat containing protein (β-TrCP) binds to hPer2 that has been phosphorylated by CKIε [29]. The resultant polyubiquitination and ensuing degradation a highly specific process requiring β-TrCP since cells that were β-TrCP-deficient resulted in a lack of ubiquitination and degradation of hPer2. Iron regulatory protein 2 (IRP2) is a HRM containing molecule which is subject to ubiquitination by Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) [30]. The ubiquitination is specific for the oxidation of IPR2 by heme and suggests that heme served as a molecular signal for redox states within the cell. We provided evidence of an HRM in the C-terminus region of Per2 which is also responsible for degradation but the E3 ligase for heme-mediated degradation has yet to be discovered. Another component, E4, has been suggested to be responsible for the polyubiquitination of the target molecule. However, in yeast, it has been deemed non-essential and therefore the E4 activity has been attributed to specific ubiquitination reactions.

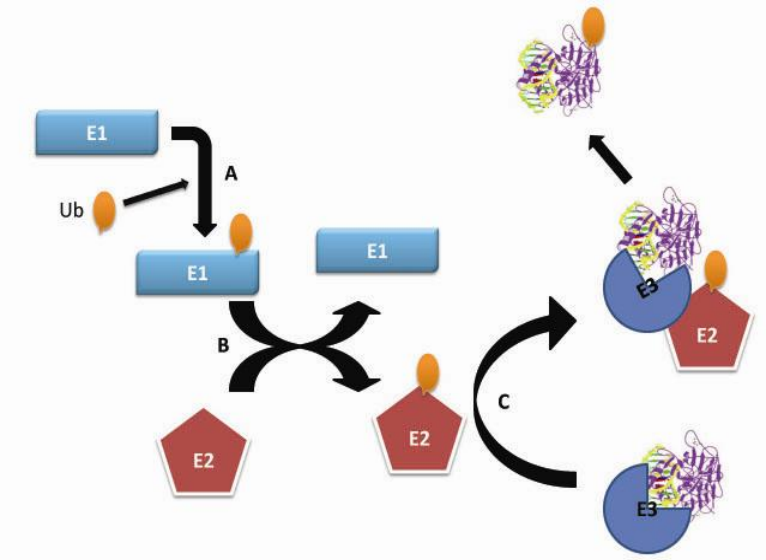


**Figure 1.5:** Ubiquitination pathway. A) Ubiquitination of a specific target begins with the E1 that activates ubiquitin through an ATP-dependent reaction binding the ubiquitin to a cysteine in the active site. B) E1 binds to one of three E2s in the human ubiquitination pathway and transfers the ubiquitin to a cystein on E2 through a *trans*-esterification process. C) E2 can bind to one of many E3s in the pathway. There are many E3s, conferring specificity out of the hundreds of targets for ubiquitination and degradation. The E3 serves as a scaffold to bring one of the three E2s into proximity with the specific substrate so the ubiquitin can be transferred.

There are several types of E3 ligases which recognize various substrates according to conserved residues or hydrophobicity. The E3a (also known as the N-recognin) binds to N-end rule substrates which contain several highly conserved amino acid residues that signal for degradation. The E3a primarily binds to basic and bulky hydrophobic residues but also contains active sites for denatured or N-acetylated proteins. The HECT (homologous to E6-AP carboxyl terminus) is a large E3 protein that primarily targets cellular proteins and shares a conserved domain as that of the E6-AP E3 ligase which targets tumor suppressor p53. The APC (anaphase promoting complex) is responsible for degradation of substrates in mitosis which utilize various sub-components to promote specificity in binding. Without its activity, cells are unable to progress to anaphase which results in forming multinucleated cells and is also associated with tumorgenesis [31]. SCF (Skp1, Cullin, and F-box) protein is another multi-component E3 that binds to a specific E2 using the Rbx1 (RING-box1) protein component. The specificity for target substrates is altered by the F-box protein that contains a degenerate F-box motif of approximately 60 amino acids. In *Arabidopsis thaliana* alone, over a dozen F-box containing proteins have been identified [32]. These examples of E3 ubiquitin ligases enable specificity in targeting substrates for degradation. This ensures the availability of the targets is limited to when they are necessary inside the cell and irregularities in its degradation can disrupt the functions in which the targets participate in.

**Ubiquitin-independent degradation pathways**

The regulation of p53, an important tumor suppressor, stability is controlled by polyubiquitination from mdm2. However, there have been studies that indicate p53 is an intrinsically unstructured molecule that result in its degradation regardless of whether or not mdm2 is present. The p53 protein escapes this default degradation through several mechanisms which involve molecules such as NADH quinine oxidoreductase 1 (NQO1) [33], protein-protein interactions, or conformational changes [34]. This means that the expression of p53 is closely regulated; p53 is produced to perform its functions and by its unstructured nature, excess p53 that is unnecessary is degraded.

The REGγ proteasome (also known as PA28 or 11S) is responsible for p21 degradation with association to 20S proteasome that works independently of ubiquitin [35]. The importance of the REGγ proteasome was suggested to function in the role of degrading unstructured proteins or lysine-less proteins. The p21 protein has a kinase inhibitory domain (KID) that is known to be intrinsically unstructured [36].

The ClpP (caseinolytic peptidase, proteolytic subunit) complex of ClpA and ClpX are ATPases that unfold target substrates before degradation by the proteasome. These units are considered molecular chaperones that unfold or "unfoldases". Another example of these unfoldases include the proteasome-activating nucleotidase (PAN) that works in concert with the 19S proteasome to unfold substrates and stimulate degradation by 20S proteasome[37].

The lon protease from *Escherichia coli* is an ATP-dependent protease capable of cleaving abnormal proteins [38] as well as specific targets [41]. Work in an archael lon protease revealed a higher protease activity independent of ATP that targeted unstructured proteins [41]. In order to target specific structured substrates, ATP was required. Particular interest is raised from the targeting of the unstructured proteins since lon-*tk* (lon from *Thermococcus kodakaraensis*) cleaves its substrates independent of ATP, ubiquitin, and the proteasome.

Ubiquitin-independent cleavage and degradation does not follow a strict model such as the ubiquitin-mediated degradation pathway. However, the various examples listed suggest that the proteins being targeted for degradation are those that contain unstructured or unordered regions that stimulate its degradation.

## Chapter 2: Specific Aims and Goals

The overall goal of our research was to identify novel interactors of hPer2 that act on its stability and to determine the alternate control mechanisms of hPer2 stability. Using a two-hybrid assay, we screened a human liver cDNA library for novel interactors of the C-terminus of hPer2. A stringent screening process was followed to select candidates that interact with hPer2 and out of the extensive number of putative interactors, TCTP was selected because of its role as a stabilizing factor of many proteins.

***Aim I. To identify novel interactors of hPer2.*** We used a bacterial two-hybrid assay with the C-terminus of hPer2 to discover possible interactors of hPer2. The C-terminus was used instead of full-length for multiple reasons: 1) the full length hPer2 cannot be expressed in bacteria with practical yields, 2) the N-terminus of hPer2 which contains the PAS domain, has already been utilized in multiple screenings in identifying new targets for heterodimerization. It was important to support the data of positive interactors with techniques not dependent on the two-hybrid assay. We performed gene ontology and identified possible pathways that have effect on hPer2 through the novel interactors found by the two-hybrid assay. TCTP was selected because of its roles in stabilizing proteins, constitutive expression, and high conservation which suggests a housekeeping role. We cloned TCTP into a mammalian expression vector and determined that TCTP binds to hPer2 not only *in vitro* through pull-down assays but also inside the mammalian cell through co-immunoprecipitations.

***Aim 2. To determine alternative methods of control in hPer2 stability.*** In collaboration, we researched a novel heme-regulatory motif in the C-terminus of hPer2 (hPer2 C). Biophysical techniques were used to isolate the binding of heme to the C-P motif on the C-terminus. *In vitro* studies in cell free lysates revealed ubiquitination and degradation of hPer2 in the presence of heme whereas with a mutant unable to bind heme, there was no degradation of hPer2. In cell culture, it was demonstrated that heme significantly decreased hPer2 levels whereas removing heme and addition of heme biosynthesis inhibitors rescues the levels of Per2.

TCTP was chosen to investigate novel methods of control in hPer2 stability due to its role in modulating the stability of antiapoptotic proteins. We used a stability assay with radiolabeled hPer2 and exogenous unlabeled TCTP to determine the effects of overexpressed TCTP on hPer2. We wanted to investigate whether the effect of hPer2 destabilization was indirectly caused by exogenous TCTP or a direct result of exogenous TCTP binding to and destabilizing hPer2. We created a cleavage assay in combination with western blots to determine that the destabilization of hPer2 was a direct effect of TCTP.

**Chapter 3: Results**

Period 2 is a core circadian protein that negatively regulates its own expression in the circadian clock but is also responsible for interactions with factors involved in multiple biological functions. Disrupted Per2 stability and degradation is implicated in Familial Advanced Sleep Phase Syndrome (FASPS), altered cardiovascular development, impaired immune response, reduced metabolism (primarily in muscle strength), and increased susceptibility to tumorgenesis. The evidence of multiple binding partners to Per2 comes as no surprise when studying the domain structure. The initial goal of the project is to screen for novel binding partners of Per2. In identifying novel interactors, we hope to gain further insight into the modulatory role of Per2.

- **Two hybrid screening**

The two-hybrid Bacteriomatch screen (Stratagene) was utilized to identify new potential binding partners of the C-terminus region of human Per2. In an effort to bait novel targets without false positives, the C-terminus region of hPer2 was selected for two reasons: i) the N-terminus region containing the PAS domain is notorious for protein-protein interactions that have been characterized in previous screenings and ii) research on novel modes of regulating hPer2 stability was investigated using the C-terminus.

The bait plasmid encoding the C-terminus of hPer2 (pBT-hPer2C) was co-transformed along with target plasmids of random cDNA clones. Two hundred and eighty-one clones were isolated and purified so that the target cDNA clones were present in the samples without the pBT-hPer2C. Each of the purified putative interactors was sequenced and the coding region analyzed with BLAST. Many of the clones were the same interactors but covering different regions (Table 1, page 24). The data from the screening was analyzed through a program called DAVID (database for annotation, visualization, and integrated discovery). We utilized the clustering aspect of the program in order to visualize the interactors in groups that were closely associated with each other; this gave us an advantage in analyzing the data since instead of investigating hundreds of individual clones, we took the entirety of the group and were able to study specific biological functions that the interactors were involved in (Figure 3.1).

Many of the putative interactors in the data clustering were involved in biological functions already associated with mPer2. Several clones were part of the immune system function, primarily in acute inflammatory response, acute-phase response, and response to wounding. The enrichment score for this particular cluster was 3.29 under a high classification stringency (any enrichment score above 1.5 is considered significant). We discovered several clones within a cluster designated for lipid transport, localization, and lipoprotein complexes. Despite the high enrichment score (5.76), the clones that make up this cluster have been identified in previous two-hybrid screenings as possible false positives. To ascertain the validity of these interactors, we validated the interaction through isolation of the clones and

co-transforming with the bait plasmid to ensure protein-protein interaction. A cluster pertaining to proteins involved in vesicles and granules, in particular proteins involved in binding capacities, had an enrichment score of 3. An interesting group was identified to be involved in apoptosis and had an enrichment score of 2.38 which is in agreement with the apoptotic role of hPer2 in tumor cells [39]. The cluster involving blood coagulation, hemostasis, and regulation of body fluid levels scored a 1.39 under high stringency conditions yet a 1.6 under medium conditions. Under the literature[40], it was deemed that the cut-off at 1.5 was a relative value and stressed discretion on the part of the investigator whether to consider the data at certain values as significant. To analyze the significance of this cluster, we will need to use other techniques of studying protein-protein interactions such as *in vitro* pull downs and in-cell co-immunoprecipitations.

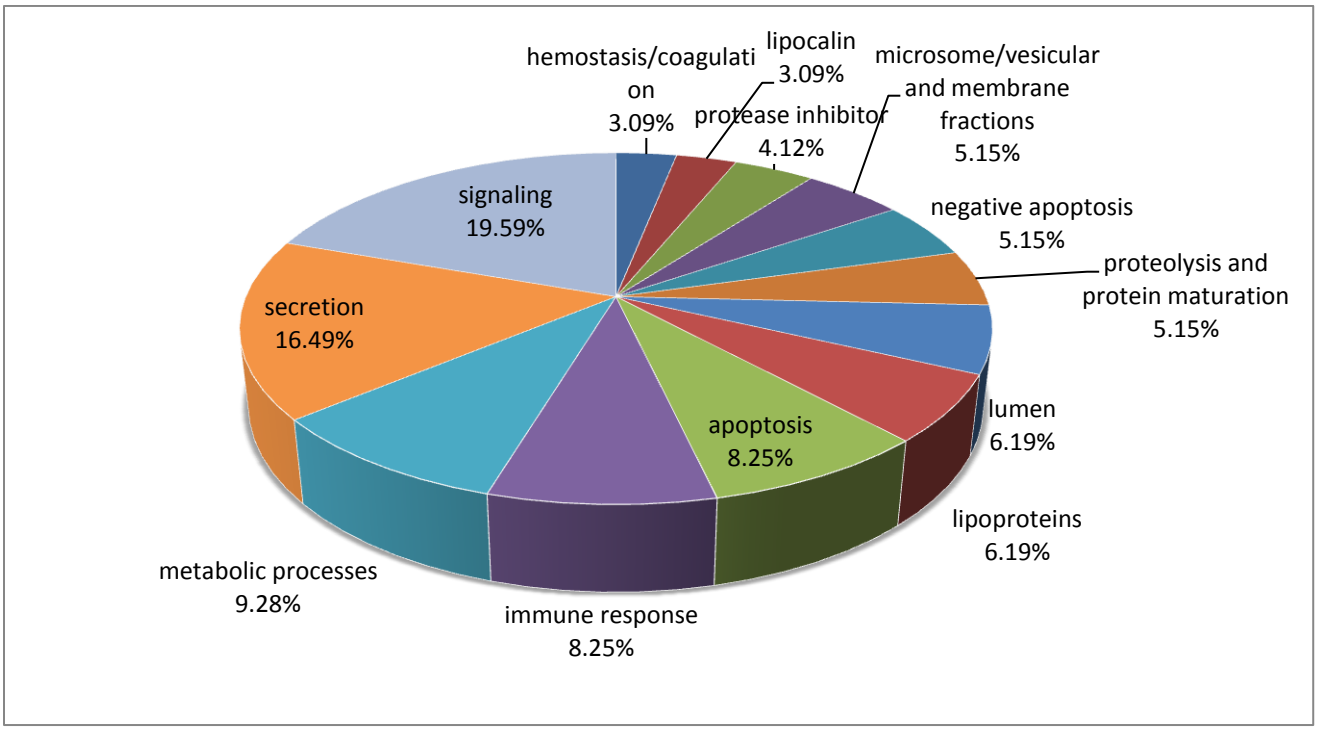


**Figure 3.1:** Functional cluster analysis. The percentages are the number of clones in each group related by biological function compared to the total number of clones listed in the groups (177). These values are not absolute because many clones in each of the functional groups overlap and are repeated. Analysis was performed using DAVID under high stringency. All clusters with an enrichment value $\geq 1.4$ and a *p*-value of $< 0.001$ were considered significant by our group.

- **TCTP binds to hPer2 in vitro**

A partial list of clones harvested from the series of screenings yielded 10 hits of the gene encoding TCTP. The TCTP protein is an interactor of hPer2 C in the two-hybrid screenings (Figure 3.2) and further verification of its interaction with pBT-hPer2 C was necessary outside of the two-hybrid system.

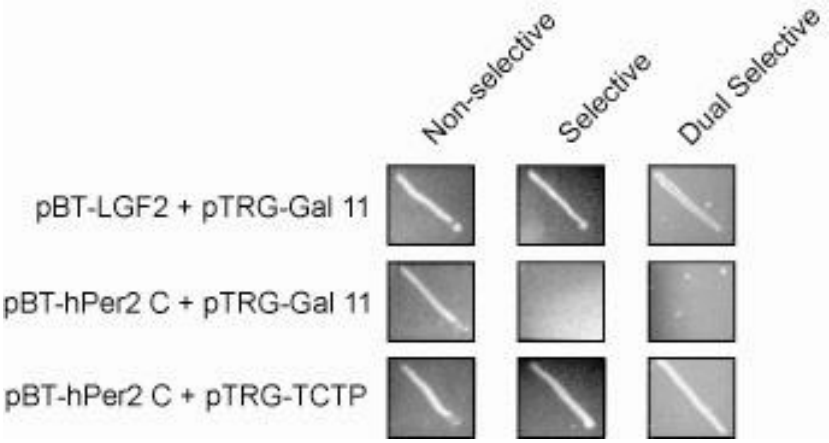


**Figure 3.2**: TCTP interacts with hPer2C in the two-hybrid system. Shown here are bacteria patched onto respective media plates. The pTRG-TCTP binds to pBT-hPer2C in order to activate the reporter genes *His3*, enabling survival in selective media, and *aadA*($Str^R$), which enables survival on dual selective media. This is further confirmed by no growth on selective and dual selective media when co-transforming bacteria with pBT-hPer2C and a control plasmid, pTRG-Gal11.

We cloned *tpt1* (the cDNA encoding TCTP) into the pGEX-4T3 vector, followed by its expression and purification from bacterial cells. The bound GST-TCTP fusion protein was incubated with radiolabeled hPer2 and subjected to a series of stringent washes which ensured no non-specific binding of proteins from the extract to GST-TCTP. The samples were then analyzed by SDS-PAGE and exposed to X-ray film for the presence of radiolabeled hPer2. The GST-TCTP bound to glutathione agarose beads were able to pull down the radiolabeled full length hPer2 *in vitro* (Figure 3.3) with specificity confirmed when GST alone did not pull down the hPer2.

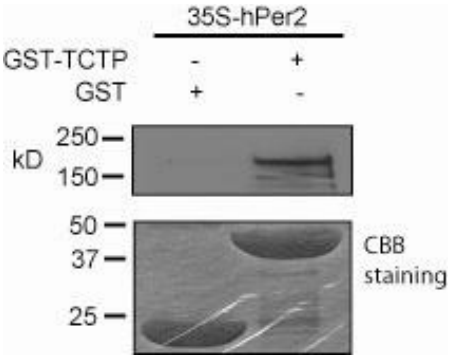


**Figure 3.3:** TCTP pulls down radiolabeled hPer2 *in vitro*. $^{[35]}$S-hPer2 is incubated in GST pull-down buffer A (Materials and methods) with respective protein (10 µg) bound to glutathione agarose beads resolved by SDS-PAGE in the bottom panel. The top panel shown is an autoradiogram of the SDS-PAGE gel. The reactions were incubated at 4°C for two hours with rocking. After incubation, the reactions were washed with buffers of increasing salt concentration to eliminate non-specific binding. GST does not pull down any $^{[35]}$S -hPer2 whereas GST-TCTP pulls down $^{[35]}$S -hPer2 confirming the interaction *in vitro*.

- **TCTP co-immunoprecipitates hPer2 in cells**

We proved that binding of TCTP and hPer2C occurs in bacterial cells and the interaction exists *in vitro* between TCTP and hPer2 full length. However, there is no evidence that the novel binding we have discovered occurred within cells, which is more physiologically relevant. The *tpt1* gene was inserted into a pCS2+FT vector for expression inside mammalian cells (pCS2+FT-TCTP). Once expressed, cells were lysed and incubated with myc tag beads to immunoprecipitate the tagged protein with endogenous Per2 and were analyzed by immunoblotting. Our results indicate FLAG-TCTP immunoprecipitates endogenous Per2 from mammalian cells (Figure 3.4 B). Due to the difficulty of manipulating endogenous proteins in mammalian cells, we wanted to verify that the interaction would still occur with overexpressed hPer2. The endogenous protein in the co-immunoprecipitation experiment discussed above was hamster Per2 and not human Per2 which was used in the *in vitro* GST pull-down assays. We used a pCS2+MT-TCTP (MT-TCTP) to co-express with pCS2+FT-hPer2 (FLAG-hPer2). Evidence supports that overexpressed MT-TCTP co-immunoprecipitates FLAG-hPer2 in cell-free lysate (Figure 3.4 A).

A. B.

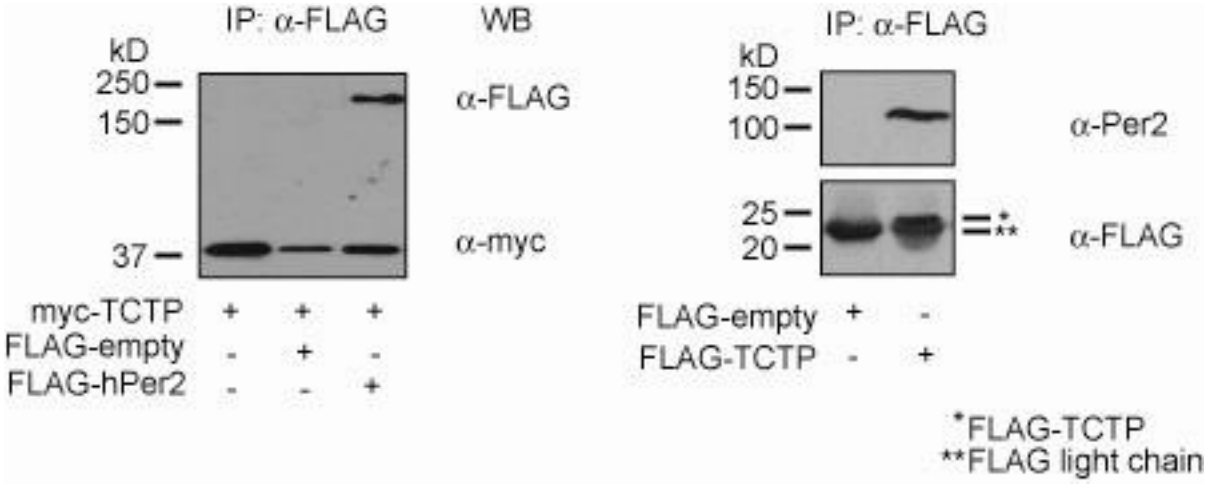


**Figure 3.4**: TCTP binds and immunoprecipitates hPer2 in cells. A) Shown here is a co-immunoprecipitation resolved by SDS-PAGE and analyzed by western blot. pCS2+myc-TCTP, pCS2+FLAG, and pCS2+FLAG-hPer2 are transfected into CHO cells. Cells are harvested, lysed using Promega lysis buffer and immunoprecipitated myc-TCTP. The samples are subjected to a series of stringent washes. MT-TCTP immunoprecipitates FLAG-hPer2 in cells that have been lysed. B) Both panels are western blots of an immunoprecipitation, immunoblotted against different antibodies. FLAG-TCTP is transfected into CHO cells. Cells are harvested, lysed and subjected to stringent washes. FLAG-TCTP immunoprecipitates endogenous Per2 and the specificity is confirmed by the control FLAG plasmid not being able to immunoprecipitate Per2.

- **hPer2 is cleaved in cell-free lysates by TCTP**

Per2 stability is a major factor in determining its functionality. There has been extensive research conducted in identifying various modes of modulating its stability, ranging from phosphorylation, acetylation, and ubiquitination. In an attempt to identify the role of TCTP binding to hPer2, a titration of exogenous human TCTP was performed in a HeLa S100 fraction containing radiolabeled hPer2. After incubation for two hours at 30°C, there was a significant reduction in the amount of $^{[35]}$S-hPer2 present in the HeLa lysate by the addition of 10 μM TCTP (Figure 3.5).

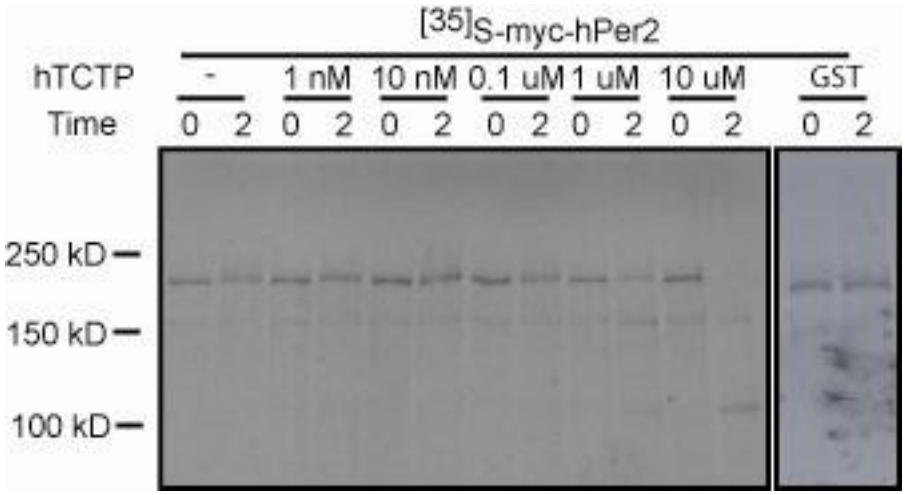


**Figure 3.5:** Exogenous hTCTP cleaves hPer2. Shown here is an autoradiogram of products from stability assays resolved through SDS-PAGE. The stability assays use HeLa S100 lysates, exogenous hTCTP at indicated concentrations, 10 µM GST, and $^{[35]}$S -hPer2. Noticeable cleavage occurs with addition of 1 µM hTCTP and pronounced at 10 µM. An equivalent molar concentration of GST causes no cleavage of $^{[35]}$S -hPer2.

The cleavage of $^{[35]}$S-hPer2 occurred in repeated experiments under the same conditions in the presence of protease inhibitor cocktail (Figure 3.6). There was an attenuation of cleavage product formed which suggested that the effect was from protease activity. This cleavage was not seen with the addition of other exogenous proteins (Figure 3.6) which indicates the cleavage is specifically by TCTP. In the same assay, we also utilized MG-132 in order to determine if the cleavage was proteasome dependent. However, the data indicates the cleavage is proteasome independent since MG-132 addition did not alter the cleavage (Figure 3.6).

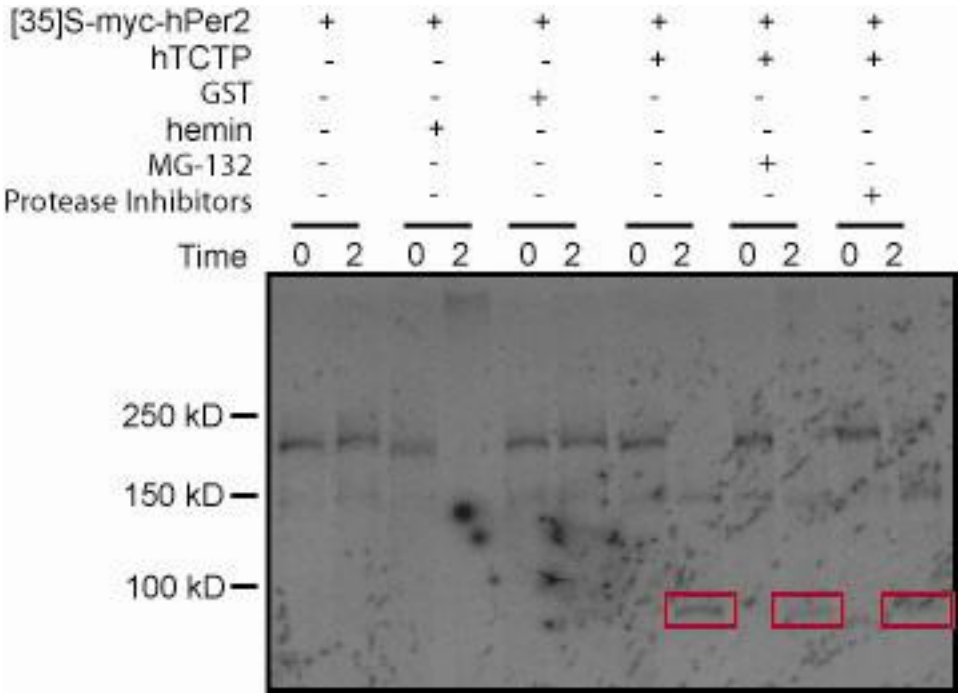


**Figure 3.6:** Cleavage of $^{[35]}$S -hPer2 is not dependent on ubiquitin or protease activity. Shown here is an autoradiogram of products from stability assays resolved through SDS-PAGE. Stability assays consist of indicated components added and both hTCTP and GST are 10 µM. Dramatic reduction of $^{[35]}$S -hPer2 still persists in the presence of protease inhibitor cocktail (materials and methods) or MG-132 (40 µM).

- **The C-terminus region of hPer2 is cleaved by TCTP**

Since the fragmentation of S$^{[35]}$-hPer2 was established as a result of the presence of TCTP, we wanted to see whether the effect of fragmentation was due to a direct effect of TCTP binding. In order to do this, we had to verify that the fragmentation effect occurred with a particular domain of hPer2; the C-terminus, since we are unable to express full length hPer2 in bacteria.

In a GST pull-down assay (Figure 3.7), we demonstrate that binding occurs in fragments consisting of both the N and C-terminal regions. In parallel experiments, it was shown that a fragmentation pattern occurred to the C-terminus of hPer2 (the fragment of hPer2 used in the two-hybrid screen) but not at the N-terminus (Figure 3.8). We concluded that although both N-terminus and C-terminus of hPer2 bind to TCTP, C-terminus responds in a manner similar to that of the full-length through its cleavage.

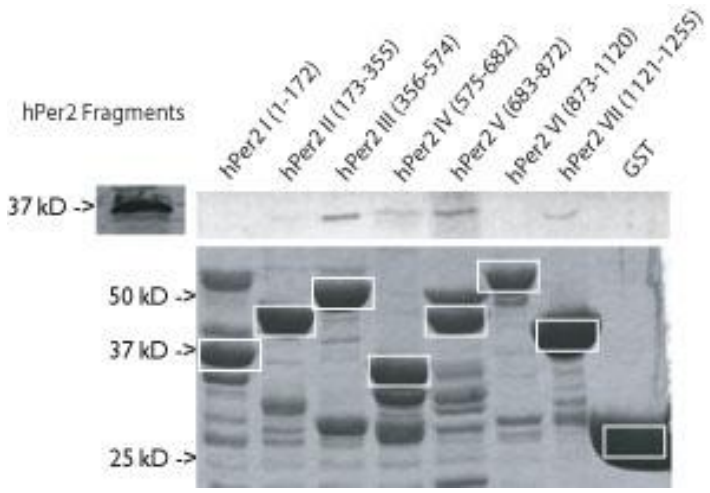


**Figure 3.7:** TCTP binds to multiple regions of hPer2. The top panel is an autoradiogram of the various hPer2 constructs resolved by SDS-PAGE gel in the bottom panel. The top left panel is the total input of $^{[35]}$S-TCTP added to each GST pull down reaction. Proteins consisting of amino acids indicated by each fragment were expressed and purified with GST tags. Each sample was incubated in buffer A and $^{[35]}$S-TCTP at 4°C for 2 hours while rocking before being subjected to stringent washes of increasing salt concentration. TCTP binds to multiple regions in hPer2.

**Figure 3.8:** TCTP cleaves the C-terminus of hPer2. Shown here is an autoradiogram of products from stability assays resolved through SDS-PAGE. Stability assays using respective radiolabeled fragments of hPer2 were incubated with 10 µM hTCTP. hTCTP incubated with the N-terminus of hPer2 does not result in cleavage or decrease in signal. hTCTP incubation with C-terminus results in cleavage of C-terminus and a reduction in signal.

- **hPer2C is cleaved directly by TCTP**

We performed a cleavage assay in which GST-hPer2C was incubated in Buffer A (see Materials and methods) with agitation at room temperature. To this reaction, increasing stoichiometric molar ratios of TCTP to GST-hPer2C were used and detected by immunoblotting against GST. At a stoichiometric ratio of 1:1, there was obvious fragmentation and lesser fragmentation at stoichiometries below 1:1. In the presence of other exogenous proteins and at the highest stoichiometric ratio studied, no cleavage of hPer2C could be detected inferring that the cleavage by TCTP was specific through its binding (Figure 3.9).

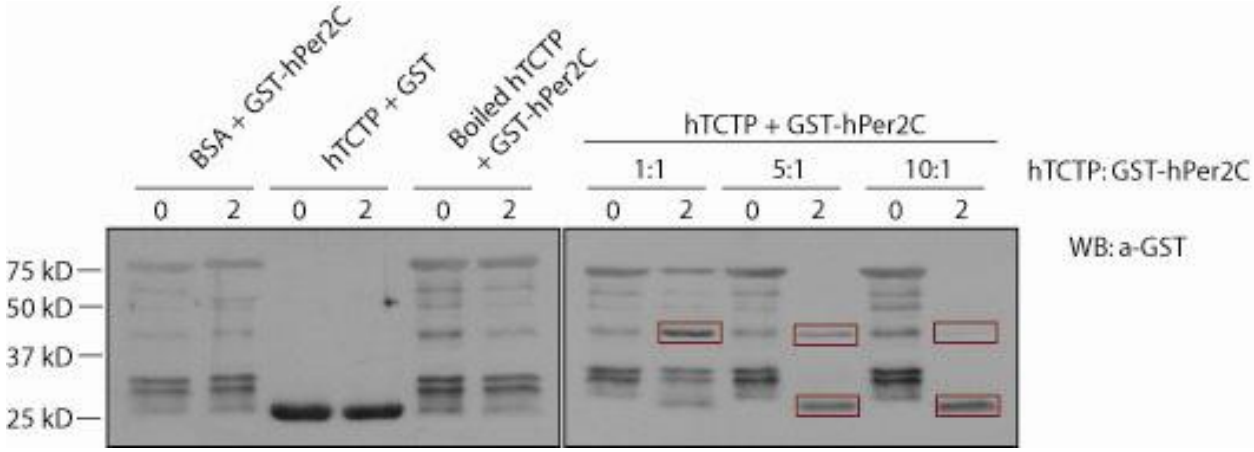


**Figure 3.9:** hPer2C is directly cleaved by hTCTP *in vitro*. Western blots of cleavage assays that were resolved through SDS-PAGE gels are shown here. Respective proteins in buffer A were incubated for indicated times at 25°C with rocking. Cleavage fragments detected by GST-antibody are highlighted in red boxes. BSA has no effect on GST-hPer2C, hTCTP does not alter GST, and boiled hTCTP does not cleave GST-hPer2C. There is a dose-dependent increase in cleavage of hPer2C with increased hTCTP.

- **TCTP does not have protease activity**

After identifying that the cleavage was a direct result of TCTP's presence, it was assumed that the fragmentation was specific cleavage by proteolysis from TCTP binding to GST-hPer2C. We performed the same cleavage assays but in the presence of a general protease inhibitor cocktail to ascertain if the cleavage was caused by protease activity. The cleavage by control protease trypsin was inhibited by the incubation with the general protease inhibitor cocktail up to 1:200 dilution (recommended use is 1:1000). As for the samples containing TCTP, the cleavage of GST-hPer2C was not attenuated until a dilution of 1:100 of protease inhibitor cocktail (Figure 3.10). This suggests that either the proteolytic activity of TCTP is weak or that the TCTP cleaves hPer2C through a different proteolytic method.

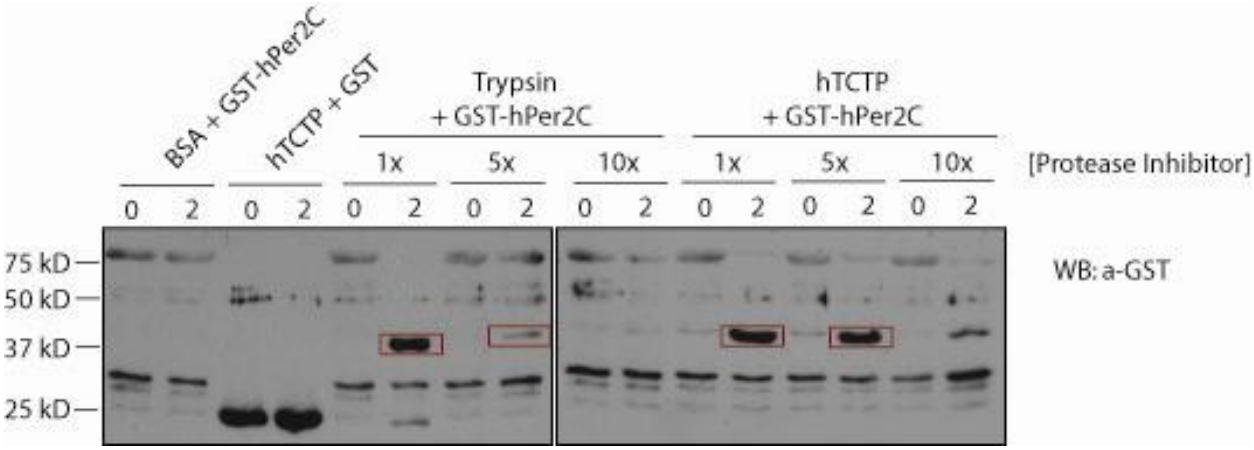


**Figure 3.10:** hTCTP cleavage of hPer2C is inhibited by high concentrations of general protease inhibitor cocktail. Western blots of cleavage assays that were resolved through SDS-PAGE gels are shown here. Trypsin cleavage of GST-hPer2C is almost abolished with increase in protease inhibitor cocktail. The hTCTP continues cleavage of GST-hPer2C with 5x concentration of inhibitor cocktail but is attenuated with higher concentrations of protease inhibitor cockatil.

In order to validate this result, a protease activity assay was performed using trypsin and GST as positive and negative controls respectively. The trypsin showed a dose dependent increase in proteolysis of casein whereas no proteolysis of α-casein was detected regardless of GST dosage. The TCTP showed no proteolysis of α-casein and was comparable to the activity detected for the GST (Figure 3.11). These results implied that any proteolytic activity, if responsible for cleavage, would have to be specific to hPer2.

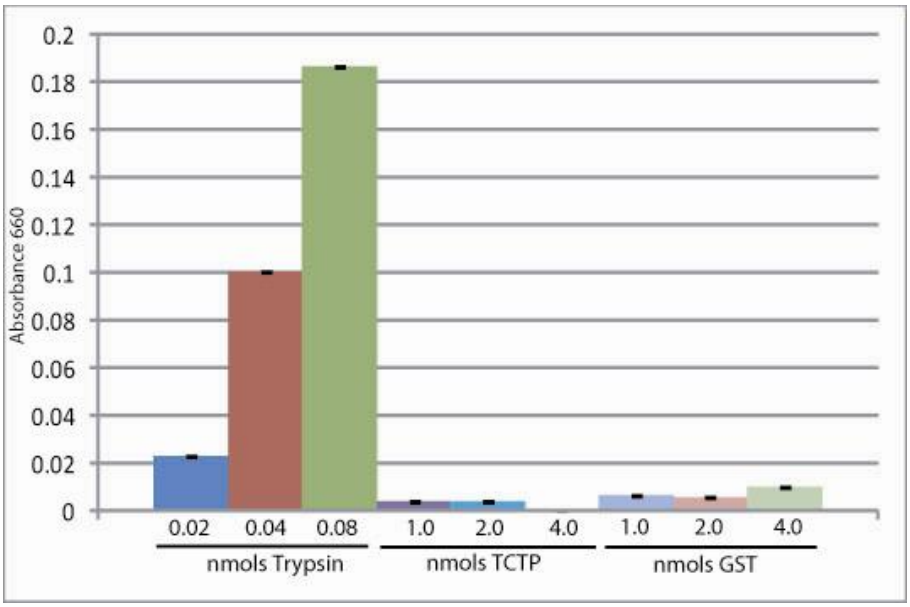


**Figure 3.11:** Protease activity assay. The reactions were incubated with substrate and molecules indicated for 10 minutes at 37°C. TCA was added to stop the reactions and filtered to remove precipitate. Addition of Folin's reagent bound free tyrosine molecules in solution and produced a chromophore measurable at $A_{660}$. Trypsin proteolysis of substrate was dose dependent while GST proteolysis detected was minimal and insignificant. Values of TCTP indicate there is no non-specific proteolysis occurring. Relative absorbance values are calculated by subtracting absolute absorbance from the absorbance measure with no active proteases.

**Chapter 4: Summary and Conclusions**

The objective of this work was to identify novel interactors of the circadian factor hPer2. We utilized the two-hybrid screening assay to determine what human proteins would bind to hPer2. From this data we have gained a list of putative interactors that have previously not been identified as being involved with hPer2. With the extensive number of clones listed, we used a program that would group the clones according to the function that each was related to. As verification that the two-hybrid was successful, we identified several clusters of biological function that has already been attributed to hPer2; apoptosis, immune response, metabolic processes, signaling. Clusters of biological function not yet attributed to hPer2 that are still tentative and hold potential to be physiologically relevant are: hemostasis/coagulation, lipocalin function, protease inhibitor activity, protein maturation/proteolysis, and secretion.

We focused our research on one clone in particular from the two-hybrid screen; TCTP. We proved that TCTP binds to hPer2 *in vitro* and in cells. Finally, we show that TCTP, when bound to hPer2, results in the cleavage of hPer2. This occurs in a cell free lysate as well as *in vitro* suggesting a direct effect from binding. This cleavage persisted in the presence of MG-132 suggesting that fragmentation is not ubiquitin-mediated. Some fragmentation was attenuated through the addition of protease inhibitors which directed our attention to investigating the possibility of a previously uncharacterized protease activity by TCTP. Using a non-specific protease activity assay, it was determined that TCTP is not a protease but is able to cleave through an alternate proteolytic activity. We suggest that the method of cleavage resembles that of lon-*tk* in that the cleavage of hPer2 by TCTP is ATP, ubiquitin and proteasome-independent [41] in the cleavage assays which contain none of these components.

Cells ubiquitously express TCTP and transcriptionally activate TCTP further under developmental stages [41]. In certain stress conditions, transcriptional activation of TCTP is increased further [27], [42]. In tumor cells where apoptosis does not occur, TCTP is overexpressed [43]and there is consistent evidence that hPer2 exists at significantly lower levels than normal cells [44]. Whether this correlation is an indirect effect or there exists a connection between the two proteins in tumorgenesis is yet to be investigated. Further work in this direction will better our understanding of the factors involved with hPer2 destabilization from stress conditions that transcriptional activates TCTP.

**Chapter 5: Discussion**

The two-hybrid screening assay reveals new binding partners and thus places hPer2 in pathways different from circadian rhythmicity. Different categories are represented in the two-hybrid screening results which imply pleiotropic effects of hPer2 that may not be linked to circadian rhythm. Examples of diversity include clones functioning in metabolic pathways, immune responses, apoptosis/anti-apoptosis, signaling, and secretion. Among the positive clones identified in our protein interaction screening is haptoglobin, which is involved in binding hemoglobin and forming a complex. The haptoglobin/hemoglobin complex enables various degradative enzymes to break down the hemoglobin [45]. This data is in agreement considering one of the criteria in the screening process was growth of the interactors in the presence of heme. In addition, clusterin and various other immune response related proteins (8.3% of interactors) bind to hPer2. Clusterin is partly responsible for identifying and clearing immunogenic molecules such as immunoglobulin G, M, and A (IgG, IgM, IgA), C1q, C3, and C9 [46]. The Per2 protein is also believed to serve an apoptotic function by its implications of being a downstream effector of p53 [17] and in our screen, we identified 8.25% of interactors to be related to apoptosis or 6.19% in negative apoptosis. A large part of our screening results indicate 19.6% of the interactors are involved with signaling which is supported by evidence that Per2 is linked to signaling pathways such as estrogen receptor signaling [47]. Lipoproteins are another cluster of interactors that bind to hPer2 in our screen and although there is no direct evidence of lipoprotein/hPer2 binding in the literature, there are studies relating circulating lipoprotein levels following circadian patterns [48], [49]. A major portion of the interactors (16.5%) also functions in secretion. Where there is no evidence that hPer2 is involved with any secretive pathways, interleukin-6 is thought to follow circadian patterns of secretion in humans [50], [51]. Hemostatic factors are influenced by circadian rhythmicity [52] and in our screen, represent 3.1% of the interactors along with factors involved with coagulation, which has also been implicated to being affected by circadian variations[53], [54]. For clusters pertaining to protein maturation, lumen, and lipocalin, the link to hPer2 remains to be established.

Our data establishes hPer2 cleavage is directly triggered by TCTP and not an indirect mechanism. Further results indicate that TCTP does not have general protease activity. We establish that TCTP cleaves hPer2 with specificity and independently of ATP, ubiquitin, and the proteasome. The Lon-*tk* (*Thermococcus kodakaraensis*) protease belongs to a family of proteases that are mainly ATP-dependent. Like TCTP, lon-*tk* itself is able to target and cleave multiple substrates independently of ATP, ubiquitin, and the proteasome [55]. The protease also specifically targets inherently unstructured or unfolded proteins whereas cleavage of folded proteins does require ATP. The hPer2 contains an unstructured proline rich sequence and the C-terminus structure has not yet been identified which, in combination with the multiple putative interaction motifs, make it a candidate as a regulatory protein [56]. This would be in

agreement with the data of hPer2 cleavage since many regulatory proteins are also degraded or cleaved in an ubiquitin-independent manner. The REGγ proteasome targets and binds to and degrades p21 (binds aa 156-161) [36]. It is proposed that the REGγ proteasome may target lysine-less substrates. Ornithine decarboxylase (ODC) is targeted by antizyme (AZ) by binding to ODC and disrupting the active homodimer complex. The heterodimeric complex of ODC/AZ is now inactive and is directed to the proteasome for degradation without ubiquitination as well. The tumor suppressor p53 is an important regulatory molecule that is degraded by ubiquitination but also in recent studies, independent of ubiquitin by the proteasome [34]. It is proposed that p53 is an unstructured protein and only by binding to its respective downstream DNA-damage response elements is it able to persist. Excess p53 is therefore regulated by degradation through its own unstructured nature.

We have uncovered an extensive number of novel interactors that bind to the C-terminus region of hPer2. The stability of hPer2 is regulated by multiple pathways; phosphorylation, acetylation, ubiquitination. In our work, we uncover two modes of modulating the stability of hPer2. One mode is ubiquitin-dependent and is heme-mediated while the second mode is ATP, proteasome, and ubiquitin-independent mediated by TCTP. The TCTP binding and cleavage of Per2 occurs at a high molar stoichiometric ratio which suggests that this mechanism pertains to conditions in which the cell is overexpressing TCTP. The TCTP is overexpressed under stress conditions such as heavy metal ions [57], heat shock [58], ammonium starvation [59], and depletion of stored Ca2+ or increase in concentration [57]. We hypothesize that these stress conditions that overexpress TCTP cleaves hPer2 in the cell. The cell will persist in conditions where the pro-apoptotic hPer2 is cleaved and the anti-apoptotic TCTP is prevalent, which may result in propagation of cells suffering damage from stress conditions.

The significance of our findings indicate that the novel mode of regulating hPer2 plays an important role in maintaining cell survival by impairing the pro-apoptotic function through cleavage. The TCTP will be up-regulated in response to stress conditions which would induce cell death under normal conditions. Up-regulating the anti-apoptotic protein TCTP enables the cell to persist although the same conditions that up-regulate TCTP may also be damaging the cell. It can also be inferred that, through the extensive number of putative interactors discovered through our two-hybrid screen, hPer2 serves a regulatory role in multiple pathways through these interactors. Other regulatory proteins, such as p53, are regulated through various modes of degradation, both ubiquitin-mediated and ubiquitin-independent. In this way, hPer2 is similar to regulatory proteins in that its stability is modulated through ubiquitin-mediated degradation and, from our *in vitro* findings, an ubiquitin-indpendent mode.

**Table 1. Two-hybrid screening results**. Each of the pTRG-cDNA clones selected for sequencing and analysis were chosen when differential growth in either of the growth conditions (aerobic vs. anaerobic, heme vs. no heme) were identified. The plasmids were re-transformed from initial screen in order to remove presence of pBT-hPer2C.

| pTRG construct | BLAST homology | Accession | Identification (%) | Query coverage (%) | Query coverage of subject |
|---|---|---|---|---|---|
| 1-A2 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II (LOC100288578) | XR_078216.1 | 97% | 99% | 2066-2096 |
| 1-B1 | *Homo sapiens* apolipoprotein A-II (APOA2), mRNA | NM_001643.1 | 100 | 99 | 21-470 |
| 1-B2 | *Homo sapiens* mitochondrion, complete genome | NC_001807.4 | 99 | 99 | 1836-2435 |
| 1-B3 | *Homo sapiens* orosomucoid 2 (ORM2), mRNA | NM_000608.2 | 99 | 95 | 101-653 |
| | *Homo sapiens* orosomucoid 1 (ORM1), mRNA | NM_000607.2 | 94 | 95 | 96-648 |
| 1-C2 | *Homo sapiens* apolipoprotein A-II (APOA2), mRNA | NM_001643.1 | 100 | 99 | 21-468 |
| 1-C4 | *Homo sapiens* apolipoprotein A-II (APOA2), mRNA | NM_001643.1 | 100 | 99 | 21-468 |
| 1-C5 | *Homo sapiens* fumarylacetoacetate hydrolase (fumarylacetoacetase) (FAH), mRNA | NM_000137.1 | 100 | 98 | 1447-903 |
| 1-D6 | *Homo sapiens* fumarylacetoacetate hydrolase (fumarylacetoacetase) (FAH), mRNA | NM_000137.1 | 100 | 98 | 1447-917 |
| 1-E2 | *Homo sapiens* angiotensinogen (serpin peptidase inhibitor, clade A, member 8) (AGT), mRNA | NM_000029.3 | 100 | 100 | 2036-2558 |
| 1-E3 | *Homo sapiens* apolipoprotein B (including Ag(x) antigen) (APOB), mRNA | NM_000384.2 | 100 | 96 | 13292-13671 |

| | | | | | |
|---|---|---|---|---|---|
| 1-E5 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1), transcript variant 11, mRNA | NM_001127707.1 | 100 | 97 | 704-1074 |
| 2-B1 | *Homo sapiens* B-cell translocation gene 1, anti-proliferative (BTG1), mRNA | NM_001731.2 | 99 | 68 | 1020-643 |
| | *Homo sapiens* haptoglobin (HP), transcript variant 2, mRNA | NM_001126102.1 | 100 | 27 | 1253-1101 |
| | *Homo sapiens* haptoglobin (HP), transcript variant 1, mRNA | NM_005143.3 | 100 | 27 | 1430-1278 |
| | *Homo sapiens* haptoglobin-related protein (HPR), mRNA | NM_020995.3 | 100 | 27 | 1242-1090 |
| 2-B2 | PREDICTED: *Homo sapiens* similar to NADH dehydrogenase subunit 2 (LOC100131754) | XR_078214.1 | 98 | 100 | 406-1056 |
| 2-B4 | *Homo sapiens* group-specific component (vitamin D binding protein) (GC) | NM_000583.2 | 100 | 84 | 1759-1634 |
| 2-B5 | | | | | |
| 2-B6 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99 | 52 | 3846086-3846400 |
| | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 52 | 9276102-9275788 |
| 2-C1 | *Homo sapiens* chromosome 2 genomic contig, GRCh37 reference primary assembly | NT_022184.15 | 100 | 98 | 41929352-41928910 |
| 2-E4 | EH binding domain? PREDICTED: *Homo sapiens* similar to DC24 (LOC100293090) | XR_078993.1 | 95 | 98 | 827-1216 |
| 3-A4 | Chrom. 2 (GRch37 reference primary assembly) | NT_022184.15 | 100% | 98% | 41929352-41928793 |

| | | | | | |
|---|---|---|---|---|---|
| 3-B6 | *Homo sapiens* chromosome 2 genomic contig, GRCh37 reference primary assembly | NT_022184.15 | 100 | 97 | 41929352-41928923 |
| 4-A5 | *Homo sapiens* stromal cell derived factor 4 (SDF4), transcript variant 1, mRNA | NM_016547.2 | 99 | 98 | 1498-1940 |
| | *Homo sapiens* stromal cell derived factor 4 (SDF4), transcript variant 2, mRNA | NM_016176.3 | 99 | 98 | 1382-1824 |
| 4-B2 | PREDICTED: *Homo sapiens* similar to DC48 (LOC100293563), mRNA | XM_002345305.1 | 98 | 99 | 684-166 |
| | PREDICTED: *Homo sapiens* similar to OK/SW-CL.16 (LOC100288418) | XM_002342023.1 | 98 | 99 | 72-590 |
| 4-B3 | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT) | NM_001127610.1 | 100 | 99 | 2588-3023 |
| | PREDICTED: *Homo sapiens* similar to zinc finger and BTB domain containing 10 (LOC100129070) | XR_039316.2 | 99 | 94 | 690-270 |
| | *Homo sapiens* zinc finger and BTB domain containing 10 (ZBTB10), transcript variant 2 | NM_023929.3 | 83 | 94 | 3106-2668 |
| 4-B6 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1), mRNA | NM_003295.2 | 99 | 50 | 829-527 |
| 4-C1 | *Homo sapiens* apolipoprotein A-II (APOA2), mRNA | NM_001643.1 | 100 | 100 | 165-472 |
| 4-C2 | PREDICTED: *Homo sapiens* hypothetical protein LOC100293548 (LOC100293548) | XM_002345671.1 | 86 | 96 | 1174-1572 |
| 4-C4 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II (LOC100293593) | XR_078889.1 | 98 | 100 | 2054-2446 |
| 5-A3 | SERPIN A-1 (multi. transcript) | NM_001127707.1 | 99% | 89% | 1463-1685 |

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|---|---|---|---|---|---|
| 5-A5 | Chrom 1 (GRch37 reference primary) | NT_032977.9 | 99% | 47% | 3846093-3846400 |
| 5-A6 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1) | NM_003295.2 | 100 | 38 | 829-645 |
| | *Homo sapiens* TPT1-like protein (FLJ44635) | NM_207422.1 | 94 | 36 | 1072-901 |
| 5-B1 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 58 | 9276102-9275788 |
| 5-B2 | Chrom 1 (GRch37 reference primary) | NT_032977.9 | 99% | 50% | 9276102-9275788 |
| 5-B4 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase sub | XR_078889.1 | 98% | 100% | 1281-1691 |
| 5-C1 | *Homo sapiens* paraoxonase 3 (PON3), mRNA | NM_000940.2 | 100 | 100 | 385-919 |
| 5-C2 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99 | 55 | 3846086-3846400 |
| 5-C4 | Apolipoprotein H | NM_000042.2 | 99% | 100% | 216-832 |
| 5-C5 | SERPIN A-1 (transcript 11) | NM_001127707.1 | 100% | 100% | 710-1208 |
| 5-D1 | Haptoglobin (transcript 2) | NM_001126102.1 | 100% | 100% | 300-812 |
| | Haptoglobin (transcript 1) | NM_005143.3 | 100% | 100% | 477-989 |
| | Haptoglobin-related protein | NM_020995.3 | 100% | 96% | 289-801 |
| 5-D2 | *Homo sapiens* serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (SERPING1), transcript variant 2 | NM_001032295.1 | 100 | 99 | 910-1432 |
| 5-D3 | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT) | NM_001127610.1 | 100 | 98 | 2588-3252 |
| | *Homo sapiens* zinc finger and BTB domain containing 10 (ZBTB10), transcript variant 2 | NM_023929.3 | 83 | 61 | 3106-2668 |
| 5-D4 | *Homo sapiens* tumor protein, translationally- | NM_003295.2 | 99 | 45 | 829-581 |

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| | controlled 1 (TPT1) | | | | |
| 5-D5 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1) MUTLIPLE VARIANTS | NM_001127707.1 | 100 | 97 | 704-1059 |
| 5-D6 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 44 | 9276102-9275788 |
| 5-E1 | *Homo sapiens* apolipoprotein A-II (APOA2), mRNA | NM_001643.1 | 100 | 99 | 21-465 |
| 5-E2 | *Homo sapiens* vasoactive intestinal peptide receptor 1 (VIPR1), mRNA | NM_004624.3 | 99 | 100 | 1569-2107 |
| 5-E6 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1), mRNA | NM_003295.2 | 99 | 52 | 829-510 |
| 5-F1 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99 | 70 | 3846086-3846400 |
| 5-F4 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1), mRNA | NM_003295.2 | 99 | 41 | 829-617 |
| 6-A2 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1) | NM_001127707.1 | 100 | 97 | 704-1113 |
| 6-A3 | PREDICTED: similar cytochrome c oxidase subunit II | XR_078889.1 | 98% | 100% | 2073-2544 |
| | PREDICTED: similar cytochrome c oxidase subunit II | XM_002345847.1 | 91% | 27% | 2074-2545 |
| 6-A4 | Albumin | NM_000477.5 | 99% | 100% | 1241-1606 |
| 6-A5 | Chrom 20 | NT_011362.10 | 100% | 100% | 13049551-13049841 |
| 6-A6 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 52 | 9276102-9275788 |

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|---|---|---|---|---|---|
| 6-B1 | mitochondrion (many multiple hits) | NC_001807.4 | 99% | 100% | 2245-2594 |
| 6-B2 | multiple variants PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078216.1 | 97 | 98 | 2192-2710 |
| 6-B3 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef) | NW_001838577.2 | 99 | 56 | 3846086-3846400 |
| 6-B4 | SERPIN A-1 (multi. transcript) | NM_001127707.1 | 100% | 100% | 710-1102 |
| 6-B6 | multiple variants alpha-1-microglobulin/bikunin precursor | NM_001633.3 | 100% | 100% | 209-744 |
| 6-C1 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98 | 98 | 1337-1837 |
| 6-C2 | *Homo sapiens* orosomucoid 2 (ORM2), mRNA | NM_000608.2 | 100 | 98 | 101-611 |
| | *Homo sapiens* orosomucoid 1 (ORM1), mRNA | NM_000607.2 | 94 | 98 | 96-606 |
| 6-C4 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1), transcript variant 11, mRNA | NM_001127707.1 | 100 | 99 | 704-1230 |
| 6-C5 | PREDICTED: *Homo sapiens* similar to NADH dehydrogenase subunit 2 (LOC100131754), miscRNA | XR_078214.1 | 98 | 99 | 405-874 |
| 6-C6 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 64 | 3846086-3846400 |
| 6-D1 | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT) | NM_001127610.1 | 100 | 100 | 2591-3065 |
| | *Homo sapiens* zinc finger and BTB domain containing 10 (ZBTB10) | NM_023929.3 | 83 | 86 | 3102-2668 |

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| | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT), transcript variant 1 | NM_001701.3 | 100 | 100 | 2692-3166 |
| 6-D2 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 57 | 9276102-9275788 |
| 6-D4 | tpt1 | NM_003295.2 | 99% | 47% | 829-565 |
| 6-E4 | leucine rich repeat containing 8 family, member A (LRRC8A) | NM_019594.3 | 99% | 98% | 3719-4283 |
| | transcript 3 | NM_001127245.1 | 99% | 98% | 3612-4176 |
| | transcript 1 | NM_001127244.1 | 99% | 98% | 3988-4552 |
| 6-F1 | similar to NADH dehydrogenase subunit 2 | XR_078214.1 | 98% | 98% | |
| | mitochondrion (many multiple hits) | NC_001807.4 | 100% | 98% | |
| 7-A1 | *Homo sapiens* clusterin (CLU), transcript variant 2 | NM_203339.1 | 100 | 67 | 1497-1883 |
| | *Homo sapiens* clusterin (CLU), transcript variant 1 | NM_001831.2 | 100 | 67 | 1377-1763 |
| 7-A2 | *Homo sapiens* apolipoprotein C-III (APOC3), mRNA | NM_000040.1 | 100 | 78 | 203-533 |
| | *Homo sapiens* ADP-ribosylation-like factor 6 interacting protein 4 (ARL6IP4), transcript variant 4, mRNA | NM_001002252.1 | 98 | 18 | 850-926 |
| 7-A4 | *Homo sapiens* stromal cell derived factor 4 (SDF4) | NM_016547.2 | 99 | 98 | 1498-1907 |
| 7-A5 | PREDICTED: *Homo sapiens* similar to DC24 (LOC100293090), m | XR_078993.1 | 95% | 100% | 59-795 |
| 7-A6 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1) multiple variants | NM_001127707.1 | 100 | 97 | 704-1122 |
| 7-B1 | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine | NM_001127610.1 | 100% | 100% | 2594-3264 |

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| | N-choloyltransferase) (BAAT), transcript variant 2, mRNA | | | | |
| | *Homo sapiens* zinc finger and BTB domain containing 10 (ZBTB1) variant 2 | NM_023929.3 | 83% | 79% | 3098-2668 |
| 7-B2 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98 | 98 | 1337-1819 |
| 7-B3 | Chrom 1 (GRch37 reference primary) | NT_032977.9 | 99% | 47% | 9276101-9275788 |
| 7-B4 | PREDICTED: *Homo sapiens* similar to DC24 (LOC100293090), miscRNA | XR_078993.1 | 95 | 100 | 166-722 |
| 7-C2 | *Homo sapiens* metallothionein 1G (MT1G) | NM_005950.1 | 99 | 96 | 15-396 |
| | MULTIPLE VARIANTS | | | | |
| 7-C3 | PREDICTED: *Homo sapiens* similar to DC24 (LOC100293090) | XR_078993.1 | 94% | 99% | 236-877 |
| 7-C5 | *Homo sapiens* orosomucoid 2 (ORM2) | NM_000608.2 | 99 | 98 | 101-758 |
| | *Homo sapiens* orosomucoid 1 (ORM1) | | | | 96-709 |
| | *Homo sapiens* TSPY-like 3 (pseudogene) (TSPYL3), non-coding RNA | | 82 | 6 | 438-476 |
| 7-D1 | PREDICTED: *Homo sapiens* similar to cytochrome b (LOC100288871) | XR_078322.1 | 85 | 98 | 388-1137 |
| 7-D2 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1) | NM_003295.2 | 100 | 32 | 829-684 |
| 7-D3 | *Homo sapiens* orosomucoid 2 (ORM2), mRNA | NM_000608.2 | 99 | 95 | 101-753 |
| | *Homo sapiens* orosomucoid 1 (ORM1), mRNA | NM_000607.2 | 95 | 99 | 96-748 |
| 7-D4 | *homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1) | NM_001127707.1 | 100 | 98 | 704-1208 |

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| | multiple variants | | | | |
| 7-D5 | *Homo sapiens* ribosomal protein S6 (RPS6) | NM_001010.2 | 99 | 99 | 827-190 |
| | *Homo sapiens* zinc finger, CCHC domain containing 6 (ZCCHC6) | NM_024617.2 | 75 | 9 | 3282-3223 |
| 7-D6 | PREDICTED: *Homo sapiens* similar to OK/SW-CL.16 | XM_002342023.1 | 99% | 43 | 1258-1434 |
| 7-E1 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99% | 55 | 3846086-3846400 |
| 8-A1 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 referenc | NT_032977.9 | 100% | 100% | 84462861-84463275 |
| 8-A2 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1) | NM_001127707.1 | 100% | 100% | 709-1240 |
| | multiple variants | | | | |
| 8-A3 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase sub | XR_078889.1 | 98% | 97% | 1337-1764 |
| 8-A4 | *Homo sapiens* metallothionein 1G (MT1G), mRNA | NM_005950.1 | 99 | 64 | 15-396 |
| | multiple variants | | | | |
| 8-A5 | *homo sapiens* aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III) (AKR1C2) | NM_205845.1 | 99% | 100% | 1498-960 |
| 8-B3 | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT) | NM_001127610.1 | 100 | 98 | 2588-3082 |
| | *Homo sapiens* zinc finger and BTB domain containing 10 (ZBTB10) | NM_023929.3 | 83 | 82 | 3106-2668 |

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| 8-B4 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99 | 64 | 3846086-3846400 |
| 8-B5 | PREDICTED: *Homo sapiens* similar to DC24 (LOC100293090) | XR_078993.1 | 95 | 97 | 827-1216 |
| 8-B6 | PREDICTED: *Homo sapiens* similar to NADH dehydrogenase subunit 2 (LOC100131754) | XR_078214.1 | 98% | 100% | 156-843 |
| 8-C2 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 referenc | NT_032977.9 | 99% | 79% | 9276101-9275788 |
| | *Homo sapiens* solute carrier family 14 (urea transporter), member 1 (Kidd blood group) (SLC14A1) | NM_001128588.1 | 90% | 5% | 3366-3396 |
| | *Homo sapiens* solute carrier family 14 (urea transporter), member 1 (Kidd blood group) (SLC14A1), transcript variant 2 | NM_015865.3 | 90% | 5% | 3208-3238 |
| 8-C3 | *Homo sapiens* fumarylacetoacetate hydrolase (fumarylacetoacetase) | NM_000137.1 | 100% | 100% | 1447-834 |
| | *Homo sapiens* methylcrotonoyl-Coenzyme A carboxylase 2 (beta) (MCCC2), nuclear gene encoding mitochondrial protein | NM_022132.4 | 93% | 4% | 1546-1574 |
| 8-C4 | *Homo sapiens* angiotensinogen (serpin peptidase inhibitor, clade A, member 8) (AGT) | NM_000029.3 | 100 | 86 | 2034-2570 |
| | *Homo sapiens* ribonuclease P/MRP 30kDa subunit (RPP30), transcript variant 2 | NM_006413.4 | 90 | 5 | 1074-1105 |
| | *Homo sapiens* protein phosphatase 1, regulatory (inhibitor) subunit 12B (PPP1R12B) | NM_032105.1 | 92 | 4 | 7371-7398 |
| | *Homo sapiens* zinc finger protein 738 (ZNF738), non-coding RNA | NR_027130.1 | 96 | 4 | 2326-2450 |

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| | *Homo sapiens* ankyrin repeat domain 53 (ANKRD53), transcript variant 1 | NM_001115116.1 | 100 | 3 | 675-654 |
| 8-C5 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1) | NM_003295.2 | 99 | 47 | 829-560 |
| | *Homo sapiens* TPT1-like protein (FLJ44635) | NM_207422.1 | 95 | 45 | 1072-816 |
| 8-D1 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II (LOC100293593), miscRNA | XR_078889.1 | 98 | 97 | 1337-1724 |
| 8-D2 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 reference primary assembly | NT_032977.9 | 99 | 58 | 9276102-9275788 |
| 8-D3 | SERPIN G-1 (transcript 2) | NM_001032295.1 | 99% | 100% | 916-1393 |
| | SERPIN G-1 (transcript 1) | NM_000062.2 | 99% | 100% | 1068-1545 |
| 8-D4 | *Homo sapiens* aquaporin 9 (AQP9) | NM_020980.3 | 99 | 58 | 2592-2996 |
| 8-D5 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 99% | 100% | 1506-1934 |
| 9-A1 | Chrom 1 (GRch37 reference primary) | NT_032977.9 | 99% | 49% | 3846086-3846400 |
| 9-A4 | *Homo sapiens* chromosome 7 genomic contig, GRCh37 reference primary assembly | NT_007933.15 | 99 | 80 | 37460530-37461010 |
| 9-A5 | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (YWHAB), transcript 1 | NM_003404.3 | 100% | 75% | 583-1088 |
| | *Homo sapiens* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (YWHAB), transcript variant 2 | NM_139323.2 | 100% | 77% | 488-993 |

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| 9-A6 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99 | 52 | 3846086-3846400 |
| 9-B1 | SERPIN A-1 (multi. transcript) multiple variants | NM_001127707.1 | 100% | 100% | 710-1069 |
| 9-B2 | similar to NADH dehydrogenase subunit 2 | XR_078214.1 | 98% | 98% | 405-1055 |
| 9-B5 | Chrom 1 (GRch37 reference primary) | NT_032977.9 | 100% | 89% | 84462861-84463354 |
| 9-B6 | *Homo sapiens* group-specific component (vitamin D binding protein) (GC) | NM_000583.2 | 100 | 77 | 1759-1634 |
| 9-C1 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98 | 97 | 1199-1536 |
| 9-C3 | *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1) | NM_001127707.1 | 100 | 98 | 704-1321 |
| 9-C6 | SERPIN A-1 (multi. transcript) multiple variants | NM_001127707.1 | 99% | 99% | 533-1182 |
| 9-D1 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1) | NM_003295.2 | 100% | 39% | 829-633 |
| | *Homo sapiens* TPT1-like protein (FLJ44635) | NM_207422.1 | 95% | 37% | 1072-889 |
| 9-D3 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98 | 98 | 1337-1839 |
| 9-D4b | PREDICTED: *Homo sapiens* similar to DC24 | XR_078993.1 | 96 | 97 | 791-1216 |
| 9-D5 | Chrom 1 (GRch37 reference primary) | NT_032977.9 | 99% | 45% | 9276100-9275788 |
| 9-D6 | *Homo sapiens* serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (SERPING1) | NM_001032295.1 | 100 | 98 | 910-1340 |

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| 10-A1 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase sub | XR_078889.1 | 98% | 97% | 1337-1840 |
| 10-A3 | *Homo sapiens* apolipoprotein A-II (APOA2) | NM_001643.1 | 100 | 97 | 165-472 |
| 10-A4 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98% | 98% | 1337-1904 |
| 10-A5 | *Homo sapiens* SEC11 homolog A (S. cerevisiae) (SEC11A) | NM_014300.2 | 100 | 98 | 1375-681 |
| 10-B1 | *Homo sapiens* apolipoprotein A-II (APOA2), mRNA | NM_001643.1 | 100% | 100% | 165-472 |
| 10-B2 | *Homo sapiens* clusterin (CLU), transcript variant 2, mRNA | NM_203339.1 | 100% | 96% | 1497-1883 |
| | *Homo sapiens* clusterin (CLU), transcript variant 1, mRNA | NM_001831.2 | 100% | 96% | 1377-1763 |
| 10-B3 | *Homo sapiens* mitochondrion, complete genome multiple chromosomes | NC_001807.4 | 100 | 97 | 1832-2130 |
| 10-B4 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98% | 100% | 2062-2600 |
| 10-B6 | *Homo sapiens* chromosome 1 genomic contig, GRCh37 referenc | NT_032977.9 | 99% | 52% | 9276102-9275788 |
| 10-C1 | *Homo sapiens* orosomucoid 2 (ORM2) | NM_000608.2 | 99 | 98 | 101-550 |
| | *Homo sapiens* orosomucoid 1 (ORM1) | NM_000607.2 | 93 | 98 | 96-545 |
| 10-C2 | *Homo sapiens* SEC13 homolog (S. cerevisiae) (SEC13) | NR_024273.1 | 100 | 97 | 1449-1237 |
| 10-C3 | UNREADABLE | | | | |
| 10-C4 | PREDICTED: *Homo sapiens* similar to cytochrome c oxidase subunit II | XR_078889.1 | 98 | 98 | 2065-2536 |
| 10-C5 | *Homo sapiens* bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT) | NM_001127610.1 | 100 | 98 | 2588-3125 |

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| | PREDICTED: *Homo sapiens* similar to zinc finger and BTB domain containing 10 (LOC100129070) | XR_039316.2 | 99 | 76 | 690-270 |
| 10-D1 | *Homo sapiens* mitochondrion, complete genome | NC_001807.4 | 99 | 98 | 15135-15676 |
| | *Homo sapiens* chromosome 5 genomic contig, GRCh37 reference primary assembly | NT_034772.6 | 91 | 64 | 42573225-42572871 |
| 10-D2 | *Homo sapiens* chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence | NW_001838577.2 | 99 | 58 | 3846086-3846400 |
| 10-D4 | *Homo sapiens* tumor protein, translationally-controlled 1 (TPT1), mRNA | NM_003295.2 | 99 | 52 | 829-510 |

## Chapter 6: Materials and Methods

### Bacterial Two-Hybrid Screen

The cDNA sequence encoding for hPer2C (GenBank accession number: NM_022817) was cloned (using primers FBTV4VII: ATAAGAATGCGGCCGCCTTGAACGCCACAGCCTGGTCA, RBTV4VII: CGGAATTCTTACGTCTGCTCTTCGATCCTG) into the bait plasmid of the Bacteriomatch© Two-Hybrid Screening System. The pBT-hPer2 was cotransformed along with the cDNA of human liver that had been cloned unidirectionally into target vector with restriction sites EcoRI and XhoI. Primary incubation was done with minimal media which contains 0.4% w/v glucose, 0.2 mM adenine HCl, 1x His-Drop out supplement (essential amino acids and nucleosides without histidine), 1 mM $MgSO_4$, 1 mM Thiamine HCl, 10 uM $ZnSO_4$, 100 uM $CaCl_2$, 50 uM IPTG, 1x M9 salts (Qbiogene, cat. # 3037-032) and antibiotics to select for pBT and pTRG vectors. After 24 hour incubation, another incubation time of 16 hours was performed in order to detect weak interactors. Any other colonies formed after the 40 hr incubation period was considered false positives (refer to Stratagene catalogue #982000). The colonies that formed on selective media were deemed interactors and individually picked to be patched on to dual selective plates (same media as minimal media mentioned above with addition of 12.5 ug/mL Str) which required a secondary reporter cassette to be activated through the expression of the streptomycin resistance gene. The bacteria containing both bait and target plasmids needed to be manipulated to only contain target plasmid. This was done by retransforming the clones that had been purified and plating onto media selective for the pTRG plasmids. A colony was regrown in LB-media with again only the antibiotic selective for pTRG. The DNA purification from each of these cultures yielded a purified pTRG plasmid with minimal or no pBT-hPer2C. The target plasmids were sequenced using the forward primer pTRGF (5' TGGCTGAACAACTGGAAGCT 3').

### Stability Assay

Reactions were comprised of HeLa S100 cell-free lysates (1 mg/mL, Enzo Life Sciences) with the addition of methylated ubiqituin, ubiquitin aldehyde, okadaic acid, casein kinase, and exogenous proteins in a total of 10 µL for every time point in 0.6 µL Eppendorf tubes. Radiolabeled proteins were prepared using a Wheat germ cell free lysate Transciption and Translation© (TnT) system with $^{[35]}$S-methinione. The reaction, without $^{[35]}$S-labeled protein, was pre-incubated at room temperature for 15 minutes and following addition of $^{[35]}$S-labeled protein, incubated at 30°C for the indicated times. The reactions had 2 µL of 6x Laemmeli sample buffer added and boiled at 95°C for 5 minutes before analysis in an SDS-PAGE gel followed by exposure to X-ray film. Protease inhibitor cocktails used in the stability assays are from Research Products International Corp. (#P50750, contains 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin, pepstatin A) and are added in a 1:1000 dilution.

**Cleavage Assay**

Each sample contains bacterially expressed and purified proteins indicated. Buffer solution is made up of (50 mM Tris pH 7.5, 250 mM NaCl, and 1 mM EDTA) and where indicated, include 5 mM EDTA and 5 mM EGTA or 10 mM ATP, 20 mM $MgCl_2$. Reactions in 1.5 µL Eppendorf tubes of 50 µL were incubated at room temperature with rotation for the times indicated. Sample buffer (described previously, 10 µL of Laemmeli buffer 6x) was added to each reaction, heated for 5 minutes at 95°C and run in an SDS-PAGE gel. Analysis was run after western blotting the gels and applying GST antibody [60].

**Mammalian Cell Culture**

Chinese Hamster Ovaries (CHO) cells were cultured in F-12K complete media (Kaighn's modification of Hams F-12 medium, ATCC). Transfections with respective plasmids were performed using Lipofectamine©. In 6-well plates, 1 ug of plasmid were incubated in 800 uL of F-12K complete media without any serum or antibiotic along with 10 uL Lipofectamine for 45 minutes. These samples were added to respective wells which had been previously washed twice with PBS to remove any residual serum. The plates were then incubated at 37°C with 5% v/v $CO_2$ for 5 hours when 800 uL of 2X serum concentration F-12K complete media was added. Twelve hours of incubation with serum and complete media is time zero and when harvesting of cells begin.

**Co-Immunoprecipitation**

Cells (in 35 mm, 6-well plates) are harvested when at 100% confluency by being washed with PBS twice after centrifugation at 4000 x g for two minutes. Samples are kept at -80°C until needed. The samples are thawed on ice followed by addition of 100 µL Lysis Buffer© (Promega) with the addition of protease inhibitor cocktail. Resuspension with a combination of pipetting and gentle vortexing to lyse cells is followed by centrifugation at 14000 x g at 4°C for 2 minutes to pellet down cellular fragments. The lysate is removed and placed into a fresh pre-cooled tube along with beads (4 µL of Santa Cruz c-myc beads, SC-40AC) containing respective antibodies to immunoprecipitate specifically tagged proteins in the cell lysate (protein concentration is ~ 3.1 µg/µL) in a total volume of 300 µL in 1.5 µL Eppendorf tube.

**GST pull-down assay**

Beads with GST or GST-TCTP attached to them are in reactions consisting of GST pull-down buffer A (40 mM Tris, 100 mM NaCl, 5 mM EDTA, 0.1% Triton-X). To each of these reactions of 300 µL, 3 uL of $^{[35]}$S-hPer2 produced by the TnT system are added. Incubation occurs at 4°C for two hours with rotational agitation in 1.5 µL Eppendorf tubes. Reactions are centrifuged at 14,000 x g for 30 seconds to collect all beads. The beads are washed with buffers of increasing salt concentration (up to 1 M NaCl) to remove non-specific binding. 20 µL of Laemmeli sample buffer 1x is then added to beads,

boiled at 95°C for 5 minutes and loaded into an SDS-PAGE gel. The resultant gel is then exposed to X-ray film for analysis.

**Protease activity assay**

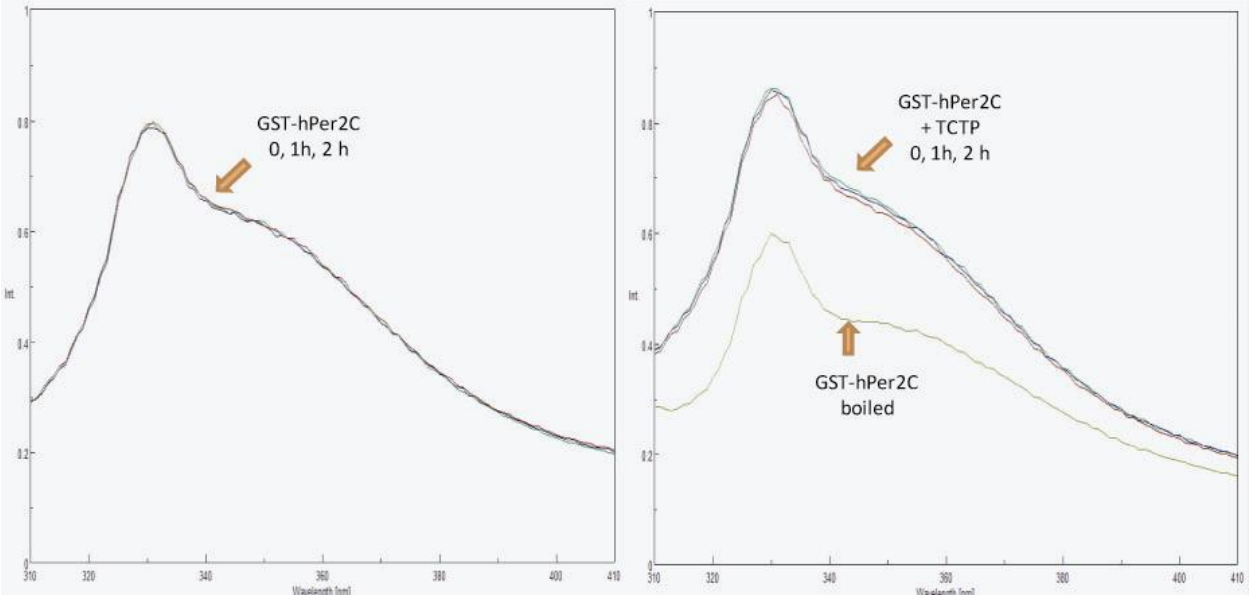
The assay used in this protocol follows the substrates and guidelines designed by Sigma-Aldrich. α-Casein, dephosphorylated, was the substrate to measure protease activity through a colorimetric assay using Folin's reagent. The The reagent reacts with free tyrosines, producing a blue chromophore detectable and measurable at 660 nm. The indicated amounts of enzyme or protein were used in reactions 500 µL of Buffer A. Reactions were stopped using tricholoroacetic acid after 10 minutes at 37°C and filtered using 0.45 µm PES syringe filters to remove any precipitated protein. After stopping the reactions, the same amount of enzyme or protein was added to each reaction to eliminate readings from tyrosines in the enzyme or protein itself. After 30 minutes incubation at 37°C, these reactions were then filtered by the previously described method and measured in 200 µL aliquots in a 96-well plate.

**Chapter 7: Collaborations**

A. **A Novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor Period 2.** Jianhua Yang, Kevin D. Kim, Andrew Lucas, Karen E. Drahos, Carlo S. Santos, Sean P. Mury, Daniel G. S. Capelluto, and Carla V. Finkielstein. *Molecular and Cellular Biology*. Aug. 2008: 4697-4711. Reprinted with permission, American Society for Microbiology License Number 2186140420519.

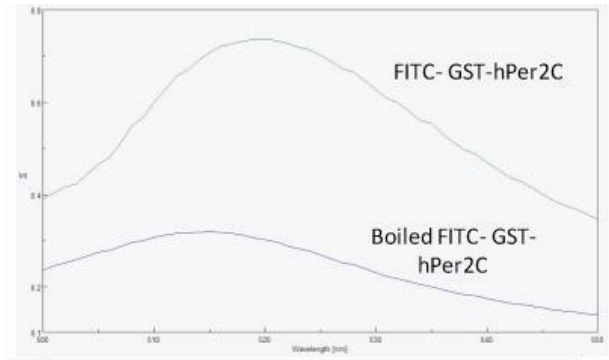
## Appendix A: Tryptophan and FITC experiments

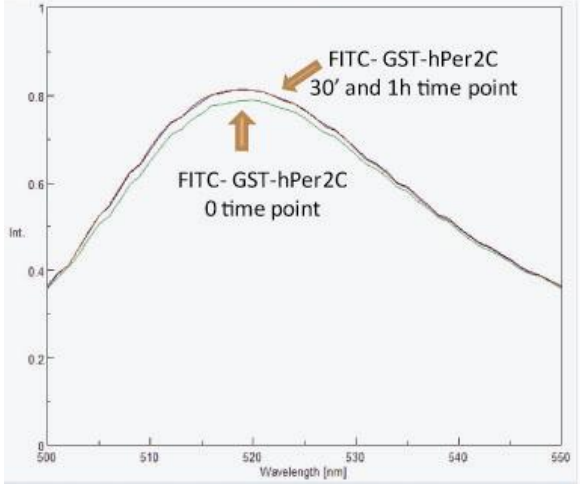
### Tryptophan quenching experiment

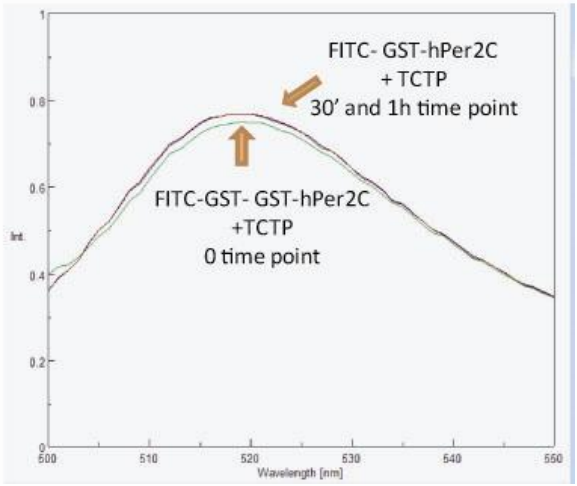



Tryptophan quenching was performed using the Jasco J-815 Spectrophotometer and 1 mm bi-directional quartz cuvette for perpendicular fluorescence detection. GST-hPer2 C (125 nM) was incubated at 25°C with mixing in buffer A, as previously described, and GST (125 nM) was used as a control. Addition of TCTP (625 nM) was done in fresh samples so as not to perturb the dilution factor of GST or GST-hPer2 C. Measurements were performed in triplicates at an emission range of 310 to 410 nm and excitation at 295 nm.

**Fluorescein Isothiocyanate (FITC) experiments**




GST (362.9 uM) and GST-hPer2 C (9.76 uM) was labeled with FITC (0.9 uM FITC) at room temperature for 1 hour. Labeling was performed adjusting the Buffer A to pH 9 after which the labeled sample was collected in a Centrifugal Filter Unit (Millipore) in order to increase the concentration of labeled protein, readjust the buffer to pH 7, and to remove free FITC. The concentration of FITC-labeled proteins were calculated taking UV/Vis readings at 260, 280 and 494 nm while taking into account the contribution of signal from intrinsic fluorophores and the absorption of signal by buffer. Approximately 25 nM FITC-labeled GST-hPer2 C or GST were used in the assays with or without 125 nM TCTP. The experiment was performed in the same method and equipment as for the tryptophan quenching experiment with the exception that the excitation wavelength was 494 nm.

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