Identification and regulatory role of E3 ligases in the time-dependent degradation of the circadian factor Period 2

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ABSTRACT

Circadian rhythms are self-sustained, 24h, biological oscillatory processes that are present in organisms ranging from bacteria to human. Circadian rhythms, which can be synchronized by external cues, are important for organisms to adjust their behavior, physiological activity, and metabolic reactions to changes in environmental conditions. Another well-established oscillatory mechanism that shares common organizational and regulatory features with the circadian system, is the cell division cycle. Recent findings reveal that some essential regulators are common to both the cell cycle and the circadian clock.

The first half of my thesis (Chapter 2-3) focuses on the function of Period 2 (Per2), a key regulatory component of the negative feedback arm of the clock and tumor suppressor protein, as a modulator of cell cycle response. We found that Per2 binds the C-terminus end of the tumor suppressor p53 thus forming a trimeric complex with p53's negative regulator Mdm2 and preventing Mdm2-mediated p53's ubiquitination and degradation. Thus, Per2 stabilizes p53 under unstressed conditions allowing for basal levels of the protein to exist and be available for a rapid response to take place in case of any stressed signals. Our experiments prove that Per2 plays an indispensible role in p53 signaling pathway.

The second half of my thesis (Chapter 4-5) focuses on how Mdm2 and Per2 interplay regulate Per2 availability and its impact on circadian clock function. My research found that Mdm2 targets Per2 for ubiquitination as Mdm2 depletion stabilizes Per2 and, conversely, Mdm2 ectopic expression shorten Per2's half-life. Accordingly, association of Per2 to Mdm2 maps C-terminus of the p53 binding region in Mdm2 and thus, the RING domain remains accessible. Next, we tested the hypothesis that Mdm2-dependent ubiquitination of Per2 directly impacts circadian clock period length. Accordingly, addition of sempervirine nitrate (SN), a specific molecular inhibitor of Mdm2, to MEF cells abrogated Per2 ubiquitination leading to the accumulation of a stable pool of Per2. By recording the oscillatory behavior of the Per2:Luc reporter system in MEF cells treated with SN at different circadian times, we found that inhibition of Mdm2 E3 ligase activity promoted phase advance only when treatment took place during the degradation period. This is in agreement with our findings that radiation, but not light pulses, causes the same phase behavior. Considering the established role of both Mdm2 and p53 in the response of cells to genotoxic stress and Per2 in modulating the clock, the existence of the Mdm2-Per2-p53 complex opens the possibility of various stimuli triggering regulatory mechanisms converging in a critical node. Overall, our work provides a holistic view of how signals are integrated at multiple levels to ensure that environmental signals are sense and responses triggered timely.

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Abbreviations

4EBP: eIF4E binding protein

ACTH: Adrenocorticotropic hormone

Akt: AKT8 virus oncogene cellular homolog

AMPK: Adenosine monophosphate activated protein kinase

APA: Alternative polyadenylation

ATM: Ataxia telangiectasia-mutated protein kinase

ATR: ATM and Rad3-related protein kinase

AVP: Vasopressin

beta-TrCP: Transductin repeat containing protein

BMAL1: Brain and muscle Arnt-like 1

cAMP: Cyclic AMP

CHK1: Checkpoint kinase 1

Chk2: Checkpoint kinase 2

CHX: Cycloheximide

CKI: Casein kinase I

CLOCK: Circadian locomotor output cycles kaput

CRE: cAMP response element

CREB: cAMP response element binding protein

CRH: Corticotropin-releasing hormone

CRY: Cryptochrome

DBP: D element-binding protein

FASPS: Familial adcance sleep-phase syndrome

G6pc: Glucose-6-phosphatase gene

GC: Glucocorticoid

GPCR: G-protein coupled receptor

GRE: Glucocorticoid response element

H1299: human non-small cell lung carcinoma-1299

HAT: Histone acetyltransferase

HAUSP: Herpevirus-associated ubiquitin-specific protease

HCT116: Human colorectal carcinoma-116

HECT: Homologous to the E6AP carboxyl teminus

hnRNP D: heterogeneous nuclear ribonucleoprotein D

HPA: Hypothalamus-pituitaty-adrenal gland

HSE: Heat shock elements

LD: Light-dark

LPS: lipopolysaccharide

MAPK: Mitogen-activated protein kinases

Mdm2: Murine double minute 2

MEF: Mouse embryonic fibroblasts

MMS: Methyl methane sulfonate

NPC: Nuclear pore complex

PABPII: Polyadenine binding protein II

PAP: Polyadenine polymerase

Pck1: Phosphoenolpyruvate carboxyl kinase gene

PER: Period

PKC: Protein kinase C

PRC: Phase response curve

PTB: Polypyriminde tract-binding protein

PVN: Paraventricular nucleus

Rb: Retinoblastoma protein

RBP: RNA-binding protein

RING: really interesting new gene

RNP: Ribonucleoprotein

SAD: Seasonal affective disorder

SCN: Suprachiasmatic nucleus

SIRT1: Sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*)

SN: Sempervirine Nitrate

StAR: Steroidogenic cute regulatory protein

TIM: Timless

Tipin: TIM binding protein

TLR: Toll-like receptor 9

TTFL: Transcription-translation feedback loop

UTR: Untranslated region

Preface

Parts of the dissertation have been published.

Chapter two was published as **The circadian factor Period 2 modulates p53 stability and transcriptional activity in unstressed cells.**

Chapter three was published as **Association of the circadian factor Period 2 to p53 influences p53's function in DNA-damage signaling**

Contributions of authors are addressed at the beginning of each chapter.

CHAPTER 1: Introduction

In this chapter, I will first introduce concepts and features of circadian rhythms that make these oscillations unique from other rhythmic behaviors that are part of our physiology and present in our environment. Then, I would summarize the current state of knowledge regarding the molecular mechanisms involved in generating and sustaining the clock. Lastly, the focus will shift towards the circadian factor Period 2 and its post-translational modifications and how these modifications impact Per2's functions. Closing this section, the reader will find a brief discussion on the innovative aspects of this work.

1.1 Essential concepts in circadian rhythmicity

Circadian rhythms are any biological processes that oscillate in about 24-hour period. Although organisms have evolved in complexity on the mechanisms that generate, sustain, and entrain to environmental cues their circadian rhythms, the fundamental feature that makes of circadian rhythms is its persistence in the absence of external cues. The clock drifts the free running 24 h periods slightly in constant conditions, while still are capable to be entrained by cyclic environmental signals.

Another typical feature of circadian clocks is compensation with respect to environmental chaos. One good example is temperature compensation, which means the rate of clock maintaining nearly constant at any stable temperature in a physiological range. The temperature relations of circadian clocks in poikilotherms are obviously interesting, at one hand clocks must be reasonably reliable to controlling various of biological processes, at the other hand, daily temperature varies in natural environments, which impacted on biochemical kinetics universally, including the kinetic reactions that regulate circadian clock components oscillation. Therefore widespread attention had been drawn on the debate of temperature compensation in 1950s. *Drosophila pseudoobscura* cultures were raised under constant temperature conditions at 16°, 21° or 26°C under Light-dark (LD) conditions, the emergence activity of a culture showed a 24-hour rhythm, no matter what the temperature conditions are (Pittendrigh, 1954). Temperature insensitive feature was also demonstrated to occur at the unicellular level by the green alga *Euglena gracilis*, the rhythm in phototactic response is always very close to 24 hours at constant temperatures ranging from 16.7°C to 33°C(Bruce and Pittendrigh, 1956). Recently the concept of metabolic compensation is proposed, suggesting temperature compensation is actually a subset of metabolic compensation, which maintains the frequency of circadian oscillators and keeps resilience to the factors that impinge on metabolism that would otherwise destabilize the clocks (Johnson and Egli, 2014). Overall the distinguishing features of circadian rhythms include self-sustained, entrainable and resistance to moderate environmental variations (Merrow et al., 2005).

1.2 Circadian rhythm discovery

As the earth rotates, light and temperature change in a 24 h cycle. In order to better adapt to environmental changes, organisms have developed circadian rhythm throughout the process of evolution. The term circadian derives from the Latin words "*circâ"* " approximately" and *di (ês)* "a day". The earliest record of a circadian process dates 400 years

B.C. when Androsthenes described the diurnal movement of the tamarind tree leaves. During the 18th century, the French scientist Jean-Jacques d'Ortous de Mairan, carried out an experiment in which a heliotrope plant was exposed to either light/dark or constant darkness for a sustained period of days (de Mairan, 1729). Remarkably, the leaves maintained their daily opening and closing behavior despite the presence of a light phase, a result that establishes the endogenous nature of the circadian clock. Further work in circadian leaf behavior from the Swiss botanist Augustin Pyramus de Candolle defined the free running period to be approximately of 22-23 h in constant light, in agreement with the hypothesis of the existence of an endogenous clock (Moore-Ede, 1986). Despite these advances, animals' circadian rhythms were not scientifically researched until much later. In fact, early findings date to the beginning of the 20th century when the period length in animal models was defined about 24h even in the absence of any external cue (Richter, 1922; Shelford, 1918).

Genetic research in circadian rhythms begun in 1935, when Dr. Erwin Bunning discovered that the period length was a heritable feature in bean plants and insects (Bunning, 1935). In 1971, Ron Konopka and Seymour Benzer mapped the *Period* gene in Drosophila (Konopka and Benzer, 1971). Shortly after that, by using a forward genetics approach, circadian genes were wildly identified from both prokaryotic and eukaryotic systems including cyanobacteria, fungi, plants, insects, and mammals (reviewed in (Dunlap, 1999)). For example, the gene *frequency* was identified in *Nerurospora crassa* (Feldman and Hoyle, 1973); *Clock* in mouse (Vitaterna et al., 1994); *KaiABC* gene cluster in *cyanobacteria* (Ishiura et al., 1998), and *CCA1* in *Arabidopsis* (Z.-Y. Wang and Tobin, 1998). Compiling all the available molecular data together, we can see common elements conserved in the logic of the oscillators and the functions in oscillatory loops from cyanobacteria to mammalian.

1.3 The mammalian circadian clock

The classic conceptual model of circadian rhythms comprises three basic components: the input pathways, pacemaker and the output pathways (Figure 1-1). Input pathways use environmental cues, most importantly light and temperature to synchronize the endogenous clock. Endogenous periodicity is used for anticipating the onset of dawn and dusk throughout the year, which impacts on organism' fitness. The pacemaker is the oscillator, which senses the temporal environmental cues from the input pathways, generates about 24 h circadian rhythms and translates into downstream output pathways. Output pathways are used to communicate temporal information from the circadian clock to a wide range of biological processes, including regulating transcription and translation, metabolic reactions, cell division and behavior(Ko, 2006).

1.3.1 Entrainment input signals

A. Circadian entrainment

Circadian entrainment is the mechanism by which internal biological clocks are synchronized to external environmental cues. It is clear that circadian entrainment represents a form of adaptation from organisms to their environment that is tightly linked to increased fitness and survival by synchronizing their physiology and behavior to the natural dark-light (LD) cycle.

External cues that can phase-set the circadian clock are defined as *zeitgeber* (German for "time giver" or "synchronizer"). In the presence of a *zeitgeber*, the internal biological clock adjusts its period and phase to the environmental cycle. Considering a lighting schedule of 12 h of light and 12 h of darkness (L/D 12:12), ZT0 is defined as "light on" and ZT12 corresponds to "lights off". Organisms kept under constant conditions by shielding them from external time cues, for example constant darkness, still display free running activity, albeit drifted from that of 24 h. Ifthe endogenous biological clock period is either longer or shorter than the cue period the organism will experience circadian phase angle shifting during the free running period (Figure 1-2).

B. Circadian phase shift

A phase shift (either advance or delay) is a change in the phasing of the rhythm due to a distinct external stimulus (*e.g.*, a light pulse). In a phase advance scenario, the shift in the activity onset occurs to an earlier position in the circadian cycle, while a phase delay results from a shift to a later position. The phase shift response is dependent on the time of the day at which the external stimulus is applied. Accordingly, exposure to light at the early subjective night results in phase delay; conversely, late night exposure causes a phase advance. A phase response curve (PRC) is used to illustrate transient phase changes when stimuli are given at different times. Here melatonin phase response curve to light pulse is demonstrated in Figure 1-3.

Phase response curves are further classified depending on their strength in terms of phase shifting. Type 0 PRCs show large phase shifts with a breaking point between phase advance and phase delay (Figure 1-4), whereas Type 1 PRCs displays relative small phase shifts (e.g. usually less than 6h phase shifts) and have a continuous transition between delays and advances (Johnson et al., 2003). Type1 and type 0 PRCs are convertible depends on the strength of stimulus. It has been shown that in flesh fly *Sarchophaga argyrostoma* that increasing the light dose coverts type1 into type 0(Saunders, 1978).

1.3.2 Feedback loops

A. Transcription-translation negative feedback loop

In mammals, the molecular clock is hierarchal in organization, comprised of a "master regulator" that resides in the suprachiasmatic nucleus (SCN) of the brain and contains about 100,000 neurons (Cassone, n.d.; Swaab et al., n.d.). The SCN synchronizes the body's daily rhythms to environmental conditions by rhythmic producing multitude of neural and hormonal signals. Slave clocks, on the other hand, are controlled by the master clock, exist in almost every single cell, and share similar molecular elements of the master regulator. Clock core components participate in transcription-translation feedback loop (Figure 1-4), including two positive regulators BMAL1 and CLOCK, two negative regulators: PER (Per1, Per2 and Per3) and CRY (Cry1 and Cry2). In mammalian cells, the BMAL1-CLOCK heterodimer activates the transcription of PER and CRY early in the day, PER and CRY degrade in cytoplasm and accumulate in nucleus in the middle of the night, which results in blocking the activator function of BMAL1-CLOCK. Negative regulators are degraded by ubiquitin-dependent pathway. This transcription-translation feedback loop based

molecular machinery sustained the circadian clock gears autonomously running in each single cell and at the same time orchestrated by SCN (Gallego and Virshup, 2007; Ko, 2006; Partch et al., 2014; Yagita et al., 2001).

B. Epigenetic mechanisms effect on pacemaker

Recently other regulation modes have been found to supplement the central transcription translation feedback loop. For example, the circadian genes expression is regulated by epigenetic mechanisms, such as histone phosphorylation, acetylation, and methylation. Light exposure caused rapid phosphorylation of histone 3 on serine 10 in SCN, resulted in transient induction of early response and clock genes, like *c-fos* and *Per1* implicated in phase-shifting the pacemaker(Crosio et al., 2000) . The well-established study of histone acetylation in circadian gene regulation is from SIRT1, which plays crucial roles in deacetylating histones at promoters of rhythmic genes counteracting the activity of histone acetyltransferase CLOCK(Nakahata et al., 2008). Circadian regulation of the mouse albumin D element-binding protein (*Dbp*) gene involves rhythmic binding of BMAL1 and CLOCK, which is regulated by chromatin modification. The repressed phase of *Dbp* expression is accompanied by dimethylation of Lys9 of histone H3 (Ripperger and Schibler, 2006).

C. Posttranscriptional modifications effect on pacemaker

After mRNA is transcribed from DNA, mRNAs can undergo different processing and regulatory steps that influence their expression by a dynamic environment of RNA-protein and RNA-RNA interactions. Ribonucleoprotein (RNP) complexes play key roles in coordinating many biological processes including: splicing, transporting, stabilizing (or degrading), localization, and translation (Keene, 2010). All posttranscriptional steps require the proper function of RNA-binding proteins (RBPs) at all steps along the post-transcriptional regulatory chain. **1)**. Alternative splicing: the SR proteins are pivotal for constitutive and alternative pre-mRNAs splicing, which are named by a characteristic domain with long repeat of serine and arginine amino acid residues (abbreviations are “S” and “R” respectively) (Long and Caceres, 2009). **2)**. Polyadenylation: alternative polyadenylation (APA) generating mRNAs with alternative 3’ untranslated region (UTR). Cleavage and polyadenylation specificity factor, polyadenine binding protein II (PABPII) and polyadenine polymerase (PAP) involved in the cleavage of the 3’ signaling region and forming the plolyadenine tail to pre-mRNAs (Elkon et al., 2013). **3).** Export: TAP/ p15 heterodimer forms a cargo-carrier complex promoting export of mRNA through the nuclear pore complex (NPC) and release the cargo into cytoplasm(Braun et al., 2002). **4).** Stability and Translation: cis-regulatory elements (CREs) are found in UTRs of various mRNAs for regulating mRNA turnover and translation (Keene, 2010). After microRNAs were discovered in 1990s, increasing of reports show that microRNAs and other small noncoding RNAs are interdependent with RBPs cooperating or competing on posttranscriptional regulation (Keene, 2010).

Although as many as 10% of genes are controlled under circadian rhythm, recent RNA-sequencing studies suggest that only about 20% of rhythmically expressed genes in liver are driven by *de novo* transcription (Hastings and O'Neill, 2015). Translational regulation and miRNA involvement were reported in circadian rhythmicity. miRNAs are small noncoding RNAs that regulate gene expression by hybrid imperfectly with target mRNAs’ at 3’ untranslated regions

to repress translation and increase mRNA turnover. Mouse miR-132 and miR-219 were found in SCN, whose expression were controlled by circadian clock. Interestingly these two miRNAs influence core clock in return: miR-219 regulates circadian period length and miR-132 modulates light-induced clock resetting(Cheng et al., 2007) . Many RNA-binding proteins are circadian in their expression and even circadian core proteins can influence clock genes' mRNA stability, for example Period 2 was proposed to affect its own mRNA stability directly (Xu et al., 2007). Other reports also show that polypyrimidine tract-binding protein (PTB) is responsible for Per2 mRNA stability and degradation kinetics by binding to the pyrimidine tract in the *mPer2* 3'-UTR end sequence (Woo et al., 2009); whereas *mCry1* 3'-UTR contains a destabilizing *cis*-acting element, which interacts with heterogeneous nuclear ribonucleoprotein D (hnRNP D) regulating *mCry1* mRNA stability and oscillation amplitude(Woo et al., 2009) .

D. Posttranslational modifications effect on pacemaker

The posttranslational modifications are involved in sustaining circadian clock ticking including: phosphorylation, ubiquitination, sumoylation and acetylation, which regulate clock proteins localization, stability and function (Cardone et al., 2005; Gallego and Virshup, 2007; Hirayama et al., 2007). Protein phosphorylation is the addition of a phosphoryl group to amino acid residues like serine, threonine and tyrosine, which plays a prominent role in a wide range of cellular processes. Phosphorylation is a reversible enzymatic process that involves kinase and phosphatase enzymes in process with ATP as phosphoryl donor. The reaction can be represented as Figure 1-6. Several important components of the mammalian circadian oscillator including CLOCK, BMAL1, PER and CRY proteins are phosphorylated in vivo(Lee et al., 2001). Phosphorylation of these circadian main components regulates their stability, subcellular localization and inhibitory activity (Vanselow and Kramer, 2007). For example, Phosphorylation of BMAL1 by Casein Kinase I (CKI) or mitogen-activated protein kinases (MAPK) modulates BMAL1-CLOCK dependent transcription (Eide et al., 2005a; Sanada et al., 2002); Likewise, nutrient-responsive adenosine monophosphate activated protein kinase (AMPK) regulates circadian pacemaker by phosphorylation and destabilization CRY1 (Lamia et al., 2009). Ubiquitination dependent degradation of both CRY and PER negative regulators governs circadian period length(Hirano et al., 2013; Shirogane, 2005; Yoo et al., 2013). Recently a study shows deubiquitinase herpesvirus-associated ubiquitin-specific protease (HAUSP) plays an essential role in CRY1 stabilization which impacts on total period length and DNA damage induced circadian phase shift (Papp et al., 2015). Similar as ubiquitination, SUMOylation is a reversible posttranslational modification that plays a role in various cellular processes. The most common role of SUMOylation is regulating protein stability. SUMOylation site was identified between two PAS domains of BMAL1, specifically at lysine 259, which tightly controls BMAL1 turn over and influences circadian period (Cardone et al., 2005). Some clock core components are either having histone acetyltransferase (HAT) properties or regulated by acetylation. CLOCK acetylates both histone and non-histone substrate like BMAL1 to regulate circadian rhythm (Hirayama et al., 2007). Sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*) (SIRT1) a deacetylase was found binding CLOCK-BMAL1 to promote the deacetylation and degradation of PER2, which affects circadian rhythm. The sophisticated balance between acetylation and deacetylation was important for sustaining circadian clock running.

Phosphorylation, acetylation, and ubiquitination were all verified post-translation modification in Per2 (Asher et al., 2008; Ohsaki et al., 2008; Reischl et al., 2007; Shirogane, 2005; Takano et al., 2004; Toh, 2001). Phosphorylation is the most common protein modification in mammals; in mouse Per2 exhibit more than 20 possible phosphorylation sites (Vanselow et al., 2006). Moreover without any transcription or translation of circadian proteins, phosphorylation oscillation itself seems enough to sustaining circadian clock ticking in *Cyanobacteria* (Nakajima et al., 2005). Studies done in *Drosophila* show a very complex role of phosphorylation on dPER stability. Dependent on the specific phosphorylation sites in dPER, some phosphorylation sites (SLIMB binding site) promote dPER degradation while other parts (per-short phosphor-cluster) delays degradation (Chiu et al., 2011). Similar results were obtained when analyzing the mammalian Per2 homolog. From familial advance sleep-phase syndrome (FASPS) patients, PER2 (S662A) mutant was identified, which have shorter circadian period and unstable PER2. Instead, S662D mutant that mimic constitutive phosphorylation leads to longer period and much more stable PER2 (Vanselow et al., 2006). It is believed that PER progressively phosphorylation over circadian day determines the speed of clock (Blau, 2008).

Several kinases are known to phosphorylate PER. Human casein kinase I ε/δ (CKI ε/δ) are well established kinases responsible of phosphorylating circadian clock proteins (Figure 1-6) and are believed to be core clock components that sustain circadian rhythm. Individual mutations in CKI ε and CKI δ change the length of circadian rhythm and also show partial redundancy of these two kinases. The golden hamster tau mutation was the first described single gene mutation that affected circadian period in vertebrate. Wild-type animals have 24 hours rhythms; heterozygous animals have 22 hours rhythms, while the homozygous animals exhibit 20 hours rhythms (Ralph and Menaker, 1988). The tau mutations, identified as CKI ε (R178C), in Syrian hamsters also show shorter period length of circadian rhythms (Lowrey et al., 2000). However CKI ε null mutation has a slight longer period (Meng et al., 2008). To explain the conflicting results: Meng et al. (2008) proposed CKI ε^{tau} as a gain function mutant, which selectively accelerates PER degradation independent of subcellular localization and CKI ε^{tau} has asymmetric effect on overall circadian organization. Mathematical modeling and *in vitro* experiment data also verified CKI ε^{tau} as a highly specific gain of function mutation (Gallego et al., 2006). FASPS-associaed CKI δ (T44A) shows lower enzyme activity *in vitro* and shorter period length in mice (Xu et al., 2005). These results show that CKI ε/δ dependent phosphorylation of PER plays a central role in setting circadian clock speed.

1.3.3. Output genes

Circadian rhythm core components CLOCK and BMAL1 are transcriptional regulators, so their action on other loci is the first step in generating overt rhythms. System biological study identified evolutionarily conserved *cis* elements from 16 clock and clock-controlled genes. It shows that circadian transcriptional circuits are governed by these three *cis* elements: E/E' boxes, DBP/E4BP4 binding elements and RevErb/ROR binding elements (Ueda et al., 2005). It is possible that genes associate with these *cis* elements are vulnerable to be regulated by circadian rhythm. DNA microarray experiments have been conducted by using brain suprachiasmatic nucleus (SCN) to address the question of how many clock controlled genes exists(Panda et al., 2002). The data shows at least 5% of the steady-state mRNA was rhythmically expressed. However only 28 out of 330 rhythmic transcripts from SCN overlapped the samples from liver (Panda et al., 2002). Another group also used microarray technology to analyze RNA samples isolated from

livers of wild-type and clock mutant mice, claiming 1216 genes fluctuated day/night and 135 of these showed reduced expression level in mutant mice(Oishi, 2003). Therefore, the output genes are not only subjected to circadian control of gene expression but also to tissue-specific control. Here we only demonstrate a subset of these rhythmic genes, which are involved in hormone fluctuation, metabolism and cell cycle regulation.

A. Output genes involved in hormone fluctuation

In mammals, many hormones such as growth hormone and prolactin have a 24-hour rhythm, which are coupled to the sleep-wake cycle. In contrast, some other hormones including adrenal steroids not only have robust 24-hour patterns, but also self-sustained. Here we use glucocorticoid (GC) as example to show that daily variations in circulating GC levels are achieved by multiple circadian clock related regulatory mechanisms. First, the suprachiasmatic nucleus regulates the adrenal rhythm by modulating hypothalamus-pituitaty-adrenal gland (HPA) axis activity. When neurochemical signals reach the hypothalamus as the result of circadian input from suprachiamatic nucleus or the result of stress, subset of the paraventricular nucleus (PVN) releases vasopressin (AVP) and corticotropin-releasing hormone (CRH) to induce adrenocorticotropic hormone (ACTH) synthesis and secretion from the pituitary. ACTH induces adrenocortical cells to produce and secrete GC. Secondly, beside the master clock exerted regulation, it is more important that adrenal local clock underlies the GC rhythm by tightly linked with the steroidogenic pathway. The expression of *steroidogenic cute regulatory protein* (StAR) is circadian rhythmic. StAR regulates the rate-limiting step of steroidgogenesis, which is directly controlled by the CLOCK: BMAL1 heterodimer *via* E-box (Alvarez et al., 2008). Consequently the resulting daily oscillation in steroidogenesis contributes to robust GC rhythm (Chung et al., n.d.).

B. Output genes involved in Metabolism

Microarray studies examined gene expression profiles throughout the circadian cycle in the mammalian liver, skeletal muscle, and brown and white adipose tissue. The results supported the idea that a large portion of the transcriptomes (ranged from 3% to 20%) are rhythmic(Kita et al., 2002; Panda et al., 2002; Reddy et al., n.d.). Among these identified rhythmic genes, many have roles in biosynthetic and metabolic processes, including cholesterol and lipid metabolism, glycolysis and glucogeogenesis, oxidative phosphorylation, and detoxification pathways (Green et al., 2008a). Hepatic glucogenogenesis is regulated by the circadian clock, which coordinates glucose metabolism with changes in the external environment (Gelling et al., 2003; Green et al., 2008b). During fasting, glucagon secreted by the pancreas binds to a G-protein coupled receptor (GPCR) in hepatocytes. This binding activates G-protein G_s alpha subunit, which increases intracellular cyclic AMP (cAMP) and phosphorylated CREB (cAMP response element binding protein). Transcriptionally active p-CREB drives some gluconeogenic genes transcription *via* CRE (cAMP response element) *cis* element, including *Pck1* (phosphoenolpyruvate carboxyl kinase), *G6pc* (Glucose-6-phosphatase) (Hatori and Panda, 2010). Interestingly, Cry1 inhibits accumulation of cAMP in response to G protein-coupled receptor activation through interaction with G-protein G_s alpha subunit, mediating circadian regulation to hepatic gluconeogenesis (Zhang et al., 2010).

C. Output genes involved in cell cycle regulation

It has been reported that up to 7% of all circadian controlled genes regulate either cell proliferation or death (Panda et al., 2002), so it's reasonable to expect that circadian factors influence the cell division cycle(Fu and Lee, 2003; Fu et al., 2002; Gery et al., 2006; X. Gu et al., 2011). The rhythmic signal is passed on to the cell cycle processes by at least two mechanisms (Figure 1-7). The first mechanism is at transcriptional level, which is mediated *via* the E-boxes in the promoters of genes involved in cell cycle regulation. The candidate genes are: 1). The rhythmic expression of *wee1* is regulated by the CLOCK-BMAL1 complex act on its three E-box (CACGTG) elements (Matsuo, 2003). The expression level of WEE1 affects M phase entry time. 2). The transcription factor c-Myc possesses a bHLH motif regulating E-box containing genes; interestingly *c-myc* itself also contains multiple consensus E-box sequences. In wild-type mouse livers, *c-myc* mRNA oscillates in a circadian pattern, however in $mPer2^{m/m}$ mouse the circadian oscillation pattern changed and mRNA amplitude elevates more than 20 folds compared with wild-type, which is in consistent with the fact that PER2 is a negative regulator inhibiting CLOCK-BMAL1 transcriptional activation function(Fu et al., 2002) .

The second mechanism is at protein level, which is mediated *via* core clock players directly interplay with cell cycle components to regulate their functions (Figure 1-7). Studies in *Neurospora* show that the clock gene *period-4* (*prd-4*) is an ortholog of mammalian *checkpoint kinase 2* (*Chk2*). While another clock protein FRQ (PER homolog) interacts with the kinase PRD4 (Chk2 homology) inducing FRQ phosphorylation after DNA-damaging reagents treatment at certain time of the day, resulted in promoting circadian phase-shift (Pregueiro et al., 2006). In mammalian cells overexpression of circadian protein Period 1 (PER1) sensitized human cancer cells to DNA damaged-induced apoptosis while inhibition of PER1 blunted apoptosis (Gery et al., 2006). In addition, PER1 interacts with checkpoint proteins ATM and Chk2. Moreover, ectopic expression of PER1 is sufficient to induce phosphorylation of Chk2 even without DNA damage which verified the studies from *Neurospora*, indicating the interaction between circadian component and checkpoint component is evolutionally conserved (Gery, Komatsu et al. 2006). The Timeless (*tim*) gene in *Drosophila* encodes dTIM, which is a critical component in the auto-regulatory feedback loop of circadian rhythm. However its mammalian homolog has an ambiguous clock function. On one hand, *Tim* knockdown altered core clock elements expression levels and disrupted SCN neuronal rhythm activity in rat suprachiasmatic nucleus(Barnes, 2003). Conversely, TIM and the TIM binding protein (Tipin) respond to DNA-damage-induced checkpoint activation in mammals (Gotter et al., 2007). Over all, TIM interacts with both the circadian clock proteins including Cry2, Per1, Per2, and the cell cycle checkpoint proteins Chk1 and the ATR-ATRIP complex (Barnes, 2003; Unsal-Kaçmaz et al., 2005). It seems TIM having dual functions in circadian rhythm and cell cycle checkpoints pathways.

DNA damage dependent circadian rhythm phase shifting verified the crosstalk between circadian rhythm and cell cycle checkpoint pathways. In 1963, an early study showed that the circadian clock in *Gonyaulax* could be reset by UV irradiation (Sweeney, 1963). Studies in *Neurospora* also show circadian phase advance depends on the treatment time and dose of DNA damage reagent MMS (Pregueiro et al., 2006). Consistent results found in mammalian cells,

IR-mediated DNA damage reset mammalian clock in Rat-1 fibroblasts and in mice; MMS or UV irradiation generated DNA damage in mouse embryonic fibroblasts or in the NIH 3T3 cell lines elicit phase-advanced responses (Gamsby et al., 2009; Oklejewicz et al., 2008). All the examples above illustrate that DNA damage dependent regulation on circadian rhythm resetting is preserved from fungi to mammals, from cultured cells to live animals. However the molecular mechanism remains elusive. Recent research shows DNA damage-induced phosphorylation of Cry1 and dephosphorylation of Hausp resulted in increasing Hausp-Cry1 interaction. Consequently, circadian clock phase shift was achived by Hausp, stabalizting Cry1 *via* its deubiquitination activity (Papp et al., 2015).

1.4 Period 2 function and regulation mechanisms

Because *Per* genes are at a central position in the auto-regulatory transcription and translation feedback loop, it is important to understand the function of these genes and proteins at the molecular level. In this section, we focus on the Period 2 in particular, although its dysfunction has effects on widespread biological processes from aging, developing cancer to mood regulation(Albrecht et al., 2007), its molecular function and how it is regulated is largely unknown.

1.4.1 Molecular architecture of Per2

Phylogenetic analyses indicate that Per2 protein sequences at least some functional domains are conserved from zebra fish to human, especially in mammalians, Per2 proteins share high similarity. Although the whole protein structure has not been dissolved, several structural domains and functional regions and motifs have already been identified in Per2 protein as illustrated in Figure 1-8.

1). PAS domain

PAS domain was named after PER (*Drosophila* PER)-ARNT (human aryl hydrocarbon receptor nuclear transporter)-SIM (*Drosophila* single minded protein), because this domain was first discovered in these three proteins. Many eukaryotic clock proteins ubiquitously share PAS domain, including CLOCK and BMAL1. i). Per2 PAS domain mediates homo- and heterodimerization of other PERIOD proteins(Hennig et al., 2009). Mice carrying homozygous mutation in PAS-B and PAS-C display a shorter circadian period and loss of circadian rhythmicity in constant darkness(B. Zheng et al., 1999). Glycogen synthase kinase-3β also interacts with PAS domains of Per2 and phosphorylates Per2, which resulted in nuclear translocation of Per2 in COS1 cells (Iitaka, 2005). ii). Per2 PAS domain serves as ligand-binding domain, heme is the identified ligand that binds to PAS domain of Per2 (Kaasik and Lee, 2004; J. Yang et al., 2008).

2). Other Protein interaction regions and functional motifs

Similar as Per1, N-terminal region of Per2 contain a helix-loop-helix (HLH) motif, which may not binding to DNA (Sun et al., 1997). Per2 carboxy-terminal end contains a coiled coil motif, which interacts with CRY (Miyazaki et al.,) and the leucin-zipper transcription factor E4BP4. Interestingly a noval heme-regulatory motif within the C terminus of Per2 overlapped with CRY binding region, exclusively binds to ferric heme, thus acting as a redox sensor and regulating Per2 stability(J. Yang et al., 2008). LxxLL (L is leucine and x is any amino acids) motif, which usually

found in protein coactivators interacting with nuclear receptors, was also found in Per2. This may account for Per2 potential roles as coactivator and corepressor (Albrecht et al., 2007).

1.4.2 Per1, 2, and 3: redundant and non-redundant roles

The early studies of genes controlling circadian rhythms were done in fruit fly; two independent groups identified first mammalian ortholog of Drosophila clock gene *period1* (*Per1)*, whose transcripts oscillated both in SCN and eyes(Sun et al., 1997; Tei et al., 1997). By searching *Per1* homolog in mammals, m*Per2* cDNA was clone and the functional study verified its circadian oscillation and light regulation characteristics (Shearman et al., 1997). However, when assessing the role of *mPer1* and *mPer2* genes in circadian clock phase resetting, mice mutant carrying *mPer1* or *mPer2* genes response to light pulse at ZT14 and ZT22 were quite different: *mPer1* mutant did not advance at ZT22 while *mPer2* mutant did not delay at ZT14 (Albrecht et al., 2001). A third mammalian homolog of the *Drosophila period* gene was found later, designated as *Per3*, although *mPer3* RNA levels exhibited a periodic pattern in the SCN, eyes and peripheral tissues, in contrast to the light induction of *mPer1* and *mPer2* in SCN at CT14 and CT23 (in free running cycle, The lighting cycle (12L: 12D) light on: CT=0, light off: CT=12), *mPer3* RNA levels were unresponsive to photic induction (Zylka et al., 1998).

Mice harboring genetic null alleles of *Per1*, *Per2* and *Per3* revealed that they had different but partially redundant function in mammals. Briefly, *Per1* null mice displayed about one hour shorter free-running periods, *Per2* null mice had about 1.5 hour shorter periods, and double null $Per1^{-/-}$; $Per2^{-/-}$ mice show a complete loss of circadian rhythmicity(Ko, 2006). However the null allele of Per3 did not exacerbate the phenotypes of Per1 or Per2 null alleles, suggesting its role in circadian rhythm is not important(Bae et al., 2001) (Shearman et al., 2000a). Although the circadian cycle length in *mPer3*-deficient mice is 0.5 hour shorter, the locomotor activity rhythms and *mPer1*, *mPer2*, *mCry1* and *Bmal1* RNA rhythms in the SCN did not differ between the wild-type mice and *mPer3* deficient mice (Shearman et al., 2000a) .

Per2 has a complicated role in the canonical transcription-translation feedback loop model. Although manipulation of *mPer2* gene expression and activity demonstrated its role as a negative regulator (B. Zheng et al., 1999), it seems CRY is the main negative regulator to inhibit CLOCK-BMAL1 activity (St John et al., 2014). Interestingly, Per2 regulates some clock genes including *Bmal1* and *Per2* expression in a positive manner(Akashi et al., 2014; Oster, 2002; Shearman et al., 2000b); another research shows PER2 up-regulating *Bmal1* by interaction with nuclear receptors, such as PPARα (Schmutz et al., 2010). In contrast, Per1 knockout leads to decreased peak amount of nuclear mPer2 and mCry1 despite unaltered transcript levels in SCN (Bae et al., 2001).

1.4.3 Relevance of Per2 in physiology and disease

Besides its prominent role in circadian clock function, Per2 has a role in tumor suppression by regulating DNA damage response pathways. Mice without Per2 function are prone to develop tumor and cell cycle regulation are messed up(Fu et al., 2002). PER2 is down regulated in sporadic and familial breast cancer and mutants were found in human breast cancer and colorectal cancers(Sjöblom et al., 2006; Q. Yang et al., 2010). Per2 function in cell cycle progression was verified by the study of its mutant S662G. This site mutant enhanced resistance to apoptosis and affects tumorigenesis

in cancer-sensitized mice. The study also shown that cycle progression genes expression profiles were changed in Per2 S662G mutant cells (X. Gu et al., 2011). It has also been reported that in the mouse Lewis lung carcinoma cell line and mammary carcinoma cell line, overexpression mPer2 reduces cell proliferation and increases apoptosis, however no similar results were found in NIH 3T3 cells(Hua et al., 2006). mPer2 overexpression induced cancer cell apoptosis and suppression was verified by other studies (Miyazaki et al., 2010; ODA et al., 2009). Per2 functions in cell cycle progression and tumor suppression maybe explained by its role in both p53 dependent cell cycle checkpoint pathway and AKT dependent cell signaling pathway (Gotoh et al., 2015; 2014; Xiaoming Yang, 2012).

The *mPer2* gene was found link the circadian clock system and innate immune response directly. *Per2*$^{-/-}$ mice lacked interferon- γ daily rhythm both at mRNA and protein level in spleen; interferon-γ daily oscillation in serum was also abolished in the mutant mice (Arjona and Sarkar, 2006). *Per2*$^{-/-}$ mice has defective NK and NKT cell function, therefore the mutant mice were more resistant to lipopolysaccharide (LPS)-induced endotoxic shock than wild-type mice. Correspondingly the levels of proinflammatory cytokines gamma interferon (IFN-γ) and inter leukin-1β in serum were dramatically down regulated in the mutant mice (Liu et al., 2006). Another evidence, which links m*Per2* to the innate immune system was that nonfunctional m*Per2* gene (*Per2*^{Brdm1}) affects Toll-like Receptor 9 (TLR9) expression, TLR9 responsiveness, and cytokine response after challenge with CpG oligodeoxynucleotides(Silver et al., 2012).

Circadian main components knockout leads to not only abnormal diurnal rhythms but also diverse pathophysiological disorders, especially metabolic syndrome. Homozygous *Clock* mutant mice exhibit obesity and severe metabolic syndrome(Turek et al., 2005). In contrast to *Clock*$^{-/-}$ mice, *Per2*$^{-/-}$ mice display altered systemic lipid metabolism and adipocyte differentiation (Grimaldi et al., 2010). Interestingly, during ischemia and reperfusion injury, *Per2* mutant mice have deficiency in myocardial fatty acid metabolism and increased myocardial inflammation, showing inflammatory markers such as IL-6 or TNFα levels increased compared with wild-type mice (Bonney et al., 2013). Moreover quantitative PCR-chromatin immunoprecipitation (qPCR-ChIP) analysis of the core clock components DNA binding sites exhibit that mPer2 binds to *Gys-2*, G_L, and *PTG* genes with a peak at refeeding, which is independent of Bmal1; *Per2*^{Brdm1} mice show altered rhythms of food intake, hepatic glycogen accumulation and reduced fasting glycaemia, which reveal Per2 important function in promoting glucose storage to liver glycogen (Zani et al., 2013).

Per2 has functions far beyond its clock gear role in the SCN in the central nervous system. First, Per2 has impacts on mood disorders. Seasonal affective disorder (SAD) or winter depression was come along with abnormalities in circadian rhythm, variations in *Per2* gene was associated with the disorders(Partonen et al., 2007). Second, Per2 affects neurotransmitter regulation. Glutamate transporter Eaat1 is expressed at low levels in *Per2*^{Brdm1} mice, which means less glutamate was uptaken by astrocytes, therefore extracellular space has increased level of glutamate, which caused increased alcohol uptake in those mutant mice. Consistent result was found in human study, PER2 variations in population are associated with alcohol consumption (Spanagel et al., 2004). It seems different clock proteins may have different role in drug abuse. For example, *Per* mutant mice show opposite results in the drug abuse study. *Per1* knockout mice, cocaine injection caused no sensitized behavioral response, which means lacking of cocaine reward,

while *Per2* knockout mice exhibited a hypersensitized response to cocaine, this fact also prove another no redundant function of Per1 and Per2 (Abarca et al., 2002).

1.5 E3 ligases involved in maintaining circadian rhythms

Ubiquitin is a small universal protein that exists in all eukaryotic cells. Ubiquitination is a covalent posttranslation modification involved in thousands of cellular pathways and diseases processes(Pickart and Eddins, 2004). Ubiquitination has been found to regulate the stability of core clock components and other clock protein function(Stojkovic et al., 2014). Mutation of genes encoding ubiquitin E3 ligases can either lengthen or shorten circadian period. Table 1. Listed all the E3 ligase involved in circadian rhythms.

The transfer of ubiquitin to a specific substrate needs the coordinated work of a group of enzymes known as ubiquitin activation enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). Ubiquitin is activated in an ATP-dependent pattern by E1, transferred to E2 and with the help of an E3 enzyme, ubiquitin is attached to a specific substrate through the ε-amino group of lysine residue (Figure 1-9). Ubiquitinated proteins are recognized and degraded by the 26s proteasome, which consists of a proteolytic core catalytic complex (20s) and two 19s regulatory subcomplexes binding to each end of the 20s cylinder. Tetra-ubiquitin chain is the minimum signal for efficient proteasome targeting, which is recognized by the 19s subcomplex (Thrower, 2000).

Substrates can be modified by one molecular of ubiquitin in one or more sites or added in the form of chains of ubiquitin molecules. Multi-ubiquitin chain, in which the carboxyl-terminal glycine of ubiquitin is linked to Lys48 of the preceding ubiquitin (K48-G76), can be appended to protein targeting for destruction by the proteasome (Hochstrasser, 1996). However, monoubiquitination is a regulator of the location and activity of diverse proteins. Beside degradation, there are other possible consequences after ubiquitination, which are dependent on the substrate subcellular localization and the number and topology of the substrate-conjugated ubiquitin. For example, K63-G76 isopeptide bonds formed ubiquitination chain is involved in DNA repair. However, much less is known about the functions of ubiquitination chains linked by K6, K27, K33, and also forked branches formed by different ubiquitin lysines (Figure 1-10) (Woelk et al., 2007).

Ubiquitination is a reversible protein modification. The human genome encodes about 79 deubiquitinase (DUBs) that are predicted to be active with opposite function of E3 ligases. They can be divided into five families: 1) Ubiquitin C-terminals hydrolases (UCHs); 2) Ubiquitin-specific proteases (USPs); 3) Ovarian tumour proteases (OUTs); 4) Josephin family; 5) JAB1/MPN/MOV34 metalloenzymes. The overall functions of DUBs including: generation of free ubiquitins from multiple ubiquitin molecules (Ubiquitin can be transcribed from several genes, either single copy of ubiquitin fused to ribosomal proteins or polyubiquitin precursor proteins), editing ubiquitination codes on modified proteins, which may stabilize the substrate proteins and recycling ubiquitin from degradative machines (Komander et al., 2009). Interestingly a deubiquitinase USP2 show rhythmicity in most examined tissues and multiple ubiquitinated core clock proteins are identified as its substrates (Table 2). Recently another deubiquitinase HAUSP is involved in regulating DNA damage induced circadian phase resetting *via* stabilizing CRY1 (Papp et al., 2015).

1.5.1 Classification of E3 ligases

E3 ligases are at the center of ubiquitination-coded signals, because E3 ligases recognize primary sequence motifs in the substrates for ubiquitination. Two different types of E3 ligases exist, either really interesting new gene (RING) or homologous to the E6AP carboxyl terminus (HECT) families have been identified. The E3 ligases of the homologous to the HECT domain family, recruit E2-ubi to generate E3-Ubi thioester intermediate with the active-site cysteine of the E3 ligase. However the RING family members recruit substrate and bind the E2 through a RING finger domain and directly transfer the ubiquitin from E2 to lysine residues on substrates (Metzger et al., 2012). Members of the RING finger family can function as monomer or multi-subunit complexes. Multi-subunit RING domains are exemplified by the cullin RING ligase super family, which includes the SCF complex. F-box proteins are the substrates specificity recognition subunits, which recognized phosphorylated motifs (Figure1-11).

1.5.2 Role of β-TrCP in circadian rhythm

The F-box proteins identified in controlling clock proteins' turn over are FBXL3, FBXL21, β-TrCP1/2. FBXL3 and FBXL21 both are engaged in CRY ubiquitination, however based on subcellular location FBXL21 antagonized FBXL3 E3 ligases activity in the nucleus, therefore $Fbxl3^{-/-}$ mice have an extremely long period phenotype while $Fbxl21^{-/-}$ mice have a slightly shorter period (Hirano et al., 2013; Yoo et al., 2013). β-TrCP1 and β-TrCP2 are F-box component of SCF E3 ligase complexes, which are responsible for Per2 ubiquitination (Ohsaki et al., 2008; Reischl et al., 2007). *Slimb* gene in *Drosophila* is the homolog of *β-TrCP1* and *β-TrCP2*, slimb mutants are behaviorally arrhythmic, with hyperphosphorylated Per and Tim proteins accumulated and degradation pathway impaired (Grima et al., 2002). Although down regulation of β-TrCP1 lengthens the circadian period in fibroblasts, β-TrCP1 heterozygous and homozygous knockout mice show no differences with wild type mice in locomotion activity (Ohsaki et al., 2008; Reischl et al., 2007), moreover, in NIH3T3 cells knockdown both *β-TrCP1* and *β-TrCP2* with siRNAs did not change mPer2-luc-PEST reporter circadian period length, only dampened oscillation amplitude(Ohsaki et al., 2008). The explanation hypothesis is that: 1). β-TrCP1 and β-TrCP2 have redundancy activity and when β-TrCP1 is absent, β-TrCP2 takes over the job to ubiquitinate Per2; 2) There is β-TrCP1 and β-TrCP2 independent pathway for Per2 degradation, which sustaining Per2 protein turn over from β-TrCP1 and β-TrCP2 disturbance. Interestingly, mPer2 protein is highly ubiquitinated even in the absence of CKI ε/δ(Akashi et al., 2014), that is conflict with the hypothesis that Per2 degradation is dependent on CKI phosphorylation subsequently ubiquitinated by β-TrCP1 and β-TrCP2. What's more the E3 ligase or the degradation pathway hasn't been resolved for heme dependent degradation of Per2 (J. Yang et al., 2008).

1.5.3 The E3 ligase Mdm2

The murine double minute 2 (*Mdm2*) gene was originally isolated and characterized with other two genes (*Mdm1* and *Mdm3*), that are amplified and overexpressed more than 50-fold in the 3T3DM cells (Fakharzadeh et al., 1991). 3T3DM is a tumorigenic mouse cell line with acentric chromatin bodies termed double minutes (DMs). Mdm2 Protein was shown to be responsible for transformation of the cells when overexpressed (Fakharzadeh et al., 1991). Before Mdm2 was identified, this chromosomal abnormalities correlates with amplification of cellular oncogenes in some tumor and tumor cell lines, such as *cKi-ras* and *myc* gene family (Cahilly-Snyder et al., 1987).

Human Mdm2 comprises multiple functional domains, signal sequences and sites of modification (Figure 1-12). The N-terminal region is essential for interacting with, and inhibiting p53 transcriptionally active. Nuclear localization signal and nuclear export signal locates between p53 binding region and central acidic domain. The central acidic domain plays critical role for regulating p53 ubiquitination. Zinc-finger domain follows central acidic domain, which is responsible for interaction with various proteins, including nucleolar proteins(Karni-Schmidt and Lokshin, 2016). The C-terminus of Mdm2 comprises a RING domain that is critical for E3 ligase activity. In particular, the zinc coordination residues C436, H455, C459, C464 and C473 dictate the formation of the RING fingers and are essential for E3 activity (Honda et al., 1998; Kannemeier et al., 2007).

Mdm2 is a multifunctional oncoprotein, best understood as E3 ubiquitin ligase responsible for the ubiquitination and degradation of p53(Haupt et al., 1997; Honda et al., 1998; Kubbutat et al., 1997). However Mdm2 mediated p53 monomeric ubiquitination on multiple lysine residues or polymeric ubiquitination is dependent on Mdm2 and p53 ratio *in vitro*(Li et al., 2003) or different signals (e.g. DNA damage) and other regulatory factors (e.g. MDM4 and p300) *in vivo* (Brooks and W. Gu, n.d.; Grossman, 2003; Grossman et al., n.d.; Marine et al., 2006). Interestingly, transcription factor ATF4, a component of the mammalian circadian clock, induced the circadian expression of p19ARF. p19ARF circadian oscillation binding pattern with Mdm2, consequently fluctuated p53 amount during day/night (Horiguchi et al., 2013). P53-independent functions of Mdm2 are revealed by interaction with other protein. Mdm2 binds to and ubiquitinates the Retinoblastoma protein (Rb). Rb degradation releases E2F1 transcription factor and promotes cell cycle progression (Hickman et al., 2002; Sdek et al., 2005; Uchida et al., 2005). Mdm2 also cooperates with Akt to promote androgen receptor (AR) ubiquitination and degradation (Lin, 2002).

There are two different promoters (P1 and P2) control *Mdm2* expression, the full-length p90 Mdm2 is generated from P1, while the shorter protein p76 Mdm2 is generated from P2, lost half of N-terminal p53 binding domain, thus cannot bind p53 and in fact antagonize p90 Mdm2 function. P2 promoter is responsive to both ionizing and UV radiation, which is dependent on p53 (Perry, 2004). Regarding to DNA damage response, ATM kinase phosphorylate Mdm2 on serine 395 and impairs the degradation and nuclear export of p53 , stabilized p53 promotes Mdm2 transcription from P2 promoter (de Toledo et al., 2000; Khosravi et al., 1999; Maya et al., 2001a). Recently, new study shows that DNA damage induced ATM and CKI phosphorylate Mdm2, which promotes β-TrCP ubiquitinates Mdm2 for degradation (Z. Wang et al., 2012). *Mdm2* is transcriptionally up-regulated by p53, however Mdm2 not only inhibits p53 activity and tightly controls p53 function by ubiquitinatination, but also ubiquitinating itself and induce its own degradation. Thus, p53 and Mdm2 forms an auto-regulatory feedback loop.

1.6 Innovation

p53 was claimed to repress BMAL1-CLOCK mediated transcription of m*Per2* and explained why wild-type mouse embryonic fibroblast (MEF) cells treated with γ-irradiation displayed a rapid decline in mPer2 transcripts (Miki et al., 2013). However in PER2::LUC MEF cells, methyl methane sulfonate (MMS) and UV-mediated DNA damage caused an acute decrease in luciferase levels and resulted in circadian phase advanced (Gamsby et al., 2009). This fact displays that DNA damage induced circadian phase resetting may rely on transiently regulation of Per2 protein stability. It is

noteworthy that all the studies about circadian phase resetting induced by DNA damage, the phasing is mostly consistent with larger advances seen when negative regulator is being synthesized (Gamsby et al., 2009; Oklejewicz et al., 2008; Papp et al., 2015; Pregueiro et al., 2006). All the examples listed above show the negative regulators especially Per2 plays a central role in transmitting signals from DNA damaged induced cell cycle checkpoint pathways to circadian rhythm pacemakers. But the molecular mechanisms are largely unknown. This thesis focus on identify new ubiquitin E3 ligases, which affect circadian rhythms by regulating Per2 stability and reset circadian phase by poising Per2 in different sensitive statuses to receive cell cycle checkpoint signals dependent on PER2 circadian phase. Moreover part of the collaboration work also shows Per2's role in controlling p53 stability and regulating p53 dependent checkpoint pathway under stressed condition.

Innovation in this project rests in: i) it is the first time at the protein level to demonstrate circadian component and cell cycle regulator influence each other functions by modulating posttranslational modification; ii) provide the molecular mechanisms to explain DNA damage induced circadian phase advance; iii) solving the puzzle about how a slowly ticking circadian rhythm system assistants cell cycle regulators rapidly response to stress by introducing the new concept that Per2 has circadian pool and "cell cycle" pool, which functioning partial independently. Our project will not only provide new therapeutic targets for circadian disorder, like insomnia, obesity, but also will bring far-reaching consequences for cancer interventions.

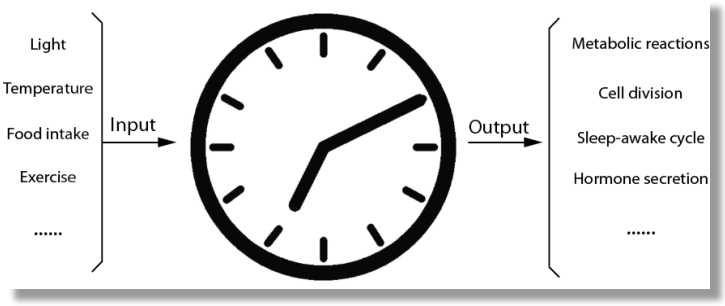
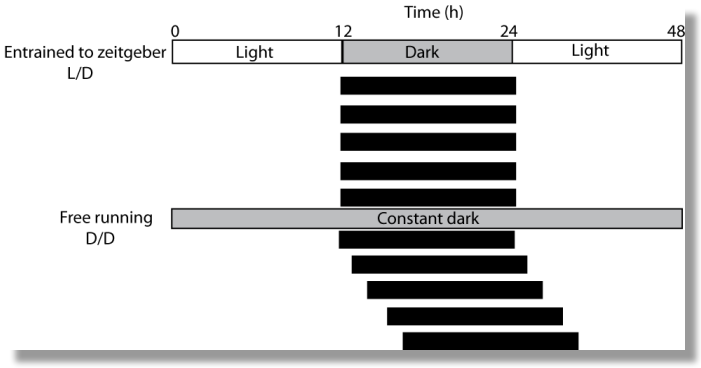


Figure 1-1. The conceptual model for circadian regulatory.

The conceptual model for circadian regulatory system. The central pacemaker generates an approximate 24-hour periodicity, which is entrained under natural conditions by input pathways and regulates many different physiological processes via output pathways.

A.



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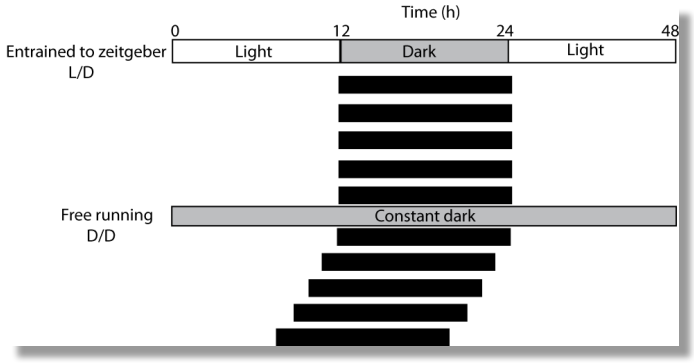

Figure 1-2. Circadian rhythm entrainment to light.

Under entrained conditions, circadian rhythms adjust their endogenous period to that of the zeitgeber. The actogram represents the rhythm of activity initially entrained to a 24 h L/D cycle, after transfer to constant dark condition, A). The activity onset shifts to right if endogenous circadian rhythm is longer than 24h; B). The activity onset shifts to left if endogenous circadian rhythm is shorter than 24h.

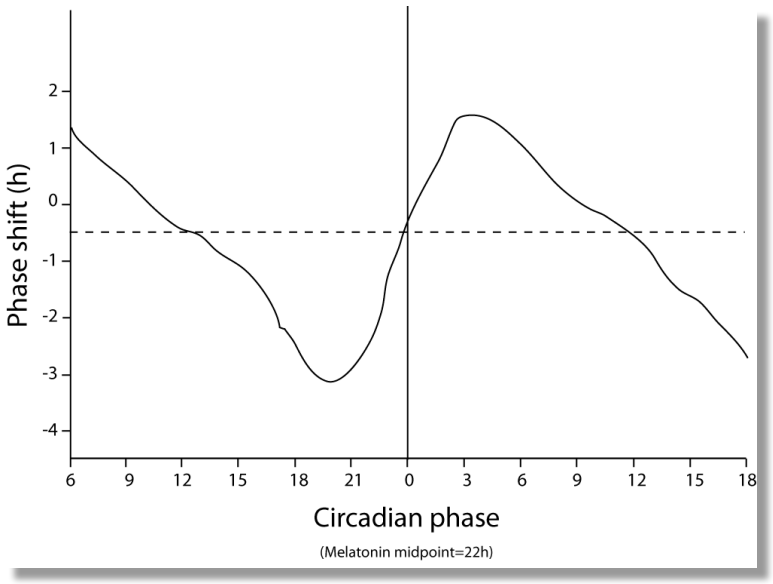

Figure 1-3. The PRC to the bright light stimulus using plasma metatonin as the circadian phase marker.

Subjects remained in an environment free of usual time cues and entrained under a pre- and post- stimulus constant routine (CR) in dim light (~2-7lx), the 6.7 h bright light stimulus consisted of alternating 6 min fixed gaze (~10000lx) and free gaze (~5000-9000lx). Different subjects were scheduled light exposures at different circadian time. Phase advances (positive values) and delays (negative values) are plotted against the timing of the center of the light exposure relative to the melatonin midpoint on the pre-stimulus CR (defined to be 22h) (Data from Khalsa et al., 2004).

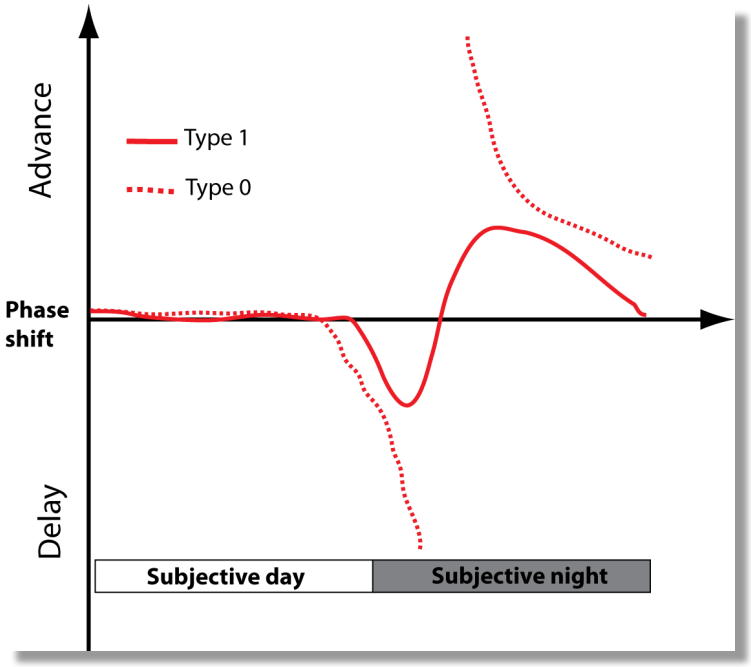

Figure 1-4. Types of phase response curves.

Strong PRC (type 0), larger amplitude changes with a breaking point. Weak PRC (type 1), small amplitude change response to the stimulus and continued between delay and advance

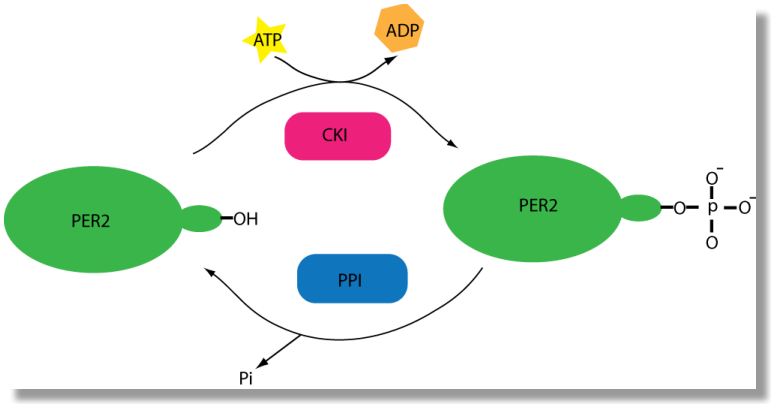

Figure 1-5. Casein Kinase I phosphorylated Per2 is a substrate for protein phosphatase I.

Casein kinase I (CKI) add phosphoryl groups to PER2 serine/ threonine residues depending on ATP, which can be removed by protein phosphatase I (PP1) (data from Gallego et al., 2006).

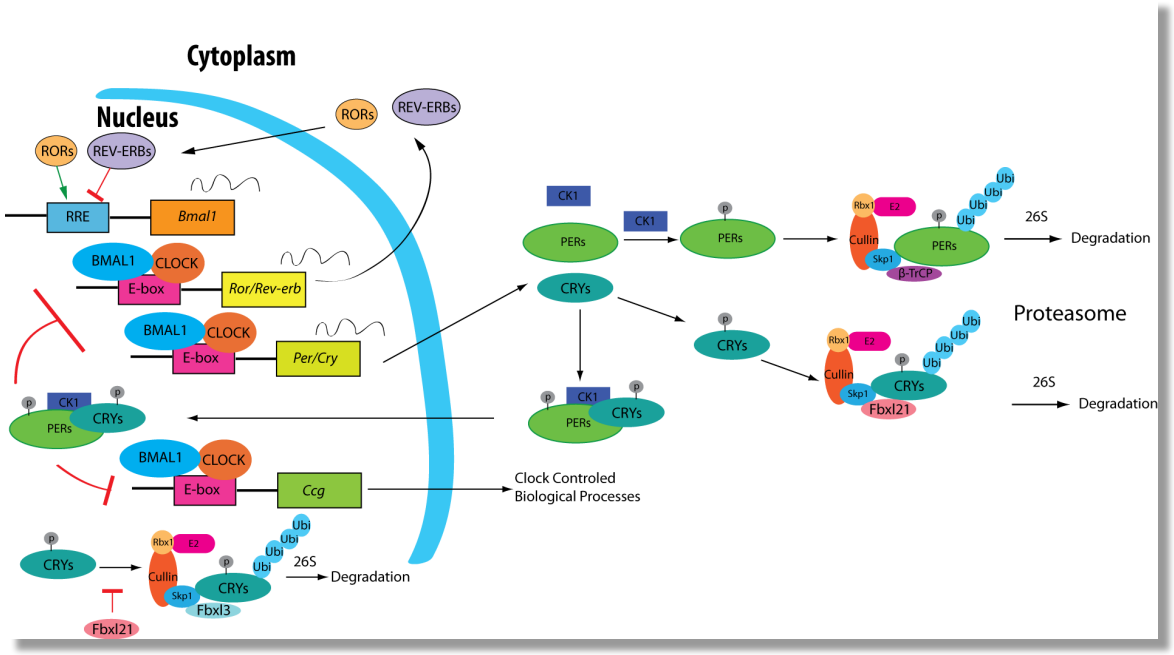

Figure 1-6. The mammalian core clock components construct a transcriptional-translational feedback loop.

The mammalian circadian core clock transcription-translation loop composed two CLOCK/BMAL1 drives the expression of their negative regulator Cry and Per. Per and Cry accumulates in the cytoplasm where Per are phosphorylated by CKI kinase, and this complex translocates back to the nucleus inhibitor the activity of CLOCK/BMAL1 heterodimer. The Per and Cry proteins are degraded in CKI-dependent manner, which release the repression of CLOCK/BMAL1. After phosphorylation Per and Cry are recognized by specific E3 ligase F-box subunits, β-TrCP and Fbxl21 respectively, undergoing ubiquitination dependent degradation. Moreover another F-box protein Fbxl21 seems stabilized nuclear Cry by competing with Fbxl3. The stabilizing feedback loop consists of BMA1's activator ROR and the inhibitor REV-ERB that control BMAL1 expression. Modified from (Takahashi et al., 2008).

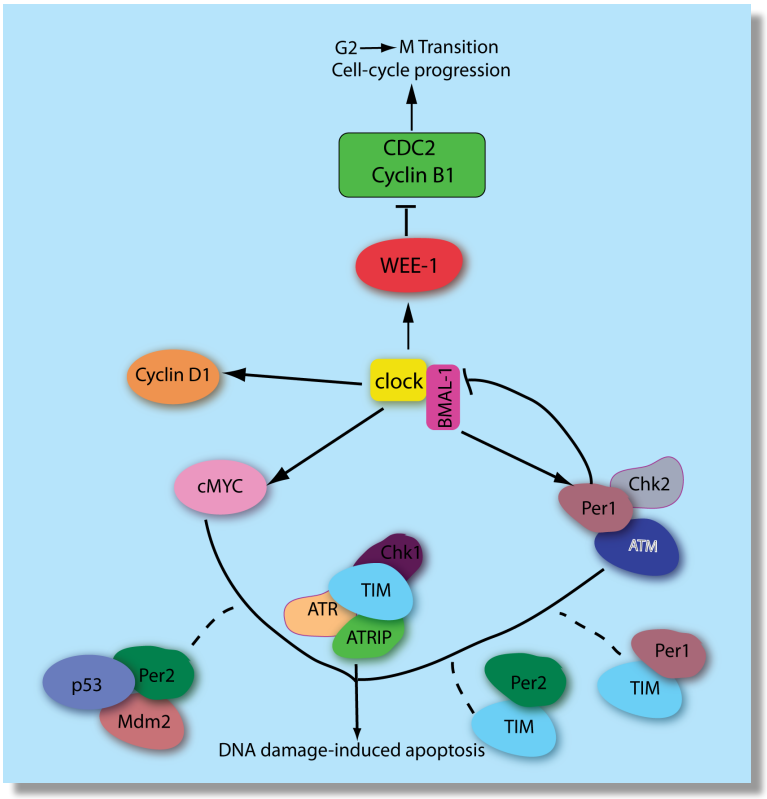

Figure 1-7. Multiple connections between the cell cycle and the circadian clock.

The CLOCK/BMAL1 heterodimer drive the expression of Wee-1, the kinase that phosphorylated CDC2/Cyclin B1 complex resulting in inhibition of G2/M transition. Cyclin D1 and c-Myc are activated by CLOCK/BMAL1 and control cell cycle progression directly. PER1 and PER2 are another two well-known circadian negative regulators, which may form complex with Chk2 and ChK1 respectively. PER2 might connect with Chk1 by TIM and also physically binding with p53 and Mdm2. Dash lines show the functions of these complexes are meager.

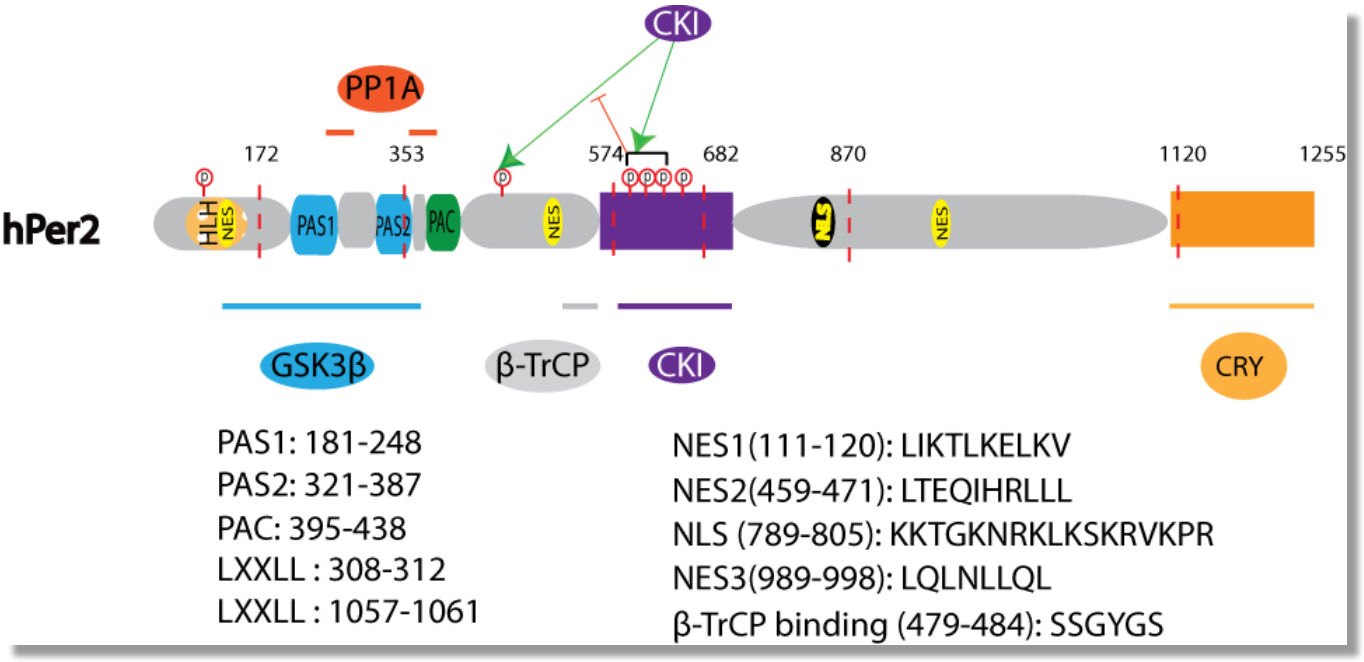

Figure 1-8. Structural domains and functional motifs in human Per2 protein.

From N-terminal to C-terminal: Helix-loop-helix motif (HLH); Nuclear export sequence (NES); PAS domains including: PAS A (PAS 1), PAS B (PAS 2), and PAC; LXXLL motif for interaction with nuclear receptors not show on the found from amino acid 308-312 and 1057-1061; Other protein binding regions including GSK3β, PP1A, β-TrCP, Casein kinase I (CKI) and CRY are shown as labeled. DBT, the CKI homolog in *Drosophila* also has multiple phosphorylation sites on dPer2, interestingly *Drosophila* Per-short cluster phosphorylation delay other DBT dependent phosphorylation site (Chiu et al., 2011). Here we show CKI phosphorylation sites by arrow, red bar shows possible human Per-short cluster.

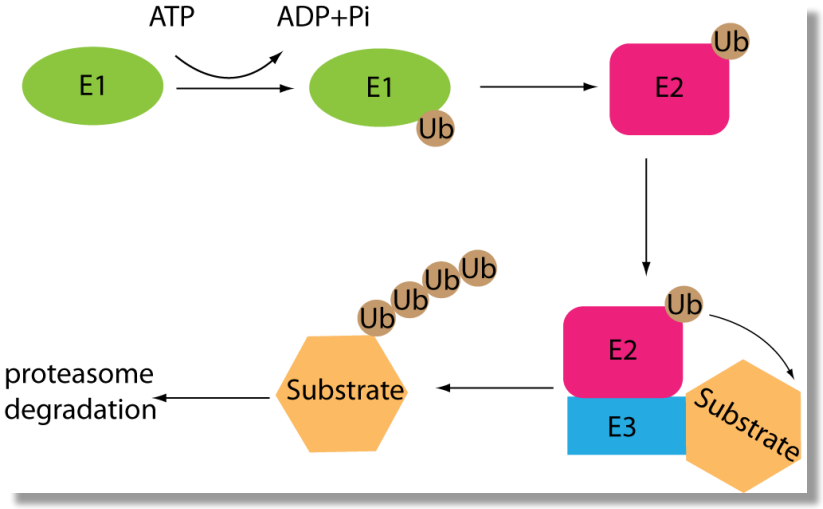

Figure 1-9. The ubiquitin/26s proteasome pathway.

Ubiquitin can be added to the substrates depending on E1, E2, and E3. The pathway begins with adenosine triphosphate (ATP)-dependent actiocation of Ub by an E1, followed by transfer of the activated Ub to and E2, and then final attachment of the activated Ub to the substrate with the help of an E3. The resulting product is an Ub-protein with C-terminal Gly carboxyl group of ubiquitin linked by an isopepteide to Lysine ε-amino group; more Ub will be added to the substrated-linked ubiquitin if polyubiquitination chains are formed. Polyubiquitinated substrates are recognized by 26s proteasome undergoing degradation (Pickart et al., 2004).

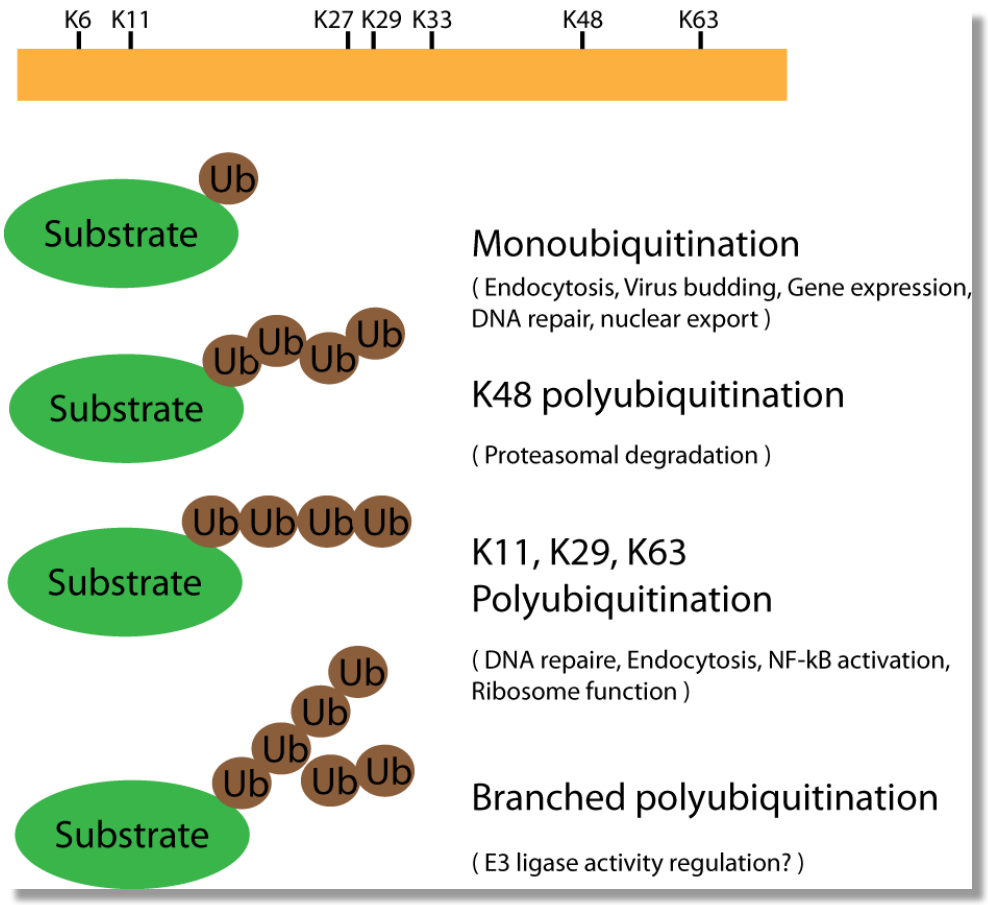

Figure 1-10. Schematic representation of the different Ubiquitination modifications and correlated functions.

Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), which are utilized for ubiquitination chains with different branches. Monoubiquitination or multi-monoubiuqitination is important for protein interaction, localization and regulating protein activity. Polyubiquitination chains formed by K48 are targeting to 26s proteasome for degradation, while K11, k29, and K63 linked polyubiquitination chains are involved in cell signaling and DNA repair. However, fork branched Ubiquitnationa chain function is still not clear.

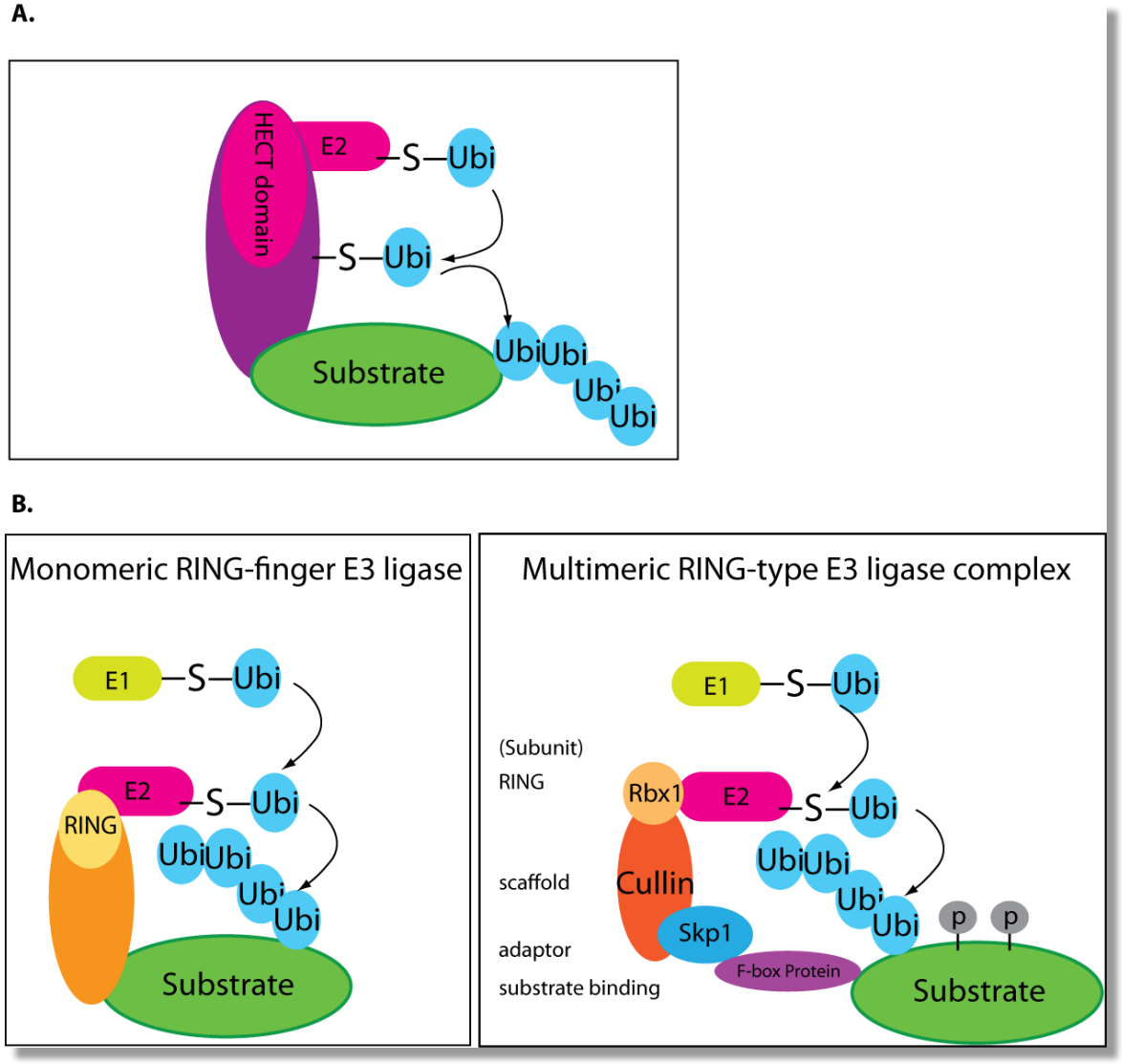

Figure 1-11. Schematic representation of E1/E2/E3 ubiquitin ligase complexes.

A. HECT family members have a HECT domain that binds to E2 and transfer ubiquitin to its active domain before conjugate to its substrates. B. RING finger family members have RING finger domains that bind to E2 enzymes and can be categorized into monomeric and multi subunit types. The mluti-subunit RING domains are exemplified by the cullin RING ligase (CRL) superfamily. There are seven cullins identified in mammals. Different collins utilizes the same Rbx (containing RING domain), as the adaptor to recruit E2, but use different adaptors (e.g. Skp1, BTB, ElonginB/C) to recruit distinct classes of substrate binding receptors (e.g. F-box proteins, WD-40 domain containing proteins).

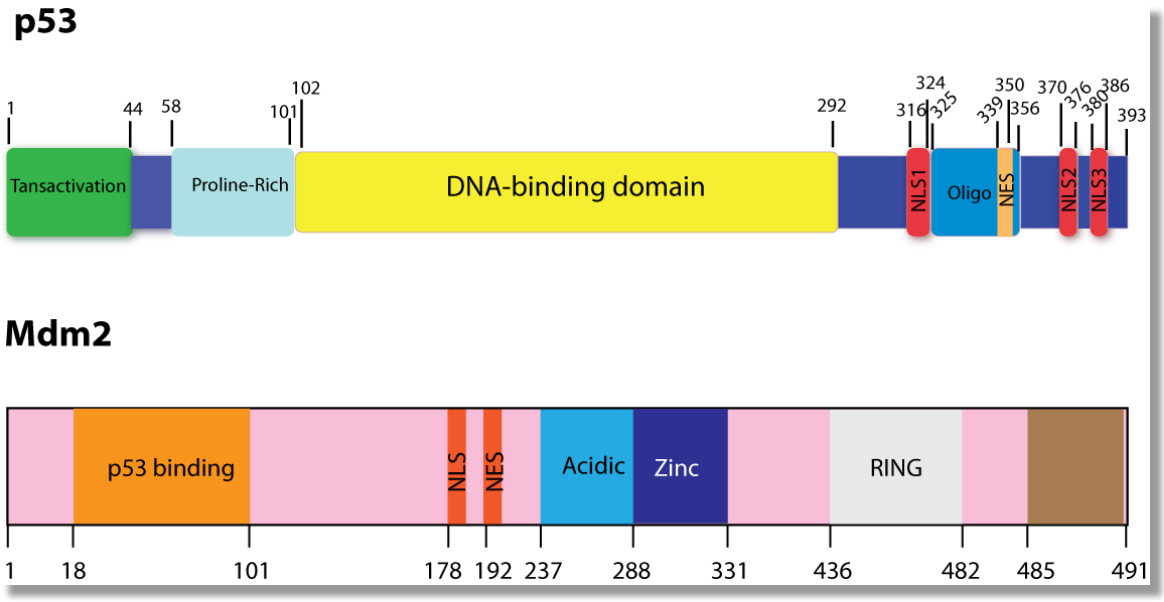

Figure 1-12. Structural domains and functional motifs of p53 and Mdm2.

The N-terminal region of p53 contains transactivation domain (1-44), which binds to Mdm2 and Proline-rich region with five PXXP motifs that are essential for inducing apoptosis; the central core domain (102-292) spans DNA-binding domain. The majority of missense mutations of *p53* gene are located within this domain. Three nuclear localization signal and one nuclear export signal is located in C terminus. The carboxyl terminus of p53 (325-356) also contains the tetramerization domain, which labeled as oligo here. N-terminal of Mdm2 spans p53 binding region (18-101); nuclear localization sequence and nuclear export sequence site right after p53 binding domain; Acidic domain that binds ARF located from residue 237 to 288; Zinc-finger motif, where ribosomal proteins interact (289-330); RING-finger domain, which is important for Mdm2 E3 ligase function, is located near C-terminus (436-482); the carboxyl terminus tail (485-491) regulates RING domain *via* Mdm2 homodimerization or heterodimerization.

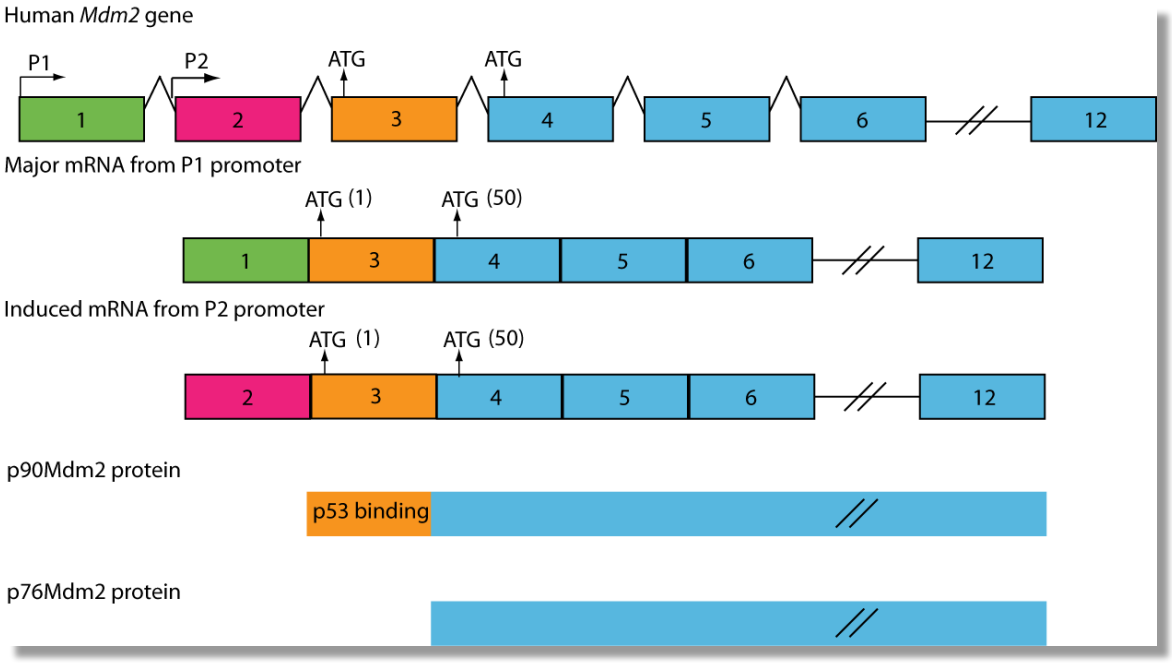

Figure 1-13. The *Mdm2* gene and two major Mdm2 proteins.

Mdm2 gene contains 12 exons and 2 promoters. P1 is a constitutive promoter and P2 is a p53-responsive promoter. Promoter 1 located upstream of exon1 while promoter 2, which contains two p53 binding sites, spans in intron1. The human Mdm2 transcripts from the P1 promoter are spliced to lack exon2. Those from P2 promoter contain exons 2-12. Full length mRNA from either P1 or P2 promoter can synthesis of both p90Mdm2 and p76Mdm2. p76Mdm2 protein is translated from alternative splicing mRNA which lacks exon 3, losing p53 binding sites. Transcripts form P2 promoter increase p76Mdm2 protein ratio.

Table 1-1 E3 Ligase involved in ubiquitination clock proteins (Modified from Stojkovic et al., 2014).

Clock protein	Mammalian E3 ligase	Phenotype of loss of function or knock-out/down E3 ligase	References
CRY1/2	FBXL3	Long free-running period of locomotor activity rhythms and long reporter luminescent period in cultures fibroblasts and SCN; stabilized CRYs; dampened and delayed rhythms of *Per* and *Cry* mRNA; phenotype partly rescued by *Fbxl21* loss of function.	(Godinho et al., 2007) (Siepka et al., 2007) (Hirano et al., 2013) (Yoo et al., 2013)
	FBXL21	Normal or short free-running period of locomotor activity rhythms; short reporter luminescent period in cultures fibroblasts, SCN and pituitary; destabilized CRYs in cytoplasm while stabilize in nucleus; mutation partly *rescues* Fbxl3 loss of function.	(Dardente et al., 2008) Hirano et al., 2013 Yoo et al., 2013
PER1/2	β-TrCP1 β-TrCP2	Dampened or lengthen reporter luminescent period in fibroblasts; stabilized PERs; β-TrCP1 KO mice have no circadian phenotype.	(Eide et al., 2005b) (Shirogane, 2005) (Reischl et al., 2007) (Ohsaki et al., 2008)
REV-ERBα	HUWE1	Stabalized REV-ERBα, decreased Bmal1/Cry1 expression in knock-down both HUWEI and PAM condition	(Yin et al., 2010)
	PAM	Stabalized REV-ERBα, decreased Bmal1/Cry1 expression in knock-down both HUWEI and PAM condition	Yin et al., 2010
BMAL1	UBE3A	Dampening and longer reporter luminescent period in cultured fibroblasts	(Gossan et al., 2014)

Table 1-2. Deubiquitiating enzymes involved in circadian clock core proteins (Modified from Cermakian et al., 2014).

Clock protein	Deubiquitinase	Phenotype of knock-down/inhibitor treated	Reference
CRY1	USP2	Decreased CRY1 protein levels in liver with *Usp2* knock-down	(Tong et al., 2012)
	USP7/HAUSP	Reduced CRY1 ubiquitination and stabilize CRY1 in cultured fibroblasts; lengthened luminescent reporter period by knocking down *Hausp*; altered circadian phase shifting after DNA damage by HAUSP inhibitor.	(Papp et al., 2015)
PER1	USP2	Slightly elongated free-running period of locomotor activity rhythms; altered response to light; increased levels of ubiquitinated PER1 in fibroblasts; not change in PER1 stability; altered in the time of PER1 intracellular localization	(Y. Yang et al., 2012) (Y. Yang et al., 2014)
BMAL1	USP2	Normal free-running period and slightly altered light response; reduced BMAL1 levels in the SCN.	(Scoma et al., 2011)

CHAPTER 2: The circadian factor Period 2 modulates p53 stability and transcriptional activity in unstressed cells

2.1 Abstract

2.2 Introduction

2.3 Materials and methods

2.4 Results

2.5 Discussion

(See Appendixes)

CHAPTER 3: Association of the circadian factor Period 2 to p53 influences p53's function in DNA-damage signaling

3.1 Abstract

3.2 Introduction

3.3 Materials and methods

3.4 Results

3.5 Discussion

(See Appendixes)

CHAPTER 4: The Mdm2 E3 ligase modulates Per2 stability and circadian period

4.1 Abstract

The mammalian circadian clock is sustained by the interplay of positive and negative transcriptional feedback loops, for which the turnover rate of CRY and PER proteins influences the clock's period length. A regulatory mechanism that controls protein stability is given by the proteasome pathway, which uses F-box β-TrCP1/2 E3 ligases to mark PER1 and PER2 substrates for degradation *via* ubiquitination. We know now that β-TrCP1/2 ligases selectively recognize phosphorylated degradation motifs (degron) in target substrates. Accordingly, overexpression of β-TrCP1/2 result in decreased phospho-PER2 levels; unexpectedly, overexpression of the dominant negative forms of the E3 ligases led to a decrease of non-phosphorylated PER2. Our results indicate that PER2 ubiquitination engages two different types of E3 ligases that timely control PER2 accumulation in the nucleus. We report that the mouse double-minute 2 homolog (Mdm2) RING finger E3 ligase binds to PER2 in multiple regions including an overlapping site with β-TrCP. We determined that non-phosphorylated PER2 is a specific substrate for Mdm2 and that Mdm2-mediated PER2's polyubiquitination occurs both *in vitro* and in cells. Accordingly, overexpression of Mdm2 impacts PER2 half-life, whereas, down-regulation of this ligase by siRNA expression results in PER2 stability. Furthermore, we found Mdm2-dependent post-translational modification of PER2 directly influences circadian period length in MEF:LUC cells. Overall, our findings support a model in which, the degradation rate of PER2 during its accumulation phase in the nucleus is strictly controlled by the interplay between Mdm2 and b-TrCP E3 ligases; however, the balance is shifted toward b-TrCP-mediated PER2 degradation as its phosphorylated form accumulates over time. As a result, the levels and activity of these ligases play a determinant role and influence period length through a direct target on a key player of the negative feedback loop.

4.2 Introduction

Circadian rhythm is a biological mechanism that regulates diverse organisms coordinating physiology and behavior with daily dark-light cycle and thereby optimizing the time of biological processes(Lowrey and Takahashi, 2004).The main clock pace maker is located in the suprachiasmatic nucleus (SCN), comprising about 10,000 neurons, receiving light input from the retina and synchronize all the peripheral clocks by endocrine and systemic cues (Buhr et al., 2010; Partch et al., 2014; Welsh et al., 2010). Beside controlled by central clock, peripheral clocks are susceptible to local metabolic status or tissue specific signals (Balsalobre et al., 2000a; Partch et al., 2014; Stratmann and Schibler, 2006). For example, although the rhythmicity in the SCN remained the same, liver cells circadian rhythm can be entrained by restricted feeding (Stokkan, 2001). However, both central and peripheral clocks share the same molecular mechanism to sustain circadian oscillation autonomously. Self-sustained circadian rhythm is controlled by transcription-translation feedback loop (TTFL), which including two positive activator CLOCK and BMAL1, drive expression of the negative regulators *Period* (*Per*1, *Per*2, *Per*3) and *Cryptochrome* (*Cry*1, *Cry*2) genes. PERs and CRYs proteins in turn interact, translocate into nucleus and inhibit BMAL1-CLOCK transcriptional activity. PERs and CRYs are cleaned out by ubiquitination-proteasome dependent degradation, resulted in releasing their repression on BMAL1-CLOCK(Ko, 2006; Shearman et al., 2000b).

Although the concept of transcription-translation feedback loop is well accepted, the molecular mechanisms that define the periodicity of the circadian clock are still unclear (X. Zheng and Sehgal, 2012). Posttranslational modifications regulate the negative regulators PER and CRY stability and turnover rates, which correlate with the period length of circadian rhythms in mutant mice or cell lines. For example, CRY proteins are the substrates of two F-box proteins: FBXL3 and FBXL21. Surprisingly, FBXL3 mainly promotes CRY proteins ubiqutination and degradation while FBXL21 competes with FBXL3 to stabilize CRY proteins. This result is verified by long-period phenotype in *Fbxl3* knockout mice was attenuated in *Fbxl3/Fbxl21* double knockout mice (Hirano et al., 2013; Yoo et al., 2013). The first mutation has been found with dramatically short period of circadian locomotor rhythm in golden hamster is occurred at *tau* gene locus (Ralph and Menaker, 1988). Later on, the molecular identification of the *tau* locus is encoded by casein kinase I ε (CKIε), a kinase for phosphorylation PER proteins(Lowrey et al., 2000). Phosphorylated PERs are recognized by F-box proteins β-TrCP1 and β-TrCP2, which are responsible for Per2 ubiquitination (Ohsaki et al., 2008; Reischl et al., 2007). Although down regulation of β-TrCP1 lengthens the circadian period in fibroblasts, β-TrCP1 heterozygous and homozygous knockout mice show no differences with wild type mice in locomotion activity (Ohsaki et al., 2008; Reischl et al., 2007), moreover, in NIH3T3 cells knockdown both *β-TrCP1* and *β-TrCP2* with siRNAs did not change mPer2-luc-PEST reporter circadian period length, only dampened oscillation amplitude(Ohsaki et al., 2008). The explanation hypothesis is that: 1). β-TrCP1 and β-TrCP2 have redundancy activity and when β-TrCP1 is absent, β-TrCP2 takes over the job to ubiquitinate Per2; 2) There is β-TrCP1 and β-TrCP2 independent pathway for Per2 degradation, which sustaining Per2 protein turn over from β-TrCP1 and β-TrCP2 disturbance. Interestingly, mPer2 protein is highly ubiquitinated even in the absence of CKI ε/δ(Akashi et al., 2014), that is conflict with the hypothesis that Per2 degradation is dependent on CKI phosphorylation subsequently ubiquitinated by β-TrCP1 and β-TrCP2.

Mdm2 was originally found as an amplified gene on murine double minute chromosome in transformed mouse 3T3 cell line. It has been characterized extensively for its function in regulating p53 transcription and stability. Here we report Mdm2 as a new E3 ligase for Per2. We describe that this RING finger E3 ligase dependent polyubiuqitination plays a pivotal role in modifying Per2 stability and cooperates with F-box E3 ligase β-TrCP regulating circadian period in cultured MEF cells.

4.3 Materials and Methods

Cell culture and transfections

The human colorectal carcinoma-116 (HCT116), and human non-small cell lung carcinoma-1299 (H1299) cell lines were purchased from the American Type Culture Collection (ATCC) and maintained according to manufacturer's recommendations. MEF cells were a gift from Dr. Shihoko Kojima and cultured in Dulbecco's Modified Egale's Medium (DMEM, MT 10-013-V, Corning) supplemented with 10% FBS (35-010-CV Corning), 50 units/ml penicillin, 50 ug/ml streptomycin and maintained at 37°C in a 5% CO_2 environment. When cells were cultured in Lumycycle for luminescence measurements, the same clulture conditions were maintained except DMEM was without phenol red, 2% FBS was added and 0.05mM luciferin was added. For transfection experiments, cells were seeded in 6- or 12-well plates until they reached 50-80% confluence. Transfections were optimized using Lipofectamine LTX (Life Technologies) for HCT116 and H1299 following manufacturer's instructions. Otherwise, transfections in all cell lines were in HyClone HyQ-RS reduced serum medium (Thermo Scientific) for 4 h for H1299 and HCT116. Proteins were then allowed to express at 37°C/5% CO2 in the appropriate media containing 10%FBS without antibiotics after which they were either collected or further synchronized. Extracts for protein analysis were prepared in NP-40 lysis buffer containing 10 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 80 mM β-glycerophosphate, 1mM Na3VO4, 10 mM NaF, and protease inhibitors (10 μM leupeptin, 1 μM aprotinin A, and 0.4 μM pepstatin).

Immunoprecipitation and immunoblot assays

For immunoprecipitation experiments, transfected cells were harvested in lysis buffer, and extracts (500 ug) were incubated with either α-FLAG M2 agarose beads (Sigma-Aldrich) or α-*myc* (9E10) beads (Santa Cruz Biotechnology, Dallas, TX) either for 2 h or over night at 4°C with rotation before washing. Where indicated, immunoprecipitations were carried out in a two-step procedure; with extracts being incubated with the antibody (α-FLAG, α-*myc*, or α-Per2) overnight at 4°C before the addition of protein A beads (50% slurry; Sigma-Aldrich). Sample beads were then washed four times with lysis buffer, resolved by SDS-PAGE, and analyzed by immunoblotting using specific primary antibodies (α-FLAG [Sigma-Aldrich], α-*myc* [Santa Cruz Biotechnology], α-Per2 [Sigma-Aldrich, Farmingdale, NY], α-Mdm2 [Santa Cruz Biotechnology], α- β-TrCP [Cell Signaling Technology], α-ubiquitin [Enzo Biomol]). For immunoprecipitation experiments of endogenous proteins, cells were harvested in lysis buffer, and extracts (~1 mg) were incubated with α-Per2 (Santa Cruz Biotechnology) overnight at 4°C before adding protein A beads (50% slurry; Sigma-Aldrich). Samples were then kept for additional 2 h at 4°C with rotation before washing and then processed as described. Horseradish peroxidase-conjugated α-Rabbit or α-Mouse IgG secondary antibodies (GE Healthcare Life Sciences, Buckinghamshire, UK; Cell signaling, Danvers, MA) were used for immunoblotting following standard procedures. Chemiluminescence reactions were performed using the SuperSignal West Pico Substrate.

In vitro binding assays

In vitro transcription and translation of pCS2+*myc*-hPer2, *myc*- β-TrCP1, FLAG-Mdm2, FLAG-β-TrCP1, Mdm2 were carried out using the SP6 high-yield TNT system (Promega) following the manufacturer's instructions, although, unlike the standard procedure, the reaction was cold. Aliquots (1-4µl) of indicated recombinant proteins were pre-incubated for 15 min at room temperature to allow the complex to form before adding NP40 lysis buffer. Immunoprecipitation of the various complexes was carried out essentially as described.

Protein pull-down assay

GST fusion proteins were expressed *in Escherichia coli* strain *Rosetta* (Novagen) and purified by glutathione-Sepharose chromatography based on the manufacturer's instructions (GE Healthcare Life Sciences). For pull-down assays, a total of 5 µg of GST-Per2 recombinant fragments (1-7)-bound beads, or an equivalent amount of glutathione beads (GST control) were washed in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100) and incubated with 4 µl of in vitro transcribed and translated [^{35}S]-FLAG-Mdm2 at 4^{o}C for 1 h. After washing of the beads with low- and high-salt binding buffer (100 mM and 1M NaCl, respectively), bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography. Mdm2 input was 3µl [^{35}S]-FLAG-Mdm2.

Ubiquitination assays

For in vitro assays, pCS2+ constructs of FLAG-hPer2, *myc*-Mdm2, *myc*-Mdm2, FLAG-hp53, or *myc*-hp53 were transcribed and translated in vitro as described. Aliquots of each tagged protein (1–4 µl), or a combination of them, were incubated at room temperature for 15 min to allow complex formation to happen before adding the reaction buffer containing 1× ubiquitination buffer (Enzo Biomol), 2 mM dithiothreitol, 20 µg/ml ubiquitin-aldehyde, 100 µg/ml ubiquitin or methylated ubiquitin (for polyubiquitination assay), 1xATP-energy regeneration system (5 mM ATP/Mg2+; Enzo Biomol), 40 µM MG132, and 1 mg/ml HeLa S100 lysate fraction (Enzo Biomol) to a final volume of 10 µl. Reactions were further incubated at 37°C water bath for 30 min before being terminated by the addition of lysis buffer. Diluted samples were incubated twice at 4°C for 1 h, first after addition of 3 µg of α-FLAG antibody (Sigma-Aldrich) and then after the addition of 7 µl of protein A beads (50% slurry; Sigma-Aldrich). Bound complex were washed four times with NP40 lysis buffer, resuspended in Laemmli buffer, and resolved by SDS–PAGE and immunoblotting.

For detection of in cell ubiquitination, HCT116 cells were transfected with pCS2+FLAG-hPer2 and either pCS2+*myc*-Mdm2, pCS2+*myc*-Mdm2 (C470A) or empty vector and maintained in complete medium for 24 h before the addition or not (–MG132) of 50 µM MG132 and ubiquitin aldehyde (5 nM). Cells were harvested 4 h later, lysates were immunoprecipitated with α-FLAG antibody as described, and the complexes were resolved in SDS–PAGE. Ubiquitinated complexes were detected by immunoblotting using an α-ubiquitin antibody.

Analysis of PER2 half-life

Extracts were from HCT116 cells treated with 100μg/ml cycloheximide. Protein levels were quantitated by immunoblot analysis using Image Lab (Bio-Rad) and values normalized to tubulin levels. The percentage of protein remaining was calculated based on $t = 0$, and the data were fitted using Prism software (GraphPad Software, La Jolla, CA).

Circadian synchronization

HCT116 cells were grown in McCoy'5A supplemented with 10% fetal bovine serum and penicillin. The serum shock was done as follows: Either siRNA Mdm2, β-TrCP1, both or mock for 48 h or seed cells 48h before serum shock. At time 0, the medium was exchanged with serum-rich medium (McCoy's 5A supplemented with 50% horse serum), and after 2 hr this medium was replaced with serum-free McCoy's 5A medium with penicillin. At the indicated times, cells were collected and kept at -80 ^{o}C until the extraction of whole cell lysate.

Lumycicle Luminescence measurement

MEFs were cultured in 35-mm Petri dishes until 100% confluent and then synchronized by dexamethasone (Balsalobre et al., 2000) for 2 hours in 37°C. Cells were washed with PBS and cultured in phenol red-free lumicycle medium. Culture dishes were sealed with coverslps and transferred to Lumicycle. At the indicated time points, treated the cells with irradiation or mock irradiation, drug or vehicle. Luminescence data were recorded and exported by Lumicycle data collection software. Period and phase was assessed by Lumicycle data analysis software and JMP by fitting to a model (Oklejewicz et al., 2008).

4.4 Results

Period 2 binds to the E3 ubiquitin ligase Mdm2. Our studies show that human Per2 (hPer2) binds p53 and Mdm2 in a stable trimeric complex that localizes in the nucleus (Gotoh et al., 2014). Interestingly, whereas Per2 interaction prevents Mdm2-mediated ubiquitination of p53 and controls p53's transcriptional activity, the relevance of Mdm2 for modulating Per2 levels and function when generating circadian rhythms remains unknown.

To investigate the relationship between Mdm2 and Per2, we first established their functional interaction in two separate *in vitro* scenarios in which p53 was incorporated or not in the system. First, *in vitro* transcribed and translated *myc*-hPer2 was incubated with either FLAG-Mdm2 or -β-TrCP1, an E3 ubiquitin ligase that targets post-translationally modified hPer2 for degradation, in the presence of a wheat germ cell extract as only source of co-factors and modifying enzymes (Figure 1A). Immunoprecipitation of complexes show Mdm2 directly associates to hPer2 in an event that was independent of Ser^{622} phosphorylation by casein kinase 1ε/δ as hPer2(S^{662}A) also associated to Mdm2 (Figure 1A). As expected, post-translational events facilitate the *in vitro* association of hPer2 to β-TrCP1 as well. Next, we evaluated whether binding occurred in cells lacking endogenous expression of p53. Because of low expression of endogenous Mdm2 in p53-deficient cell lines (p53 transcriptionally activates *MDM2* gene, ref), experiments using human non-small cell lung carcinoma and colon carcinoma cell lines deficient in p53 expression (NCI-H1299 $p53^{-/-}$ and HCT116 $p53^{-/-}$, respectively) were co-transfected with tagged forms of hPer2, Mdm2, Mdm2 dominant negative form Mdm2(C^{470}A), and β-TrCP1 (Figure 1B). Moreover, immunoprecipitation of endogenous hPer2 from Mdm2-transfected HCT116 $p53^{-/-}$ cells provided further evidence of the existence of the Per2:Mdm2 endogenous complex (Figure 1C). Overall, our data show that binding of hPer2 to either E3 ubiquitin ligase occurs even in the absence of p53 expression in cells suggesting a distinct regulatory role for the hPer2:Mdm2 complex.

To define the regions in hPer2 and Mdm2 responsible for their interaction, we first generated a number of GST-tagged recombinant constructs based on sequence homology, secondary structure prediction, and molecular modeling for each protein and determined their binding capacity to its full-length FLAG-Mdm2 counterpart. Each of the seven recombinant fragments of hPer2 [GST-hPer2(1-172), GST-hPer2(173-355), GST-hPer2(356-574), GST-hPer2(575-682), GST-hPer2(683-872), GST-hPer2(873-1120), GST-hPer2(1121-1255)] was incubated with in vitro transcribed and translated FLAG-Mdm2 and protein association was evaluated by pull-down assays and immunoblotting (Figure 1D). Results showed that Mdm2 interacts with the N-terminus of hPer2 and mainly within its central region comprising residues 356-574 (C-terminus of the PAS domain) and 683-872, a stretch of sequence predicted to be structurally flexible and strongly post-translationally processed (Albrecht et al., 2007; Gotoh et al., 2014). Conversely, the identification of the hPer2 binding region within Mdm2 was carried out using epitope mapping. Antibodies directed to conformational native epitopes in Mdm2 comprising residues 19-50, 154-167, and 383-491 (named Ab-5, SMP14, and 4B11, respectively) located within the p53 binding domain, a central unstructured region, and the C-terminus end of Mdm2 containing the RING domain, respectively were used in pull-down experiments. *In vitro* transcribed and translated FLAG-Mdm2(C^{470}A) was pre-incubated with either IgG (control) or each of the epitope antibodies (Ab-5, SMP14, and 4B11) before adding *myc*-hPer2. After complexes were allowed to form, samples were exhaustively washed and bound components were analyzed by immunoblotting (Figure 1E). Results show pre-incubation of

dominant negative Mdm2 with α-SMP14, but not α-Ab-5 or -4B11) prevents hPer2 binding. These results further support the existence of the hPer2:p53:Mdm2 complex as the binding sites for hPer2 and p53 in Mdm2 take place at different locations. In addition, because hPer2 binding does not involve the C-terminus RING domain in Mdm2, it is expected that the E3 ligase activity of Mdm2 would remain unaltered even when the complex is formed.

Mdm2 targets hPer2 for ubiquitination. Next, we evaluated whether binding of Mdm2 to hPer2 results in hPer2's polyubiquitination. *In vitro* transcribed and translated FLAG-hPer2 and *myc*-Mdm2 were pre-incubated and the complex allowed to form in the presence of an ubiquitin reaction mixture containing a source of E1 and E2 ligases, ubiquitin, an energy regenerating system, and the proteasome inhibitor MG132 (Figure 2A). Ubiquitination of p53 by Mdm2 was a positive control. Complexes were immunoprecipitated and both binding and ubiquitination were confirmed by immunoblotting (Figure 2A). Results show hPer2 is an effective substrate of Mdm2 as poly-ubiquitinated forms of hPer2 were detected associated to the ligase. This result was further confirmed in cells as HCT116 cells co-transfected with FLAG-hPer2 and either Mdm2 or Mdm2($C^{470}A$) were analyzed for the formation of poly-ubiquitinated complexes in the presence of MG132 (Figure 2B). Immunoprecipitation of FLAG-hPer2 from cell extracts showed an increased level of poly-ubiquitination that resulted from overexpression of the wild-type, but not the dominant negative, Mdm2 ligase. As expected, poly-ubiquitinated complexes were undetected in sample extracts from non-MG132-treated cells confirming the role of the proteasome in Mdm2-mediated degradation of hPer2. We then reasoned that Mdm2-mediated ubiquitination of hPer2 should impact hPer2 half-life. To test this possibility, HCT116 cells were transfected, or not (control), with FLAG-Mdm2 and were allowed to grow for 24h before adding cycloheximide (an inhibitor of *de novo* protein synthesis) to the media (t=0h). Cells were harvested at different times after treatment and extracts were analyzed for expression of endogenous hPer2 (Figure 2C). Results show hPer2 levels decreased rapidly in transfected cells compared to control and reflects in the two-fold decrease on hPer2 half-life (Figure 2C, lower panels). In summary, our results support a model in which Mdm2 is a novel E3 ligase of hPer2, it modulates hPer2 ubiquitination, and impacts its rate of proteasome degradation.

Interplay between E3 ligases influence their stability and ultimately the fate of hPer2. Further experiments were devoted to identify the region of hPer2 targeted for ubiquitination. We focused our efforts on the two regions in hPer2 that bind Mdm2: hPer2(356-574) and hPer2(683-872). Accordingly, HCT116 cells were either mock or siRNA Mdm2-transfected and therefore depleted of endogenous Mdm2 (Figure 3A). Twenty-four hours after knockdown, cells were co-transfected with either construct of hPer2 [*myc*-hPer2(356-574), *myc*-hPer2(682-872)] and Mdm2 wild type or its E3 mutant form. Ubiquitination of hPer2 fragments was evaluated by immunoprecipitation and blotting using specific antibodies (Figure 2C, upper panel). Interestingly, whereas *myc*-hPer2(356-574) showed unnoticeable levels of ubiquitination (data not shown); ubiquitinated forms of *myc*-hPer2(682-872) accumulated in mock samples treated with MG132 and transfected with wild type, but not dominant negative, Mdm2 (Figure 2C, lanes 5 *vs.* 6). Unexpectedly, knockdown expression of Mdm2 did not completely abrogate *myc*-hPer2(682-872) polyubiquitination, instead, it enhanced (Figure 3A, lanes 4 vs 10). Initially puzzling, this result suggested i) other E3 ligases are likely to target hPer2 (*i.e.*, β-TrCP1) and that ii) one of those enzymes could also act as Mdm2 substrate itself. As β-TrCP1 has been previously identified as a type of E3 ligase that recognizes post-translationally modified hPer2 for ubiquitination (Ohsaki et al., 2008; Reischl et al., 2007) and co-localizes with Mdm2, we focused our efforts in understanding a

possible interplay between Mdm2 and β-TrCP1. First, we generated a form of β-TrCP that, like Mdm2($C^{470}A$), lacks of ubiquitin ligase activity and cannot self-ubiquitinate. This isoform 2 of β-TrCP1 lacks the F-box region comprising residues 154 to 192 rendering the enzyme inactive (FLAG-Δ-β-TrCP, (Cenciarelli et al., 1999; Margottin et al., 1998)). Binding and ubiquitination experiments were carried out using *in vitro* transcribed and translated tagged proteins and modified substrates were identified by immunoblotting (Figure 3B). Binding was confirmed by pre-incubating wild type *myc*-Mdm2 or *myc*-Mdm2(C470A) with FLAG-Δ-β-TrCP and immunoprecipitating the complex (Figure 3B, lanes 1-2). Specific ubiquitination of β-TrCP by Mdm2 was detected when binding reactions took place in the presence of an ubiquitin reaction mixture and the modified β-TrCP protein detected using an α-ubiquitin antibody (Figure 3B, lanes 9-10). Substrate specificity was confirmed enzymatically as ubiquitination did not take place in the presence of *myc*-Mdm2(C470A) (Figure 3B, lane 10).

To evaluate the impact of Mdm2 on β-TrCP1 half-life and stability, HCT116 cells were transfected with either FLAG-Mdm2 or FLAG-Mdm2($C^{470}A$) and treated with cycloheximide 24h later. Cells were collected at different times after treatment and extracts analyzed for the expression of β-TrCP1 and Mdm2 (Figure 4A). In agreement with our *in vitro* data, results show that the total amount of β-TrCP1 is lower in Mdm2 wide type, but not dominant negative, transfected samples; interestingly, there is no a significant difference in β-TrCP1 half-life when mock and Mdm2-transfected samples are compared (~3h in both cases) (Figure 4A, upper panel). When HCT116 cells were knock down for β-TrCP1, the half-life of Mdm2 and its overall amount remained unaltered when compared with mock samples (Figure 4B, middle panel). Conversely, when HCT116 cells were knockdown for Mdm2 both half-life and accumulation of β-TrCP1 increased (Figure 4B, middle panel). This suggest a unique scenario in which substrate specificity and stability may be directly linked to post-translational events as β-TrCP1, but not Mdm2, only target substrates for degradation when marked by phosphorylation. This results certainly merit further investigation (*e.g.*, note that the β-TrCP2 isoform has not been knocked down in any of these experiments and may play an additional role in regulating protein stability) and goes beyond the scope of the current thesis presentation. At this point, we are seeking input from mathematical modelers to help us understand better the interplay between these E3 ligases and the potential scenarios that lead to protein accumulation, but increased half-life, in one case (for β-TrCP when Mdm2 is knocked down) or unaltered levels and half-life for the other (for Mdm2 when β-TrCP is knocked down).

The relevance of both E3 ligases for regulating hPer2 accumulation is evident in HCT116 knockdown experiments (Figure 4B, upper panel). As shown, both hPer2 levels and half-life are increased when either Mdm2 or β-TrCP1 are knocked down and compared to mock-treated samples. Remarkably, the sole knocks down of each E3 ligase caused essentially same alterations in hPer2 when compared to each other (Figure 4B, lanes 7-12 *vs.* 13-18). We interpret these results as both E3 ligases influencing hPer2 directly but propose that there a temporal regulation that it is not being considered in these experiments but that we believe play a role in modulating hPer2 availability. This is largely supported by unpublished preliminary data from our group that show β-TrCP1 levels oscillate in circadian synchronized cells whereas Mdm2 remains largely steady throughout the time course analyzed.

Mdm2-mediated modulation of hPer2 influences circadian period length. Despite the relevance of identifying both hPer2 as a novel target for Mdm2 and Mdm2 as a novel E3 ligase for hPer2, it is the functional relevance of this interaction for the functioning of the circadian cycle that makes it particularly intriguing. Accordingly, we hypothesized that Mdm2-mediated polyubiquitination and proteasomal degradation of hPer2 should impact circadian period length. To test this possibility, we used MEF system in which the Per2 gene has been fused to a luciferase reporter for luminescence detection (MEF Per2::Luc). Cells were transfected with myc-Mdm2 and circadian synchronized by dexamethasone addition before placing them in the lumicycle (Figure 5A). Expression of the *myc*-transfected construct was validated by immunoblotting and recordings overlapped and compared with those of mock samples for analyses. As shown, overexpression of Mdm2 shortened period length by about 40 min as expected from a model in which Mdm2 targets hPer2 for degradation (Figure 5A graph). Conversely, knock down expression of Mdm2 bu siRNA treatment, synchronization, and later recording show that the period length of treated cells is 36 min longer when compared to mock-treated cells (Figure 5B). This result establishes Mdm2 as modulator of clock rhythms by directly targeting hPer2 for degradation.

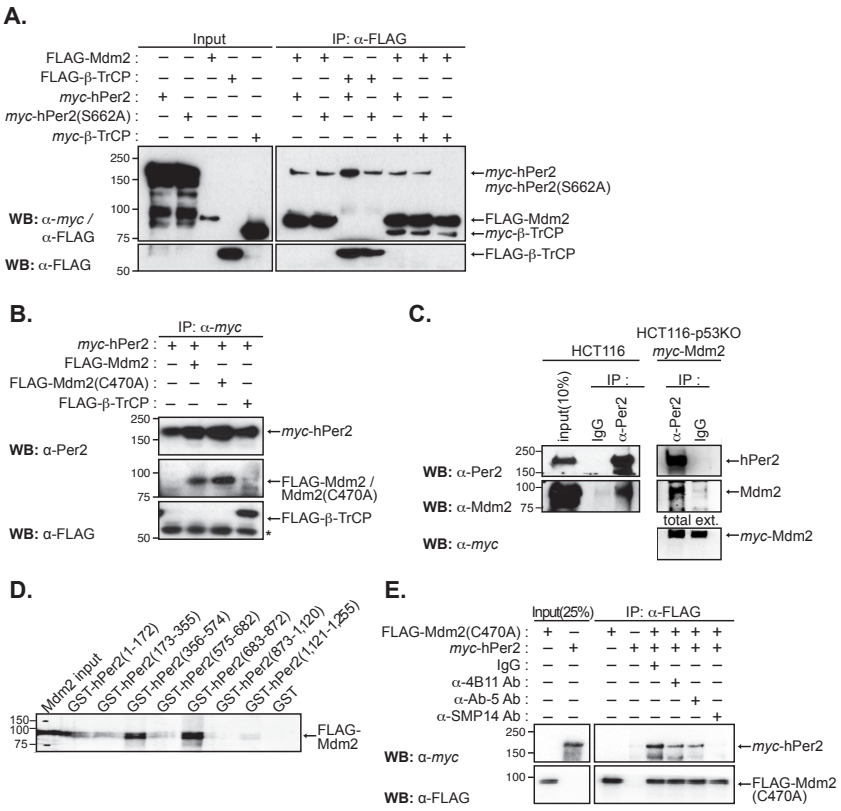

Figure 4-1. Period 2 binds to the E3 ubiquitin ligase Mdm2.

A. In vitro transcribed and translated *myc*-hPer2/hPer2 (S662A), *myc*-β-TrCP1 and FLAG-Mdm2/β-TrCP1 proteins were pre-incubated with different combination as indicated. Samples were then subjected to immunoprecipitation of the FLAG tagged proteins using α-FLAG antibody conjugated beads. Bound proteins were detected by immunoblotting and are indicated with arrows on the right. **B.** H1299 cells were transfected with different combination of pCS2+*myc*-hPer2, pCS2+FLAG-Mdm2/Mdm2 (C470A)/β-TrCP1 using Lipofectamine LTX and harvested 24h later. Cell pellets were lysed in 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100 and extracts (~500 ug) incubated with α-*myc* beads and complexes were resolved by SDS-PAGE and immunoblotting using α-myc and α-FLAG antibodies. **C.** HCT116 p53 KO cells were transfected with pCS2+*myc*-Mdm2. 1200ug of HCT116 and 1400ug of HCT116 p53 KO extracts were incubated with either α-Per2 or IgG control antibody. Complexes were immunoprecipitated by protein A beads, resolved by SDS-PAGE and immunoblotted by α-Per2, α-Mdm2 and α-myc antibodies. **D.** GST-tagged hPer2 fragments were purified using affinity chromatography and bounded beads were incubated with [35S]-labeled FLAG-Mdm2 and association radiolabeled protein were detected by autoradiography. **E.** In vitro transcribed and translated FLAG-Mdm2 (C470A) was pre-incubated with different Mdm2 antibodies. Samples were subjected to immunoprecipitation of FLAG-Mdm2 (C470A) using α-FLAG antibody and protein A beads and followed secondary immunoprecipitation by adding In vitro transcribed and translated *myc*-hPer2. FLAG-Mdm2 (C470A) bounded hPer2 were detected by immunoblotting with α-*myc* antibody.

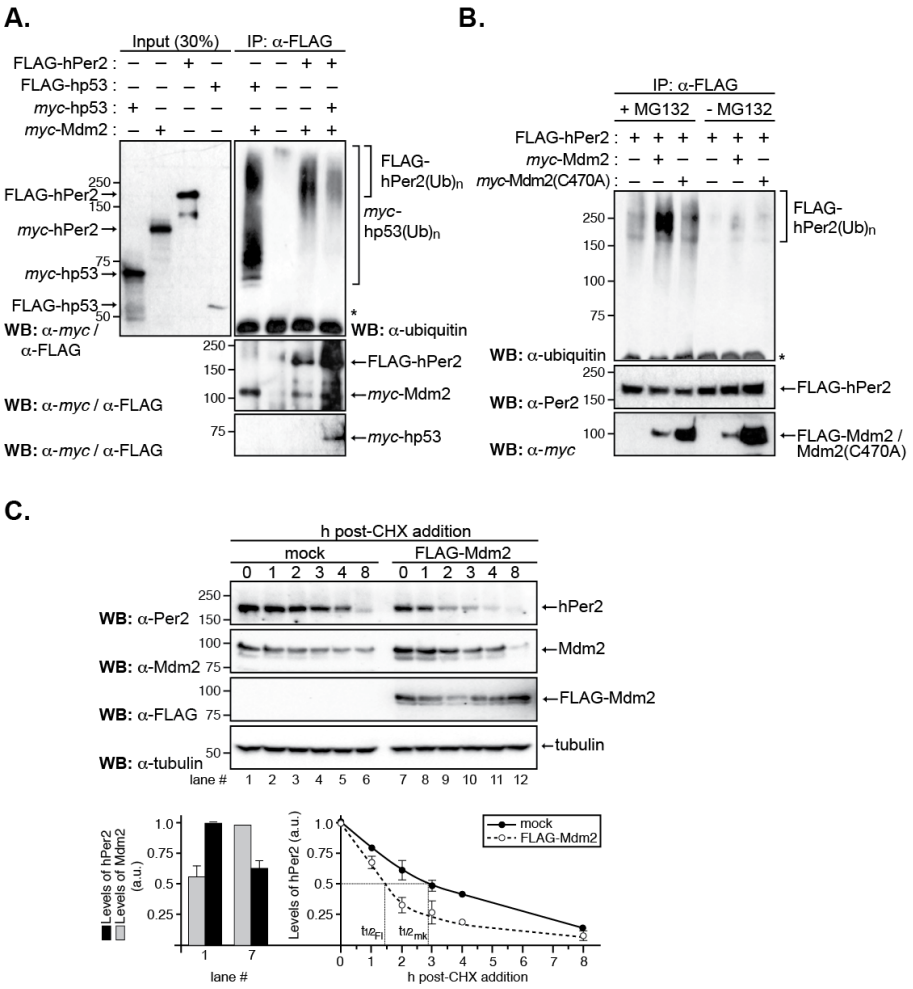

Figure 4-2 Mdm2 targets hPer2 for ubiquitination.

A. In vitro synthesized FLAG-hPer2, *myc*-Mdm2, FLAG-hp53 and *myc*-hp53 were incubated as different combinations (hp53: Mdm2: hPer2 ratio 1:2:5) and then subjected to in vitro ubiquitination followed by immunoprecipitation of FLAG-hPer2 or FLAG-hp53 (positive control) bounded proteins by α -FLAG antibody and protein A beads. Ubiquitinated hPer2 and p53 were detected by α-ubiquitin antibody. **B.** HCT116 cells were transfected with combinations of pCS2+FLAG-hPer2, and pCS2+*myc*-Mdm2 or pCS2+*myc*-Mdm2 (C470A), 24 hours after transfection, treating cells with 100uM MG132 for 4 hours before harvesting. Cell lysates were incubated with α -FLAG antibody and protein A beads. Immunoblotting with α-ubiquitin antibody showed ubiquitinated hPer2 signals. **C.** HCT116 cells were transfected with pCS2+3FLAG-Mdm2(1ug) or pCS2+ empty vector (mock) by Lipofectamine LTX, in complete media and maintained for 24h. Add cycloheximide (CHX, 100 ug/ml) to the cells, samples were harvested according to the indicated time. Extracts were resolved by SDS-PAGE and analyzed by immunoblotting using α-Per2, -Mdm2, -FLAG and -tubulin (loading control) antibodies. Protein levels of hPer2 from mock and 3FLAG-Mdm2 overexpressed samples were quantified by densitometry using ImageJ Software v1.45 and values normalized to tubulin levels.

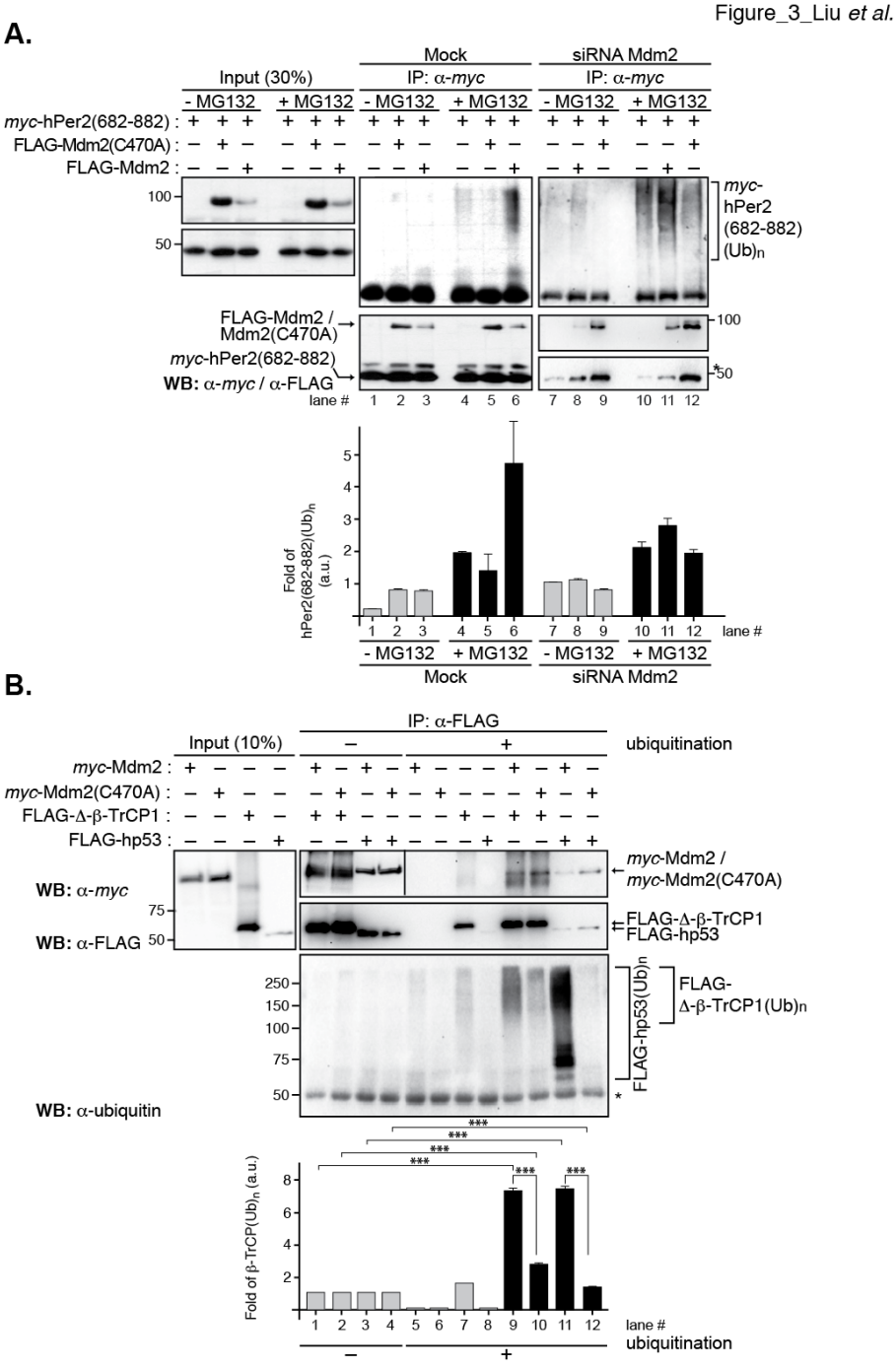

Figure 4-3 Interplay between E3 ligases influence their stability and ultimately the fate of hPer2.

A. Mdm2 is knockdown in HCT116 cells by siRNA (25nM), 24h later followed by transfected with *myc*-GST-Per2 (682-872) fragment V, which binds to Mdm2, in the presence of either FLAG-Mdm2 or FLAG-Mdm2 (C470A) mutant, incubated for 24 hours. Treated cells with 100uM MG132 for 4 hours before harvesting. After immunoprecipitation with α -FLAG antibody and protein A beads, samples were analyzed by immunobloting with α-ubiquitin antibody. Bands were quantified by densitometry using ImageJ Software v1.45 and plotted in arbitrary units. **B.** *In vitro* synthesized *myc*-Mdm2, *myc*-Mdm2 (C470A), FLAG-Δ-β-TrCP1, and FLAG-hp53 were incubated as different combinations and then subjected to in vitro ubiquitination or not (as control) followed by immunoprecipitation of FLAG-Δ-β-TrCP1or FLAG-hp53 (positive control) bounded proteins by α-FLAG antibody and protein A beads. Ubiquitinated FLAG-Δ-β-TrCP1 and FLAG-hp53 were detected by α-ubiquitin antibody.

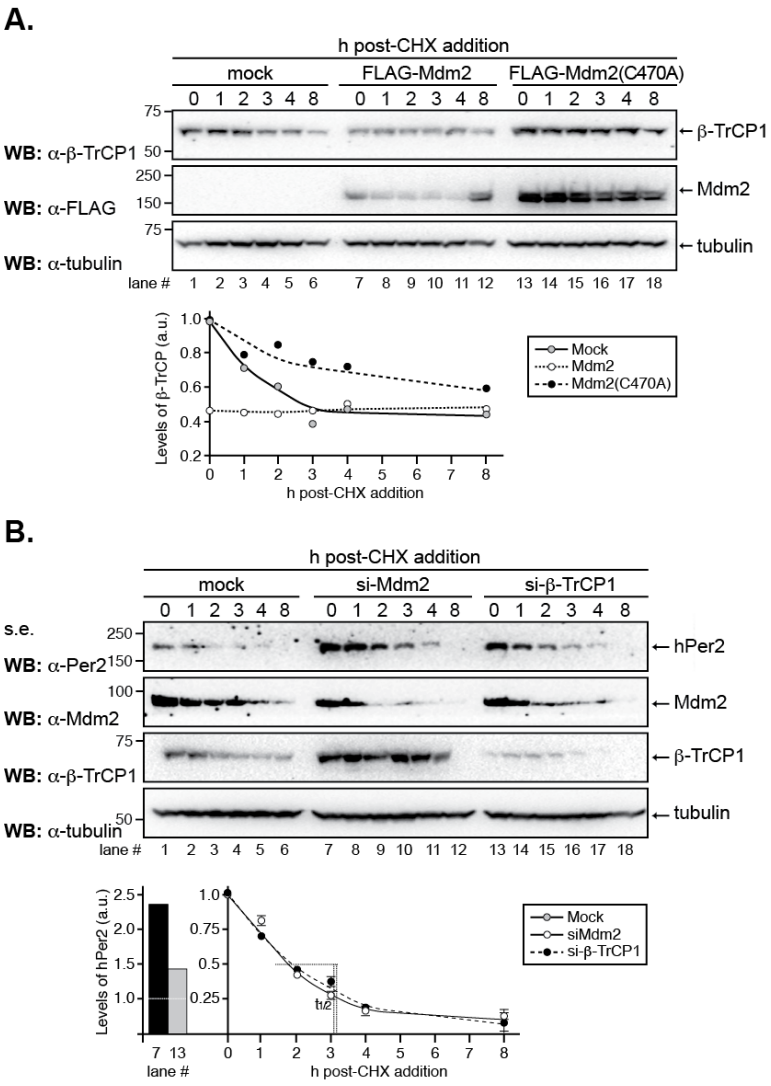

Figure 4-4 Mdm2 regulates the stability of β-TrCP1 and hPer2

A. HCT116 cells were transfected by pCS2+3FLAG-Mdm2(1ug), pCS2+3FLAG-Mdm2(C470A) (1ug), or pCS2+ empty vector (mock) by Lipofectamine LTX, in complete media and maintained for 24h. Add cycloheximide (CHX, 100 ug/ml) to the cells, samples were harvested according to the indicated time. Extracts were resolved by SDS-PAGE and analyzed by immunoblotting using α-TrCP1, -Mdm2 and -tubulin (loading control) antibodies. Protein levels of TrCP1 from mock, 3FLAG-Mdm2 or 3FLAG-Mdm2(C470A) overexpressed samples were quantified by densitometry using ImageJ Software v1.45 and values normalized to tubulin levels. **B.** HCT116 cells were transfected with siRNA hMdm2 (25nM), β-TrCP1 (25nM), or mock, in complete media and maintained for 48 h. Add cycloheximide (CHX, 100 ug/ml) to the cells, samples were harvested according to the indicated time. Extracts were resolved by SDS-PAGE and analyzed by immunoblotting using α-Per2, -Mdm2, -β-TrCP1 and -tubulin (loading control) antibodies. Protein levels of hPer2 from siRNA Mdm2 and siRNA β-TrCP1-treated samples were quantified by densitometry using ImageJ Software v1.45 and values normalized to tubulin levels.

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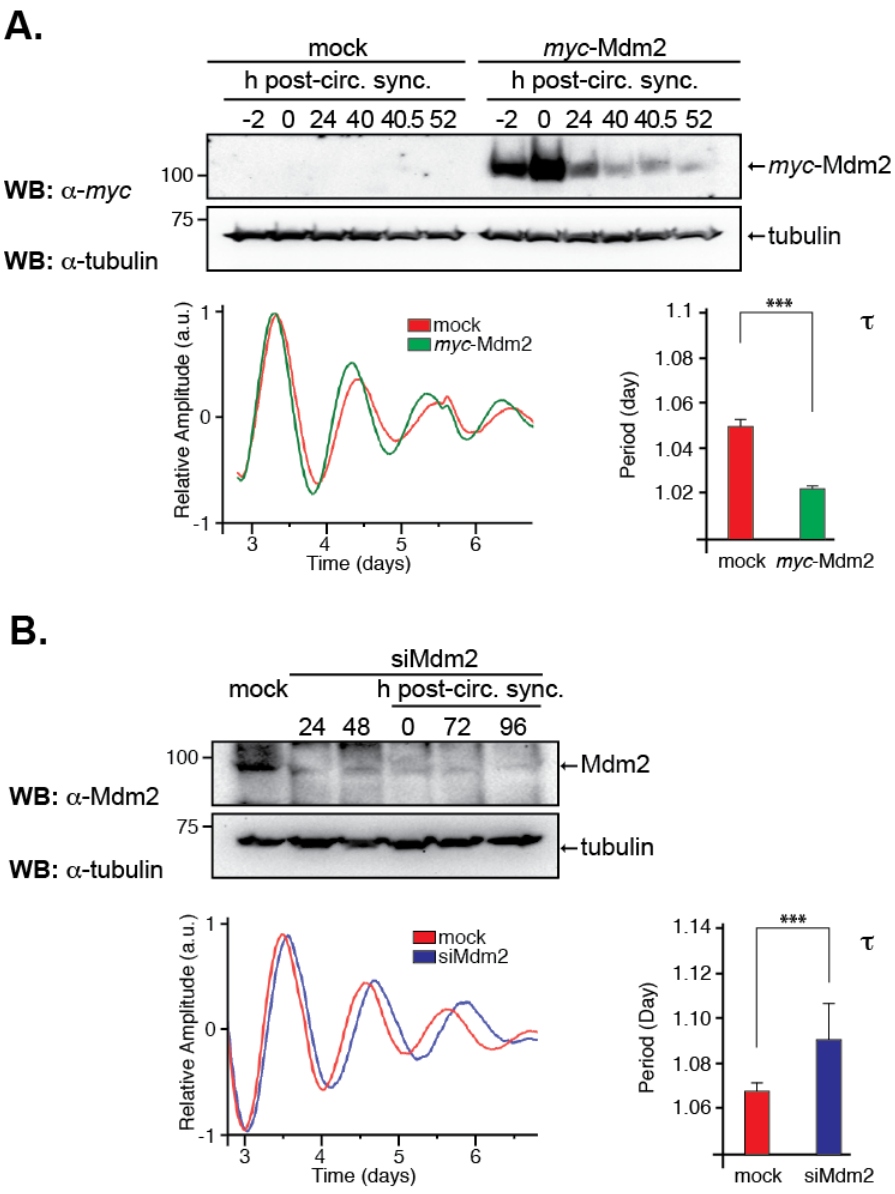

Figure 4-5 Mdm2-mediated modulation of hPer2 influences circadian period length.

A. MEF cells (Per2-luc) were transfected by pCS2+3FLAG-Mdm2(1ug), or pCS2+ empty vector (mock) by Lipofectamine LTX, in complete media and maintained for 24h. Synchronize the cell by Dexamethasone (Dex, 200nM) for 2h before either set into Lumicycle or colleting cells at corresponding time point. -2h is before adding Dex; 0h is right after 2h Dex synchronization and so forth. Luminecent data were recording for more than 6 days, the first 1.5 days data were cut. Period length was calculated by fitting a model (Oklejewicz et al., 2008) in JMP. Quantified data show on right (t-test $P<0.05$). **B.** MEF cells (Per2-luc) were transfected with siRNA hMdm2 (25nM) or mock, in complete media and maintained for 48 h. Did the same treatment as described in **A.** Different time point samples were collected for western blot. Data were recoded for more than 6 days in Lumicycle and same quantification as described in **A.** T-test $P<0.05$.

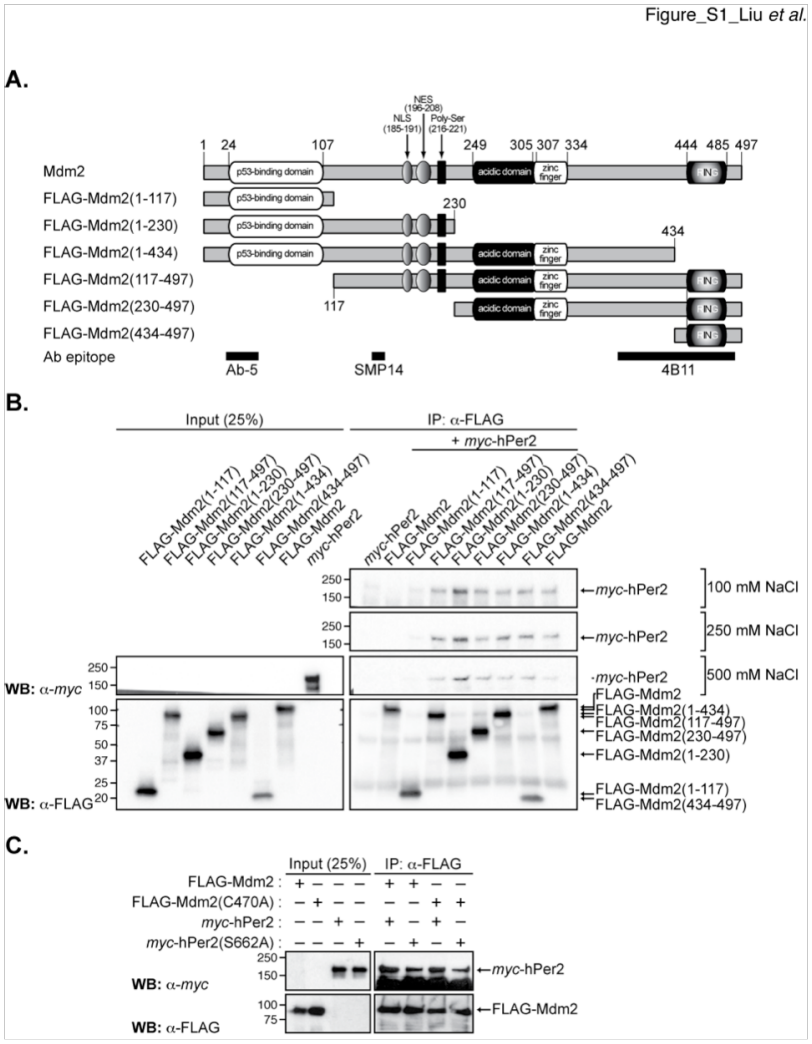

Figure 4-6 S1. Thecircadian factor Per2 interacts with Mdm2.

A. Schematic representation of 3FLAG-Mdm2 fragements in pCS2+ plasmids. Different Mdm2 antibody epitopes were labeled. **B.** *In vitro* transcribed and translated *myc*-hPer2 and FLAG-Mdm2 or FLAG-Mdm2 fragments were pre-incubated with different combination as indicated. Samples were then subjected to immunoprecipitation of the FLAG tagged proteins using α-FLAG antibody and protein A beads. The immunoprecipitated complexes were washed by different sodium chloride concentration buffers (100mM, 250mM and 500mM). Bound proteins were detected by immunoblotting and are indicated with arrows on the right. **C.** HCT116 cells were co-transfected with different combinations of pCS2+FLAG-Mdm2, pCS2+FLAG-Mdm2(C470A), pCS2+*myc*-hPer2, and pCS2+*myc*-hPer2 (S662A). Maintained in complete medium for 24 hours before harvesting. Cell lysates were incubated with α -FLAG antibody and protein A beads, resovled in SAS-PAGE, and immunobloted by α-FLAG and α- *myc* antibodies.

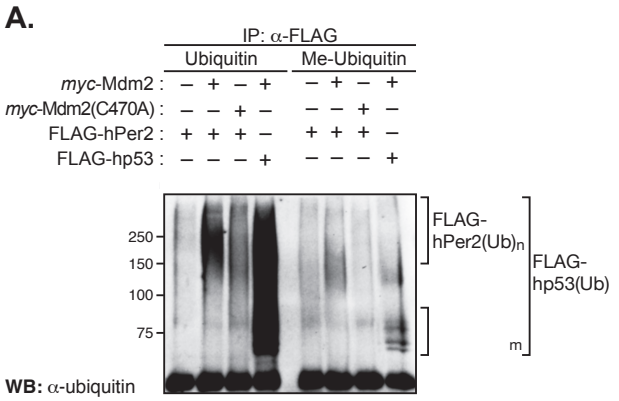

Figure 4-7 S2. Mdm2 induces polyubiquitination of Per2 *in vitro*.

A. An *in vitro* ubiquitination reaction was conducted as Figure 2. A with wild-type ubiquitin or methylated (Me) ubiquitin. Each reaction mixture was subjected to α -FLAG antibody and protein A beads, followed by immunoblotting with α-ubiquitin antibody.

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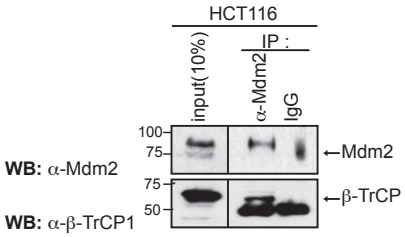

Figure 4-8 S3. The interaction between Mdm2 and β-TrCP1 *in vivo*.

A.1000ug of HCT116 extracts were incubated with either α-Mdm2 or IgG control antibody. Complexes were immunoprecipitated by protein A beads, resolved by SDS-PAGE and immunoblotted by α-Mdm2 and α-β-TrCP1 antibodies.

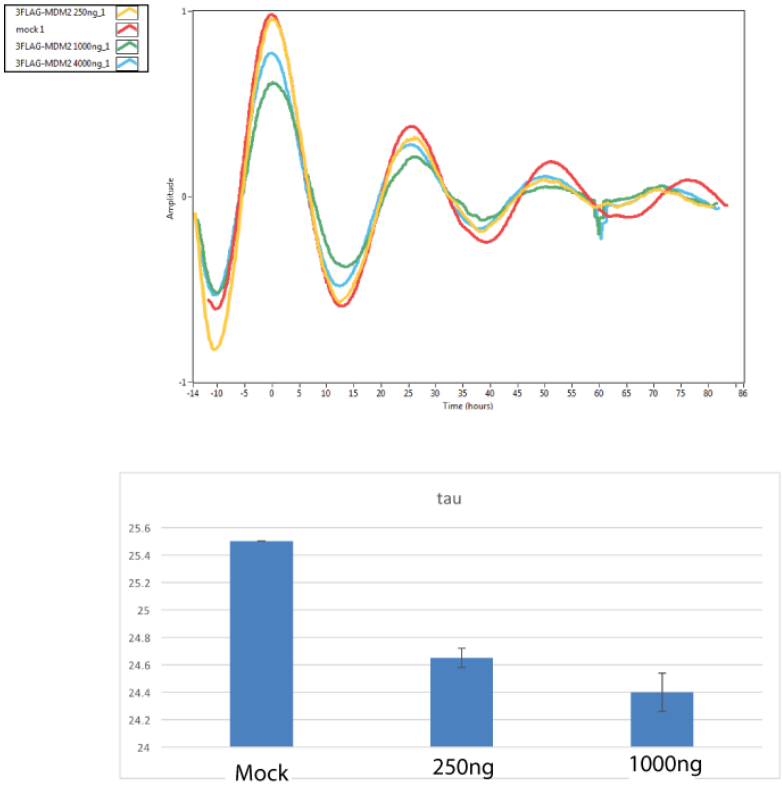

Figure 4-9 S4. Titration of ectopic Mdm2 expression regulates the circadian period.

A. MEF cells were transfected by pCS2+3FLAG-Mdm2 250ng, 1000ng, 4000ng or pCS2+ empty vector (mock) by Lipofectamine LTX, in complete media and maintained for 24h. Synchronize the cell by Dexamethasone (Dex, 200nM) for 2h before set into Lumicycle. Luminecent data were recording for more than 85 hours, the first 24h data were cut and all aligned to the second peak of oscillation. **B.** Quantification of the Periods from **A**. With the transfected amount of Mdm2 plasmids increasing, circadian periods decrease.

4.5 Discussion

We have previously established the existence of a hPer2:Mdm2:p53 complex that play multiple regulatory roles in unstressed cells and in response to genotoxic stimuli (Gotoh et al., 2015; 2014). In this work, we establish the presence of a p53-independent complex, hPer2:Mdm2 whose function under physiological conditions may be tightly linked to the circadian circuit by influencing period clock length. This is in addition to the proposed regulatory function of the trimeric complex for modulating cell cycle progression and survival responses thus, integrating all regulatory components in distinct, yet linked, regulatory loops ((Gotoh et al., 2015)Figure 6).

The finding of hPer2 acting as Mdm2 substrate is not only novel from a biochemical standpoint but also for its potentially functional implications at various regulatory levels. We now know that Mdm2 function is largely allocated to the nuclear compartment (Mayo and Donner, 2001a; Ogawara et al., 2002) and that low levels of Mdm2 activity induces mono-, rather than poly, ubiquitination of substrates (Li et al., 2003). Unpublished results from our laboratory establish that the level of Mdm2 protein and mRNA expression remains low and fairly constant over a 24h cycle and that it is β-TrCP1 protein that exhibit an oscillatory behavior. Thus, whereas our results show that overexpression of Mdm2 triggers hPer2 degradation *in vitro*, we are currently considering a role for this interaction in larger spatio-temporal scale in which β-TrCP1 does necessarily play a critical role. Although further experiments need to be performed, we work on the basis that Mdm2 targeting of hPer2 results at times of the day when hPer2 translocate and accumulates in the nucleus *via* its well-established association with Cry and casein kinase 1ε/δ (CK1 ε/δ, REF). However, we propose that the relative basal amounts of Mdm2 in this compartment under normal physiological conditions only allows for mono-ubiquitination and CK1 ε/δ-mediated phosphorylation of hPer2 to occur (experiments are ongoing to evaluate this possibility) until its accumulation surpasses a threshold level at which β-TrCP triggers the degradation phase of hPer2 and a new round of Clock/Bmal-mediated transcriptional activation of clock responsive genes. Experiments are in progress to evaluated the presented model. In conclusion, our work establishes the existence of a new component in the clock pathway, Mdm2, whose function directly impacts period length.

CHAPTER 5: DNA-damage promotes circadian rhythm phase shift *via* a Mdm2-dependent Per2 degradation mechanism

5.1 Abstract

Most organisms developed internal circadian rhythms in metabolism, physiology and behavior to anticipate the time of the day and prepare for the environmental changes(Ko, 2006). Recent studies show cell cycle progression and DNA damage response pathways are not only circadian outpouts(Fu et al., 2002; Fu and Lee, 2003; Gery et al., 2006; Gotoh et al., 2015; X. Gu et al., 2011; Matsuo, 2003), but also feedback to circadian rhythms(Gamsby et al., 2009; Oklejewicz et al., 2008; Papp et al., 2015). One interested thing is that DNA damage only induces phase advance. Our previous work show Mdm2 is a E3 ligase for Per2, moreover Mdm2 is a DNA damage responsive element in p53 pathway, phosphorylation by ATM reduced its E3 ligase function(de Toledo et al., 2000; Maya et al., 2001b; Meulmeester et al., 2005; Z. Wang et al., 2012). So we investigated whether DNA damage induced circadian phase shift *via* Mdm2 dependent Per2 degradation mechanism. By using MEF (Per2-luc) cells, expressing endogenous Per2-luciferase fusion protein, we show that Sempervirine Nitrate (SN, Mdm2 inhibitor) treatment pulse induced circadian phase advance in a dose- and time- dependent manner, which is very similar as DNA damage by irradiation. SN prevents Mdm2 ubiquitinating Per2 *in vitro* and stabilizing Per2 *in cells*. Consistent with previous results by knock down Mdm2, SN continues treatment increased circadian period by about 2 hours.

5.2 Introduction

In the process of evolution, a lot of organisms developed internal clocks to drive circadian rhythms of metabolism, physiology, and behavior. However, the internal clock is not exactly 24 h, so it must be adjusted every day to ensure synchronization of an organism's physiology to the environmental light-dark cycle. Internal clocks adopt a specific and stable phase relationship to the local time by entrainment to the light/dark cycle, which allows organisms to adapt efficiently to the external environment. Light is the main cue influencing circadian phase resetting, *Per* genes were identified as light responsive component, which are essential for regulating phase advance or delay dependent on when the exposure to light takes place(Albrecht et al., 2001). Studies show that isolated peripheral tissues(Yoo et al., 2004) and even immortalized mammalian cultured cells such as Rat-1 fibroblasts(Balsalobre et al., 1998) and NIH3T3(Akashi and Nishida, 2000) cells are capable of resetting circadian rhythm *in vitro* using chemical cues. High concentrations of serum shock resulted in *Per* genes expression pattern being similar to those observed in the SCNs of animals receiving light pulse(Balsalobre et al., 1998). Other stimuli such as dexamethasone, high potency analog of glucocorticoid(Balsalobre et al., 2000b) and forksolin, an activator of adenylate cyclase and raising levels of cyclic AMP (cAMP) (Balsalobre et al., 2000b; Yagita and Okamura, 2000) were commonly used to synchronize circadian rhythms in peripheral tissues, similar as light, the treatment time of these chemicals effects on circadian phase shift(Izumo et al., 2006).

Although the mechanisms and phase resetting types are different, from the standpoint that DNA damage also resets the circadian clock in a time of day dependent manner, which is similar to the light and drug cues. Early work by

Sweeney showed that the length of the phase depends on when irradiation takes place within the circadian cycle in *Gonyaulax* with the greatest shifts occurring in cells irradiated early in the subjective night phase (Sweeney, 1963). DNA damage caused circadian phase advance when *Neurospora* were treated with the radiomimetic drug methyl methane sulfonate (MMS) at various circadian time. Accordingly, maximum phase advance coincided with FRQ (mammalian negative regulator Period homolog) accumulation during the day (Pregueiro et al., 2006). IR mediated DNA damage can also phase advance the mammalian clock in Rat-1 fibroblasts and in mice, however, different form the Neurospora study, the minimal advances occur during negative regulator early accumulation phase (Oklejewicz et al., 2008). A similar response to DNA damage in mouse embryonic fibroblast cells (PER2::LUC, which expressing a PER2 Luciferase fusion protein from the endogenous *Per2* locus) was observed, the magnitude of the phase advance depended on the status of PER2. Maximum phase advance resulted fromirradiated MEF cells during PER2 *de novo* synthesis (Gamsby et al., 2009). It is noteworthy that i) All DNA damage dependent circadian phase response curves only reports phase advance; ii) Unlike light and drug cues, DNA damage induced circadian rhythm resetting is not dependent on de novo circadian genes expression, but posttranslational modification or circadian components relocation maybe involved (Oklejewicz et al., 2008).

Although the property of DNA damage induced clock resetting is evolutionarily conserved from lower eukaryotes to mammals, the mechanism why DNA damage affects circadian clock phase shifting is still ambiguous. Some circadian components may not function in the core clock mechanism but has been shown to regulate cell cycle progression. For example, TIM physical interacts with ATM-related kinase ATR and CHK1, a checkpoint kinase activated by ATR(Gotter et al., 2007; Unsal-Kaçmaz et al., 2005). TIM downregulation by RNAi attenuates DNA-damage dependent phase advance (Engelen et al., 2013). In addition, DNA damage triggers Cryptochrome 1 binding to a deubiquitinase called Hausp, resulted in Cryptochrome stabilization by preventing its ubiquitination and proteasome-dependent degradation (Papp et al., 2015). However, the hypothesis that DNA damage shifts circadian clock time via Hausp dependent Cry 1 stabilization only explains why during negative regulators (PERs and CRYs) increasing phase, DNA damage leads phase advance, it is untenable to explain the conflict that stabilized CRY resulted in phased advance during negative regulator decreasing phase.

Here, we report that Mdm2 is a new E3 ligase for PER2 that promotes PER2 degradation, as both siRNA and inhibitor treatment blocked its degradation. Mdm2 induced Per2 degradation is important for sustaining period length and circadian robustness. Interestingly, Mdm2 seems to mediate the DNA damage response by resetting the circadian phase angle *via*. To accomplish this, Mdm2 cooperates with another SCF ubiquitin ligase β-TrCP. We propose Mdm2-dependent ubiquitination of PER2 plays a dual role in modulating the circadian phase angle: i). Mdm2 is responsible for ensuring a pool of Per2, which do not function as circadian repressor with Cry; ii) Irradiation reduces the activity of Mdm2 as result of its degradation elevating Per2 levels transiently, if it happens during Per2 accumulation phase, Per2 peak comes in advance; if it happens during Per2 decreasing phase, Per2 trough level increases and leads to shorten Per2 accumulation phase which also makes phase advance. Our hypothesis provides a testable explanation by which DNA damage only causes circadian phase advance, which is not dependent on transcription.

5.3 Materials and Methods

Cells and cell culture

The human colorectal carcinoma-116 (HCT116) $p53^{-/-}$ cell line were provided by Dr. B. Volgestein, Johns Hopkins University. The MEF (PER2::LUCIFERASE) and NIH 3T3 (mBmal-1:LUCIFERASE) cells were a gift from Dr. S. Kojima and were cultured in Dulbecco's Modified Egale's Medium (DMEM, MT 10-013-V, Corning) supplemented with 10% FBS (35-010-CV Corning), 50 units/ml penicillin, 50 ug/ml streptomycin and maintained at 37°C and 5% CO_2. For recordings, cells were maintained in DMEM without phenol red, 2% FBS, and 0.05mM luciferin.

Preparation of protein extracts, and immunoblotting

Cell extracts were prepared in NP-40 lysis buffer containing 10 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 80 mM β-glycerophosphate, 1mM Na3VO4, 10 mM NaF, and protease inhibitors (10 μM leupeptin, 1 μM aprotinin A, and 0.4 μM pepstatin). Prepared samples were resolved by SDS-PAGE, and analyzed by immunoblotting using specific primary antibodies (α-Per2 [Sigma-Aldrich, Farmingdale, NY], α-Mdm2 [cell signaling], α-luciferase [cell signaling]). Chemiluminescence reactions were performed using the SuperSignal West Pico.

Lumycicle Luminescence measurement

MEFs were cultured in 35-mm Petri dishes until 100% confluent and then synchronized by treating cells with dexamethasone for 2 h in 37°C (Balsalobre et al., 2000) followed by washes in PBS. Cells were then cultured in phenol red-free Lumicycle medium in sealed dishes. When indicated, cells were exposed to irradiation or treated with specific chemicals. For period length experiment, cells were treated with 1ug/ml sempervirine nitrate (SN) 24h after the beginning of recording. For SN pulse experiments, sealed dishes were taken out from Lumicycle at the indicated times, and treated them with medium containing either 1ug/ml SN or DMSO (control). Period and phase was assessed by Lumicycle data analysis software and JMP by fitting to a model (Oklejewicz et al., 2008) .

In vitro ubiquitination assay

For in vitro assays, pCS2+ constructs of FLAG-hMdm2, FLAG-hPer2, *myc*-Mdm2, FLAG-hp53 were transcribed and translated *in vitro* as described (Gotoh et al., 2014). Aliquots of each tagged protein (1–4 μl), or a combination of them with or without SN, were incubated at room temperature for 15 min to allow complex formation to happen before adding the reaction buffer containing 1× ubiquitination buffer (Enzo Biomol), 2 mM dithiothreitol, 20 μg/ml ubiquitin-aldehyde, 100 μg/ml ubiquitin or methylated ubiquitin (for polyubiquitination assay), 1xATP-energy regeneration system (5 mM ATP/Mg^{2+}; Enzo Biomol), 40 μM MG132, and 1 mg/ml HeLa S100 lysate fraction (Enzo Biomol) to a final volume of 10 μl. Reactions were further incubated at 37°C for 30 min before being terminated by the addition of lysis buffer. Diluted samples were incubated twice at 4°C for 1 h, first after addition of 3 μg of α-FLAG antibody (Sigma-Aldrich) and then after the addition of 7 μl of protein A beads (50% slurry; Sigma-Aldrich). Bound complex

were washed four times using NP40 lysis buffer, resuspended in Laemmli buffer, and resolved by SDS–PAGE and immunoblotting using specific antibodies.

5.4 Results

Sempervirine nitrate (SN) inhibits Mdm2 E3 ligase activity and stabilized Per2 in cells.

Sempervirine nitrate (SN) was screened out as a new Mdm2 E3 ligase inhibitor by a high throughput electrochemiluminescent assay from a large natural product extracts library, which contains National Cancer Institute (NCI) repository extracts and 4048 other previouly furified natural products (Sasiela et al., 2008). SN was shown to inhibit Mdm2 auto-ubiquitination with an IC_{50} of 8ug/ml *in vitro*. As expected, treatment of retinal pigment epithelium (RPE) cells, embryonic fibroblasts (MEF) cells, and cancer cell model U20S cells with SN led to a dose-dependent increase in the level of p53 and Mdm2 protein, while ubiquitinating p53 and Mdm2 levels decreased (Sasiela et al., 2008).

We verified the effect of SN on Mdm2 auto-ubiquitination in our modified *in vitro* ubiquitination system (Gotoh et al., 2014). First we pre-incubate Mdm2 with SN before adding the ubiquitination assay regents. After the ubiquitination took place, Mdm2 was immunoprecipitated and auto-ubiquitination evaluated using an α-mono/poly ubiquitination antibody (Figure 1A). Results show that SN efficiently prevented Mdm2 auto-ubiquitination in a dose-dependent manner. The Mdm2 ubiquination signal intensity decreased as the SN concentration increases. To evaluate the effect of SN in Mdm2 substrates (i.e., p53 and Per2), Mdm2 and SN were pre-incubated together before the substrate was added. Accordingly, inhibition of Mdm2 prevented both p53 and Per2 ubiquitination (Figure 1B).

Since polyubiqutination leads to proteasome dependent degradation (Pickart, 2004) (Pickart et al., and our previous results), we hypothesized that SN treatment will stabilize Per2 in cells. We tested this hypothesis in two different cell lines: 1). Embryonic fibroblast cell (MEF) expressing a PER2-luciferase fusion protein (Per2::Luc) and, 2) HCT116 $p53^{-/-}$ (p53 null isogenic human colon cancer cells). These cell lines were chosen because a). MEF (PER2::LUC) is a well-established cell line for studying circadian rhythm, b). p53 was published as the negative transcriptional factor controlling *Per2* gene expression (Miki et al., 2013). c) Per2 is known to control TP53 expression (Gotoh et al., 2014), d) p53 stability was also affected by sempervirine nitrate (Sasiela et al., 2008). To rule out p53 effects on Per2, we used this p53 null background cell line. It is interesting to see whether Per2 stability in normal cell line and cancer cell line response to SN is different. SN treatment enhances endogenous Mdm2 and Per2 levels in both MEF cells and HCT116 $p53^{-/-}$ cells (Figure 1C). Our results indicate that sempervirine nitrate efficiently prevents Per2 ubiquitination from Mdm2 and increases Per2 stability in cells.

Sempervirine nitrate (SN) lengthen the circadian period.

To evaluate whether Mdm2 activity influences circadian period length we analyzed the effect of SN on circadian rhythms of MEF cell lines harboring a knock-in Per2-luciferase reporter(Yoo et al., 2004). Continuous treatment of cells with SN resulted in period lengthening and slightly amplitude increase in MEF cells (Figure 2A). Quantification of these data shows that SN increases period length by about 2h compared with DMSO treated control (Figure 2B). To rule out the possibility that SN treatment impacted cell viability, cells were treated with 0.1 to 1 ug/ml of SN for 7

days, harvested, and analyzed by Trypan-blue staining. Results show no significant difference among control and SN-treated cells (Figure 2C).

Next, we hypothesized that changes in the transient amounts of Per2 would impact in a circadian phase change. Confluent MEF cells were first treated with dexamethasone (200uM) to synchronize clock gene expression between individual cells. We take MEF cell dishes out of Lumicycle and applied 1ug/ml SN treatment pulse at 42 hours after Dex synchronization. After added SN, we leave the MEF cell in incubator for 15mintues, 30 minutes and 1 hour before reseal the dishes and put them back to the Lumicycle. Different pulses (based on incubation time) elicited a clear phase advance of bioluminescence rhythms in dose-dependent manner (Figure 3A), with maximum shift of about 7 hours being reached at 1 hr treatment of SN. 30 minutes SN treatment pulse slightly advanced the circadian phase by about 1 hour; while 15 minutes treatment did change the phase much.

Then, we examined whether SN-induced phase change is dependent on the time at which the treatment is applied (i.e., Per2 increasing phase or Per2 decreasing phase). Treatments were applied at 26 and 42h after synchronization, which corresponds to the increasing and decreasing Per2 phases, respectively. Results show a clear dose-dependent phase advance of bioluminescence rhythms at 42h while no significant change at 26h (Figure 3C, 3D). So we conclude that SN treatment induced circadian phase shift is dependent on treatment time (Per2 increasing vs decreasing phase) and dose (how long is the SN treatment).

5.5 Discussion

Here we reported semperviring nitrate (SN), the Mdm2 E3 ligase inhibitor, not only inhibits Mdm2 autoubiquitination and p53 ubiquiitnaiton, but also prevents Per2 from ubiquitination *in vitro*. As expected, SN treatment cause more stable Per2 and leads to longer circadian period, which is consistent with our previous siMdm2 results in Chapter 4. Interestingly, SN pulse introduced at different circadian time (Per2 increasing phase vs Per2 decreasing phase) resulted in altered phase shift response, showing no phase change at the beginning of Per2 accumulation phase while significant phase advance at later Per2 degradation phase. SN dependent phase shift is very similar as DNA damage induced circadian phase shift. Moreover, it was reported that after DNA damage Per proteins (Gery et al., 2006) became more stable, matched with SN treatment increased Per2 stability. Ectopic expression Mdm2 disturbed irradiation induced circadian phase shift (Data not show here). Therefore, these results supported our hypothesis that DNA-damage promotes circadian rhythm phase shift *via* a Mdm2-dependent Per2 degradation mechanism.

The experiments in this chapter are in progression. First, We will use NIH 3T3 cells, which harbors stable BMAL1 luciferase report to test whether sempervirine nitrate (SN) lengthen the circadian period from the circadian positive regulator perspective, also rule out the possibility that Per2 signal is not a reliable circadian oscillation reporter in this scenario, because Per2 protein stability directly changed by the compound. Secondly, we will conduct the MEF cell phase response curve to SN pulse, demonstrating the effects of SN on phase shifting in a whole picture. Finally, we will compile DNA-damage induce phase advance date with the SN induced phase response date together to generate an elegant model, which highlights the role of Mdm2 fine-tuning Per2 stability in the center of circadian phase shifting.

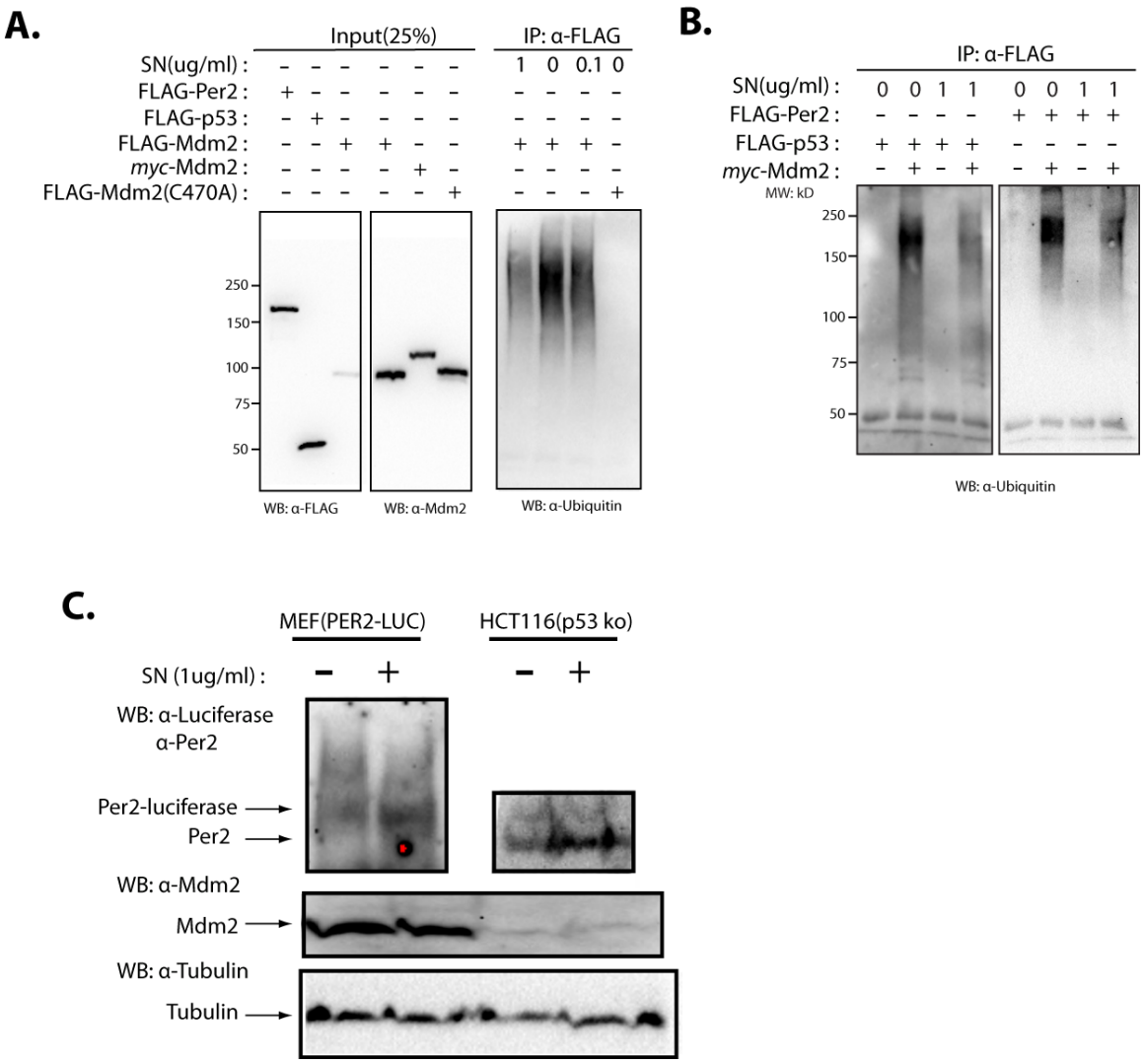

Figure 5-1 Sempervirine nitrate (SN) inhibits Mdm2 E3 ligase activity and stabilizes Per2 in cells.

A. And B. *In vitro* transcribed recombinant proteins were incubated at room temperature with or without sempervirine nitrate (SN) to form the complex first. All the samples were subjected to in vitro ubiquitination followed by immunoprecipitation of FLAG tagged Mdm2, Per2 or p53 using α-FLAG (Rabbit) antibody and protein A-beads. Ubiquitinated Proteins were detected by α-Ubiquitin antibody. Input proteins are shown on A. the left panel. A. SN inhibits Mdm2 autoubiquitination. B. SN prevents p53 and Per2 ubiquitination from Mdm2. C. SN stabilize endogenous Per2 in cells. Treat MEF cells and HCT116 p53-/- cells with SN final concentration 1ug/ml for 24 hours before harvesting. 100ug of MEF or HCT116 p53-/- extracts were resolved by SDS-PAGE and immunoblotting by Mdm2, luciferase, and Per2 antibodies. Tubulin was used as loading control.

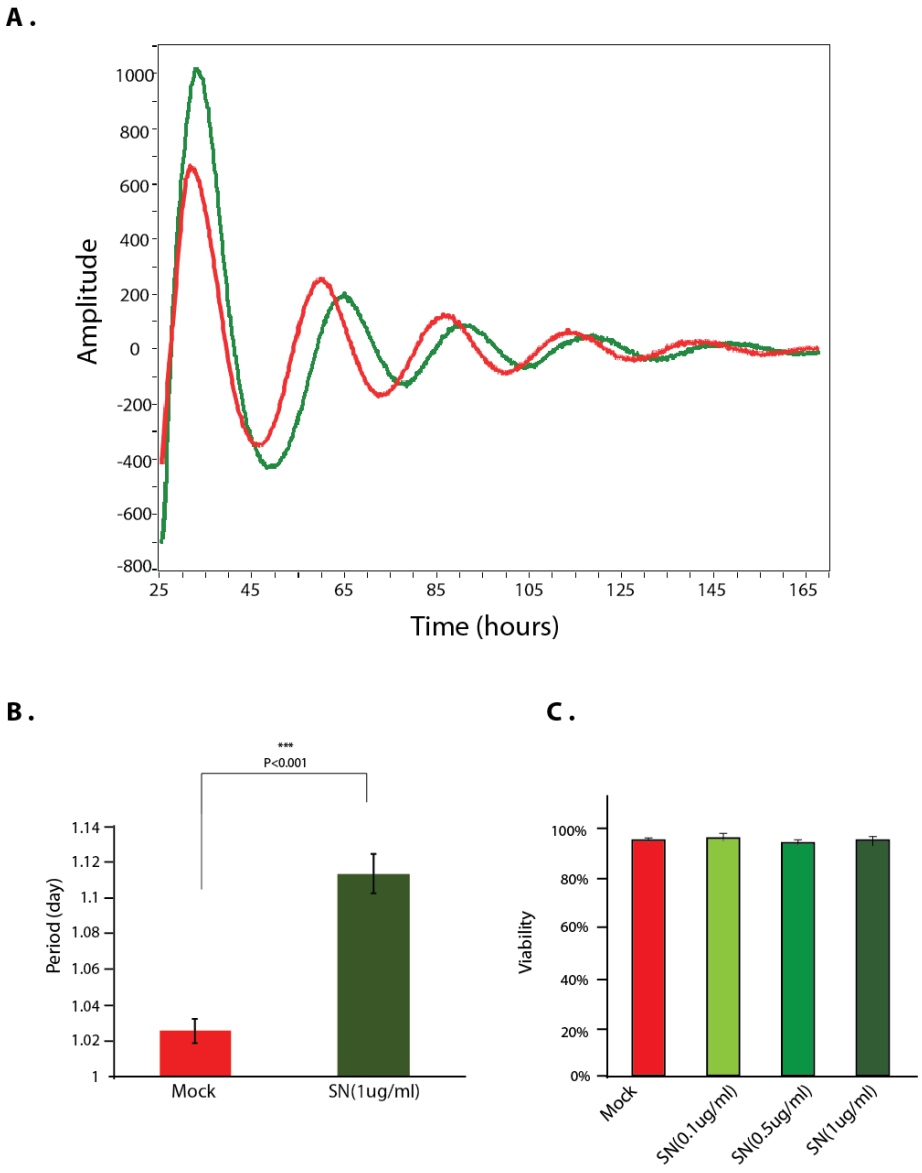

Figure 5-2 Spempervirine nitrate (SN) lengthen the circadian period in MEF cells.

A. Representative results of monitoring luciferase activity form MEF cells with knock-in Per2-luciferase fusion protein. Red line: DMSO treatment, Green line: continues treatment with SN (1ug/ml) at 24 hours after circadian synchronization. **B.** Quantitation of the circadian period of luciferase activity form experiment performed as described in **A.** Data represents the mean ± s.d. for 6-8 samples per condition. **C.** Cell viability after different SN concentration treatment for 7 days.

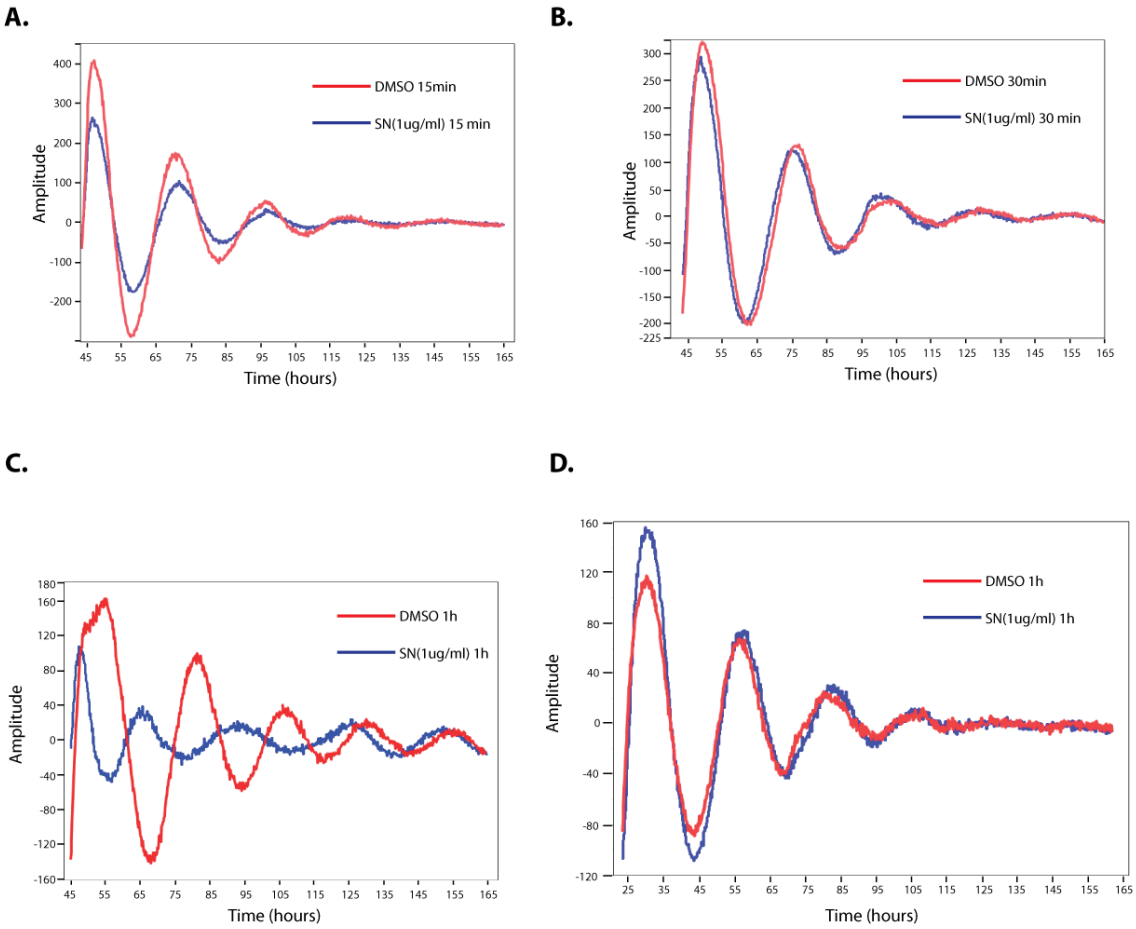

Figure 5-3 Spempervirine nitrate (SN) pulse resets the circadian clock phase.

A. Representative results of luminescence rhythms form MEF cells with knock-in Per2-luciferase fusion protein, exposed to a single pulse of SN (1ug/ml) for 15 minutes at 42 hours after circadian synchronization by Dexamethasone. Red line: DMSO treatment for 15 minutes, Blue line: 15 minutes of SN (1ug/ml) pulse. **B.** Similar experiment as **A.** except SN pulse increased to 30 minutes**. C.** SN pulse raise up to 1 hour, all the other treatment as **A.** and **B. D.**SN treatment pulse for 1h at 24 hours after circadian synchronization by Dexamethasone.

CHARPTER 6: Conclusions and Future Perspectives

6.1 Conclusions

First of all, we identified a direct interaction at protein level between the circadian rhythm core factor and tumor suppressor Per2 and the checkpoint regulator p53. We uncovered a regulatory role for the Per2:p53 complex for maintaining basal levels of p53 in unstressed cells thus, pre-conditioning the system for a rapid response to stress. Period 2 buffered Mdm2 negative regulator function by inhibits Mdm2-mediated ubiquitination in p53. We concluded that Per2 plays an essential role in the p53 signaling pathway. Then, we estudied how Per2 turnover is regulated by ubiquitination. Ubiquitination is involved in multiple biological processes, including cell cycle regulation, cell growth, signaling, and circadian rhythms. Identifying novel ubiquitin-mediated mechanisms among circadian core components promise to open up new opportunities for drug targeting and chronotherapeutics for a range of diseases spanning from cancer to neurodegenerative disorders. In this thesis, we report the identification of a novel substrate, Per2, for the E3 ligase Mdm2. Regulation of Mdm2-mediated ubiquitination of Per2 impacts on circadian period and phase resetting in cultured MEF cells by regulating Per2 ubiquitination. As in Chapter4, we propose the existence of two pools of Per2: one involved in controlling the p53 circuit, the other reserved for circadian circuit and modulated by Mdm2. Supporting this model, we found the treatment of cells with the Mdm2 inhibitor sempervirine nitrate (SN) alters period length and causes phase resetting when applied at different times. Interestingly, treatment of cells with either SN- or DNA damage cause phase advance and difference in amplitude that depend on the time of the day at which the stimuli is applied. We hypothesize that during Per2 accumulation phase, SN treatment or DNA damage results in the stabilization of Per2 and causes phase advance; whereas treatment during Per2 decreasing phase, DNA damage or SN treatment leads to increased Per2 trough level which shortened following Per2 accumulation phase.

6.2 Future Perspectives

To understand the regulatory processes in depth , we should identify the ubiquitination sites on Per2 and determine how DNA damage triggers the release of Per2 from Mdm2. More broadly, we should expand our understanding on how the Per2:Mdm2 complex integrates different signaling pathways to influence both circadian rhythms and cell cycle progression.

Mdm2 and Per2 story

The Mdm2 dependent ubiquitination of Per2 needs further investigation: 1) We verified hPer2(356-574), hPer2(682-872) fragment 3 and 5 are the regions in hPer2 that bind to and are ubiquitinated by Mdm2 (Chapter 4); however, we have not attempted to identify the specific sites on Per2. 2) Considering that the binding region for Mdm2 in Per2 overlaps with that for β-TrCP [(Reischl et al., 2007)and Chapter 4), it would be useful to know whether there is a temporal switch between these two enzymes or function simultaneously in regulating Per2 levels. 3) When considering DNA damage , Mdm2 mediates circadian phase shifting by regulating Per2 ubiquitination, interestingly, Mdm2 itself is down regulated by β-TrCP in response to this stimuli. How does this event lead to circadian phase resetting? 4)

Despite β-TrCP1 is a class of F-box component of the E3 ligase system that only recognizes phosphorylated Per2 and, Mdm2 recognition is not dependent on substrate phosphorylation, it is worth to explore the possibility that phosphorylation acts as priming signaling for sorting Per2 to different E3 ligases. If so, what kind of kinases are involved? 5) As multiple kinases have been identified to regulate Mdm2 function and localization by phosphorylation (Mayo and Donner, 2001b; Meek and Knippschild, 2003; Ogawara et al., 2002; Stommel and Wahl, 2004), in our model we supposed Mdm2 and Per2 interact in nucleus, however Mdm2 translocate to nucleus is regulated by protein kinase B(PKB)/Akt. Akt dependent phosphorylation on Mdm2 delivering Mdm2 located to nuclear(Mayo and Donner, 2001b). What's more Akt phosphorylation sites on Mdm2 (Ser166, Ser186) overlaps with Per2 binding site. So, it would be interesting to study how phosphorylation status of Mdm2 regulates its function on Per2.

Per2 is an integrator of different signaling pathways

Generally speaking, there are distinct regulation levels that influence PER2 expression, stability, and subcellular localization. First, transcriptional regulation of the *Per2* gene results from a complex interplay in which E-box enhancers drive the circadian expression of *Per2* through Clock and BMAL1 mediated transcriptional activation(Ripperger and Schibler, 2006); DBP/E4BP binding element (D-box) influences the amplitude of *Per2* oscillation by repression Per2 expression(Ueda et al., 2005); 3). cAMP-resposive element (CRE) binds CREB protein and elicits Per2 expression is triggered by multiple environmental cues, like light, calcium, and some drugs (i.e., protein kinase C activator) (Gau et al., 2002; Ginty et al., 1993; Hirota and Fukada, 2004; Travnickova-Bendova and Cermakian, 2002); heat shock elements (HSE) drives *Per2* expression by HSF1(Buhr et al., 2010; Reinke et al., 2008); 5). Glucocorticoid response element (GRE) overlaps with the E-box in the proximal Per2 promoter, which is responsible for glucocorticoid induced *Per2* expression (Cheon et al., 2013). In addition, a p53 response element was also found on *Per2*, located at -45 bp Per2 DNA promoter sequence, p53 negatively regulates *Per2* expression(Miki et al., 2013) (Figure 6-1). Moreover, crosstalk generally exists in these signling pathways. Glucocorticoid receptors were involved in regulating rhythmicity expression of circadian genes(Al-Safadi et al., 2015; Lamia et al., 2011; Segall and Amir, 2010; Segall et al., 2006), surprisingly glucocorticoids cause an inhibition of heat shock factor protein 1 mediated transcription, moreover, heat shock protein 90 and 70, which are chaperones, inactivate glucocorticoid receptor transcription activity (Grad and Picard, 2007; Wadekar et al., 2001; 2004). Thus temperature pulse induced activation of the heat shock pathway controls resetting circadian clocks, which maybe obstructed by glucocorticoid pathway. Beside the antagonistic regulation between heat shock pathway and glucocorticoid signaling pathway, synergistic regulation between different signaling pathways also plays a role in regulating circadian genes rhythmicity expression. For example, pathways involving cyclic adenosine monophosphate and calcium, regulate circadian oscillators(Balsalobre et al., 2000b; O'Neill and Reddy, 2012) by activating protein kinase C (PKC) activity. PKC phosphorylates cAMP response element-binding protein (CREB), which recognized the cAMP response element and serves as a transcription factor for *Per* genes.

Evidences also point to posttranscriptional regulation of Per2 stability and its subcellular location although the molecular mechanism are not completely elucidated. Our work shows that Mdm2 mediates Per2 ubiquitination and that is disrupted by DNA damage signals resulted in circadian phase shifting. GSK 3β phosphorylates PER2 and

promotes the nuclear translocation of Per2 in cells (Iitaka et al., 2005). Interestingly, protein kinase C inhibits GSK 3β activity by phosphorylation, which indirectly delayed the circadian negative regulator move to the nucleus.

PER2 integrates signals from different pathways first, and then transmits the signals to the circadian system indirectly by the transcriptional-translational negative feedback loop. PER2 plays a significant role in controlling circadian phase response type. Based on the signal strength some may trigger type 0 circadian phase resetting, which usually resynchronize circadian clocks oscillation *via* transcriptional regulation; others may lead to type1 circadian phase resetting, with only changes phase angels *via* posttranscriptional regulation. Transcription dependent phase resetting likes the coarse focus knob while posttranscriptional modification dependent phase resetting works more likely as the fine focus knob. It would be interesting to study: i). Whether these are cross talks between these two regulation levels? ii). What kind of signaling pathways are involved; iii). What kind of conditions triggers the cross talks; iv). What are the functions of this cross talk?

Per2 plays functions other than sustaining circadian oscillations (discussed in introduction section). Our recent work shows Per2 protects p53 from Mdm2 mediated ubiquitination and primes the system for a rapid p53 response to stress. Per2 plays a pivotal role in p53 signaling pathway as it also regulates p53's transcriptional activity. Interestingly, Per2 overexpression seems to block cell cycle progression (Data not published). Thus, Per2 integrates different signaling pathways that maintain cellular homeostasis and include metabolic pathways, surviving signals, growth factors, redox status to circadian rhythm oscillation and cell cycle regulation (Balsalobre et al., 2000b; Bass and Takahashi, 2011; Cheon et al., 2013; Fukuhara et al., 2001; Gau et al., 2002; Green et al., 2008a; Hirota and Fukada, 2004).

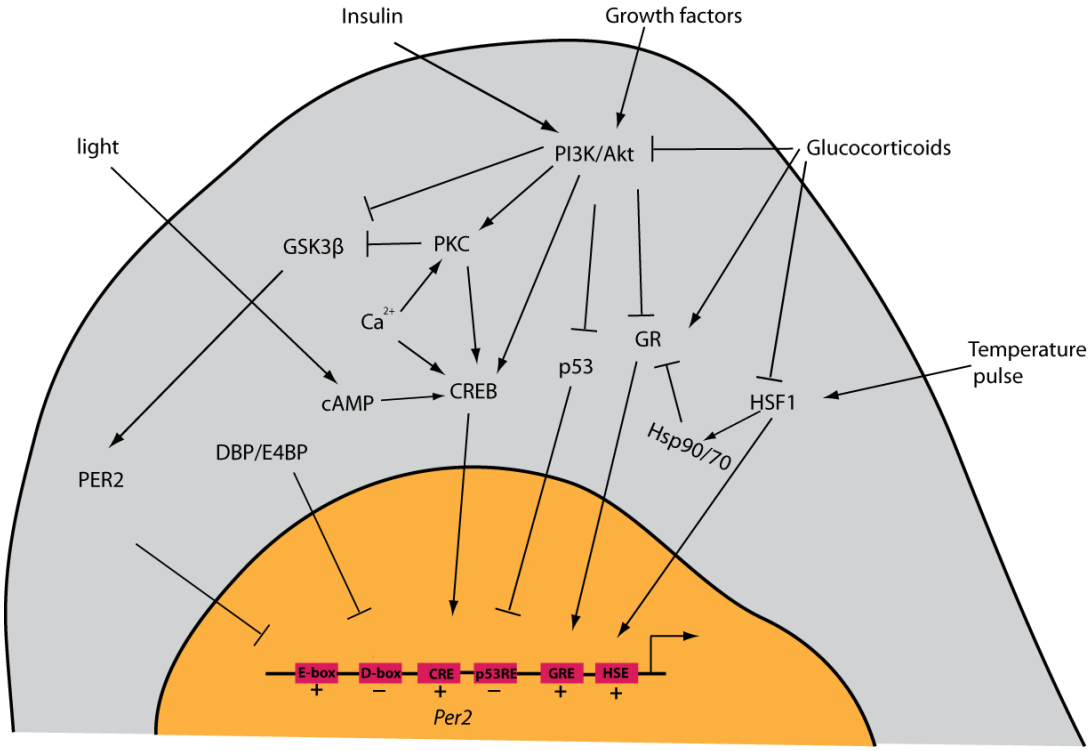

Figure 6-1 The responsive elements on Per2 promoter region.

The different responsive elements integrate signals from multiple pathways to regulate *Per2* expression, including E-box, D-box, p53-responsive elements, cAMP-resposive element (CRE), Glucocorticoid response element (GRE), and heat shock elements (HSE).

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Appendixes:

MBoC | ARTICLE

The circadian factor Period 2 modulates p53 stability and transcriptional activity in unstressed cells

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ABSTRACT Human Period 2 (hPer2) is a transcriptional regulator at the core of the circadian clock mechanism that is responsible for generating the negative feedback loop that sustains the clock. Its relevance to human disease is underlined by alterations in its function that affect numerous biochemical and physiological processes. When absent, it results in the development of various cancers and an increase in the cell's susceptibility to genotoxic stress. Thus we sought to define a yet-uncharacterized checkpoint node in which circadian components integrate environmental stress signals to the DNA-damage response. We found that hPer2 binds the C-terminal half of human p53 (hp53) and forms a stable trimeric complex with hp53's negative regulator, Mdm2. We determined that hPer2 binding to hp53 prevents Mdm2 from being ubiquitinated and targeting hp53 by the proteasome. Down-regulation of hPer2 expression directly affects hp53 levels, whereas its overexpression influences both hp53 protein stability and transcription of targeted genes. Overall our findings place hPer2 directly at the heart of the hp53-mediated response by ensuring that basal levels of hp53 are available to precondition the cell when a rapid, hp53-mediated, transcriptional response is needed.

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INTRODUCTION

Circadian rhythms are conserved mechanisms of disparate phylogenetic origin and complexity that measure time on a scale of about 24 h and adjust the organism's physiology to external environmental signals (for review, see Bell-Pedersen *et al.*, 2005). Accordingly, core circadian clock genes are genes whose protein products are necessary components for the generation and regulation of circadian rhythms. In addition to defining the core of the molecular circadian clock, high-throughput studies permitted identification of a large number of circadian molecular outputs that control many aspects of an organism's physiology, ranging from organ function and cognitive performance to systems-level behavior (Duffield *et al.*, 2002; Duffield, 2003; Panda *et al.*, 2002; Storch *et al.*, 2002). Several of those studies showed that an estimated 10% of genes in a given tissue exhibit a pattern of circadian expression and ~7% of all

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T.G. performed all experiments except for those mentioned in what follows. M.V.-C. provided data in Figures 3C, S2A, and 4, A and C, and performed all statistical analyses. J.J.L. provided data in Figure 4B and Supplemental Figure S2B and blots in Figure 4C and contributed to many stages of this research. C.S.S. performed the research included in Figure 1, A, E, and F, and Supplemental Figure S1. J.Y. contributed data shown in Figure 1, B and C. C.S.S. and J.Y. generated reagents used throughout this research. C.V.F. and C.S.S. conceived this project. C.V.F., T.G., and M.V.-C. analyzed the overall data, refined the hypothesis, and proposed the model. C.V.F. supervised and coordinated all investigators for the project and wrote the manuscript.

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Abbreviations used: *BAX*, encodes Bcl-2–associated X protein (Bax); *CDKN1a*, encodes cyclin-dependent kinase inhibitor human p21 (hp21$^{WAF1/CIP1}$); *GADD45α*, encodes the growth arrest and DNA-damage-inducible protein 45 α (Gadd45α); H1299, human non–small cell lung carcinoma cells; HCT116, human colon carcinoma cells; HEK293, human embryonic kidney 293 cells; hp53, human p53 transcription factor; hPer2, human Period 2; Mdm2, murine (human) double minute-2; mPer2, mouse Period 2; *NR1D1*, encodes orphan nuclear receptor Rev-erbα; *SFN*, encodes 14-3-3σ.

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circadian-controlled genes regulate either cell-cycle progression or cell death processes (Duffield *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002; Lowrey and Takahashi, 2004). These observations led to the hypothesis that the circadian and cell cycle systems operating within an individual cell are interlocked through the sharing of some critical elements. An emerging relationship among these mechanisms arises from the observation that cell-cycle progression is impaired in circadian gene–deficient animals, providing the first direct link between circadian gene regulation and cell division (Matsuo *et al.*, 2003). A less predictable discovery was that mutation of one of the circadian clock genes, *period 2*, resulted in a cancer-prone animal that developed spontaneous tumors more rapidly than normal animals. Moreover, this tumor-rate difference increased when animals were exposed to γ-irradiation (γ-IR), arguing for a role of the *per2* gene in tumor suppression and DNA damage response through control of cell proliferation and death (Fu *et al.*, 2002).

We focus our studies on Per2, a molecule whose function and structural organization is viewed as pivotal for its role as a "molecular integrator" of the organism's physiology and biochemical network (Albrecht *et al.*, 2007). However, Per2's potential pleiotropic functions remain to be established. Not surprisingly, regulation of *per2* gene expression is tightly controlled by a plethora of transcription factors that either interact directly within regulatory regions of *per2*, such as p53 (Miki *et al.*, 2013), or modulate the activity of other components of the transcription machinery (Horikawa *et al.*, 2000; Fukuhara *et al.*, 2001; Cajochen *et al.*, 2006; Segall *et al.*, 2006). Accordingly, fine-tuning of Per2 stability, localization, and posttranscriptional and posttranslational modifications is relevant for Per2 interaction with various proteins and ligands, all of which have an effect on numerous regulatory signaling pathways (for review, see Albrecht *et al.*, 2007, and references therein).

To understand fully how Per2's function and oscillatory behavior modulate signaling events that influence critical aspects of disease development, we first looked for novel partners of human Period2 (hPer2) that were essential components of cell proliferation and death pathways. We then investigated the regulatory consequences of their association in cell signaling. In the present study, we report the identification of the human tumor suppressor p53 (hp53 hereafter) as a novel direct interactor of hPer2, a finding supported by two-hybrid screening studies and further validated in cells. We hypothesize that hPer2, a core circadian component and tumor suppressor protein, is a novel downstream effector of the DNA-damage checkpoint pathway and is an important regulatory factor that selectively modulates p53 function. Remarkably, hPer2 association with hp53 occurs within p53's DNA-binding and C-terminal end. This region has a critical regulatory role for its tumor suppression function by virtue of containing the tetramerization domain required for hp53 oligomerization, the nuclear localization signals needed for shuttling, and various residues targeted for posttranslational modification, including those that are polyubiquitinylated (Kruse and Gu, 2009). Moreover, our findings establish the presence of a trimeric complex in cells in which the murine double minute-2 (Mdm2) E3 ubiquitin ligase, a negative regulator of hp53 that acts by modifying the hp53's C-terminus and promoting its proteasomal degradation, binds to the N-terminus region of hp53. Conversely, hPer2 modulates the extent of Mdm2-mediated ubiquitination by binding to the C-terminus portion of hp53. Furthermore, our studies show that hPer2 binding to hp53 directly controls hp53-mediated transcriptional activity, suggesting a novel multilevel mechanism for regulating hp53 function to include circadian components. These findings not only provide important insights into the already complex mechanism of hp53 regulation, but also shed light on the emerging role of circadian components as modulators of checkpoint molecules, thus supporting a potential role for circadian factors in tumorigenesis.

RESULTS

Mice without functional mouse Period2 (mPer2) show increased sensitivity to γ-IR exposure and reduced apoptotic response, all of which are phenotypically manifested by premature hair graying and hair loss, early onset of hyperplastic growth, increased tumor occurrence, and severe morbidity (Fu *et al.*, 2002). Despite the lack of a detailed mechanistic foundation for these observations, a number of cell cycle and tumor suppressor genes known to be under circadian control (*CCND1*, *CCNA2*, and *MYC*, which encode cyclin D1, A, and c-myc, respectively) were found to be deregulated in *per2*-deficient animals (Fu *et al.*, 2002). This suggests a role of Per2 in tumor suppression by modulating DNA-damage-responsive pathways. Therefore we focused our initial studies on identifying hPer2 partners acting on key nodes of the protein–protein interaction network whose circadian deregulation can directly affect cellular homeostasis.

The hPer2 transcription factor interacts with the tumor suppressor protein p53

As part of an effort to define novel factors important for hPer2 regulation, we used a bacterial two-hybrid system to screen a human liver cDNA library to search for interacting partners. We chose this library because liver is known to be a peripheral oscillator tissue, and there are comprehensive studies on how circadian components are interlocked and operate in liver tissue (Lamia *et al.*, 2008). Three baits were independently used for screening: full-length hPer2 and two fragments of cDNA encoding the N-terminal (residues 1–821) and C-terminal (residues 822–1255) regions of hPer2. These regions were chosen because of their relevance to Per2 function in various cellular processes and the presence of functional and structural domains known to bind protein counterparts (Griffin *et al.*, 1999; Kume *et al.*, 1999) or small ligand molecules (Yang *et al.*, 2008). The human liver cDNA library (primary size, 6.9×10^6) was screened with the generated pBT recombinant plasmids. Approximately 4×10^6 clones were screened, and 120 were identified as putative positive interactors (67 strong and 53 weak interactors). These clones were maintained in nonselective media containing antibiotics and later patched on selective screening medium containing 5 mM 3-amino-1,2,4-triazole (3-AT) (Figure 1A). All putative clones were then subjected to further screening on a dual selective screening medium (5 mM 3-AT and streptomycin) and confirmed positive. To validate protein–protein interactions, we performed retransformation of the reporter strain using the pTRG-positive clones and recombinant pBT baits. Our results show that 17 clones, which include proteins involved in cellular metabolic processes, RNA binding, regulation of programmed cell death, transcriptional activation, response to stress, and cell cycle progression, reproducibly grow on selective screening medium when cotransformed with the bait plasmid but failed to grow under the same conditions when cotransformed with the empty pBT vector. Among the clones identified in the screening were hp53, the translationally controlled tumor protein (TCTP), and various fragments encoding open reading frame regions of the circadian factor crytochrome (Cry), a known direct interactor of hPer2 (Figure 1A). Remarkably, hp53 was also identified as a positive interactor when screenings were carried out using the sequences encompassing the N- and C-terminal fragments of hPer2, suggesting that more than one interaction site for hp53 might exist within the circadian factor.

To investigate whether Per2 forms a complex with p53 in cells, we examined various scenarios in which recombinantly expressed proteins and their endogenous counterparts were monitored for direct interaction in mammalian cells and extracts (Figure 1, B–D, and Supplemental Figure S1). We analyzed diverse cell lines (CHO cells, human embryonic kidney 293 [HEK293], and human colon carcinoma [HCT116] cells) to probe the prevalence of the complex in different backgrounds. These cell lines, besides being relatively easy to manipulate, were chosen because 1) CHO cells can be circadian synchronized, and mPer2 has been shown to be functional in this system (Yang *et al.*, 2008), 2) human HEK293 cells have been an effective model to study the dynamic process of shuttling among hPer proteins as a result of posttranslational modifications (Vielhaber *et al.*, 2001), the transcriptional regulation of genes by Clock/NPAS2 and Bmal dimers (Shi *et al.*, 2010), and the validation of neurotransmitters regulating circadian locomotor rhythms (Mertens *et al.*, 2005), and, finally, 3) human carcinoma HCT116 cells were used to study the role of Per1 in cell growth (Gery *et al.*, 2006), the contribution of specific SCF ubiquitin E3 ligases for circadian protein turnover (Yang *et al.*, 2009a), and the role of hPer2 for expression of cell cycle genes (Yang *et al.*, 2009b). In addition, all cell lines express the wild-type forms of p53 and Per2.

To examine the interaction between p53 and Per2, we first transfected CHO cells with *myc*-hp53 and evaluated the presence of endogenous mPer2 within the complex by immunoprecipitation (Figure 1B). Results show mPer2 associates with the recombinantly expressed protein, further supporting our two-hybrid data, whereas unbound proteins remained available in the soluble fraction (Figure 1B, top). Endogenous mPer2 is usually detected as a doublet by its specific antibody in CHO cells, as previously described (Yang *et al.*, 2008). We also performed reciprocal coimmunoprecipitation assays using CHO extracts from cells cotransfected with *myc*-hPer2, FLAG-hp53, or both plasmids. As shown in Figure 1C, immunoprecipitation of recombinant hPer2 specifically associates with hp53, a result of relevance for validating further in vitro experiments. The prevalence of this interaction was then assessed in both HEK293 and HCT116 cells by immunoprecipitating both endogenous components of the hp53/hPer2 complex (Figure 1D). Thus cell extracts were immunoprecipitated with either α-Per2 antibody or immunoglobulin G (IgG). As expected, immunoblot analysis revealed that hp53 is only detected in immunoprecipitates obtained using the α-Per2 antibody but not control IgG (Figure 1D). In sum, these results indicate that p53 and Per2 can be coimmunoprecipitated from extracts of either transfected cells or endogenous pools and are consistent with direct interaction between both proteins. To further test for this latter possibility directly, we incubated recombinant glutathione *S*-transferase (GST)-hp53–bound beads with labeled *myc*-hPer2 in the presence of increasing concentrations of untagged hp53 (Supplemental Figure S1A). Our results indicate that hPer2 is efficiently displaced from the complex as a result of adding increasing amounts of hp53, which support a model of direct interaction among these two protein components.

The hPer2 protein associates with the C-terminal half of hp53

To define the regions in hp53 and hPer2 responsible for their interaction, we first generated a number of recombinant constructs based on sequence homology, secondary structure prediction, and molecular modeling for each protein and determined their binding capacity to its full-length radiolabeled counterpart. Each of the seven recombinant fragments of hPer2—GST-hPer2(1-172), GST-hPer2(173-355), GST-hPer2(356-574), GST-hPer2(575-682), GST-hPer2(683-872), GST-hPer2(873-1120), and GST-hPer2(1121-1255)—was incubated with ^{35}S-labeled *myc*-hp53 and protein association evaluated by pull-down assays (Figure 1E and Supplemental Figure S1B). Results showed that hp53 interacts within the central region of hPer2 comprising residues 356–574 (C-terminus of the PAS domain) and 683–872, a stretch of sequence predicted to be structurally flexible and strongly posttranslationally processed (www.expasy.org/structural_bioinformatics and www.expasy.org/proteomics/post-translational_modification). Similar studies were carried out in the presence of ^{35}S-labeled *myc*-hPer2 using various constructs of hp53, including the recombinant GST-hp53(1-200), GST-hp53(1-296), GST-hp53(1-325), GST-hp53Δ30, GST-hp53(100-310), GST-hp53(200-393), and GST-hp53(300-393) fragments (Figure 1F and Supplemental Figure S1C). Pull-down assays showed that hPer2 binds hp53 on its C-terminal half, comprising most of the DNA-binding domain and oligomerization regions. Current models establish the DNA-binding functionality of hp53 to be dependent on both the core DNA-binding domain to provide for sequence specificity and the C-terminal region to recognize topological structural features of the target DNA (Kim *et al.*, 1999; Ayed *et al.*, 2001). Thus our results support a model of direct association between hPer2 and hp53 and predict a functional regulatory role for these proteins when acting as a complex.

The hPer2 protein forms a ternary complex with hp53 and its negative regulator, the Mdm2 oncogene protein

The Mdm2 protein acts as a bona fide E3-ubiquitin ligase for p53 by binding to its N-terminus and promoting either p53 monoubiquitination and nuclear export or p53 polyubiquitination and degradation through the proteasomal pathway (Honda *et al.*, 1997; Li *et al.*, 2003). Our results show that hPer2 primarily interacts within the C-terminal half of hp53. Thus it is possible that Mdm2 would also be sequestered into the complex. Extracts from HEK293 and HCT116 cells were immunoprecipitated with α-p53 and α-Per2 antibodies, respectively, and complex molecules were detected by immunoblotting (Figure 2A). Consistent with this hypothesis, coimmunoprecipitation and immunoblotting showed that, in both cases and under physiological conditions, Mdm2 associates with hp53/hPer2. This reveals the presence of the hp53/hPer2/Mdm2 endogenous complex in cells (Figure 2A). This result is in line with observations that all three proteins colocalize in similar cellular compartments and thus their interaction could readily occur (Supplemental Figure S2A; Giannakakou *et al.*, 2000; Lohrum *et al.*, 2000; Miyazaki *et al.*, 2001).

To study the functional relevance of interactions of these molecules, we devoted further experiments to establishing an "in-cell" system that allowed us to manipulate the complex components. HEK293 cells were transfected with various tagged forms of hPer2, hp53, and Mdm2 and complexes were monitored by immunoprecipitation and blotting (Figure 2B). Results show ternary complexes that included Mdm2 were detected using reciprocally tagged forms of both hp53 and hPer2 proteins (Figure 2B, left dashed box). Furthermore, an additional finding established that hPer2 is able to interact and form a stable complex with Mdm2 (Figure 2B, right dashed box). To rule out the contribution of endogenous hp53 in bridging hPer2/Mdm2 association, we transfected human non–small cell lung carcinoma cells (H1299 cells, p53-null; they contain a homozygous partial deletion of the p53 protein and lack expression of p53) with FLAG-hPer2 and *myc*-Mdm2 and evaluated their interaction by coimmunoprecipitation (Supplemental Figure S2B). Interaction between hPer2 and its E3-ubiquitin ligase β-TrCP was

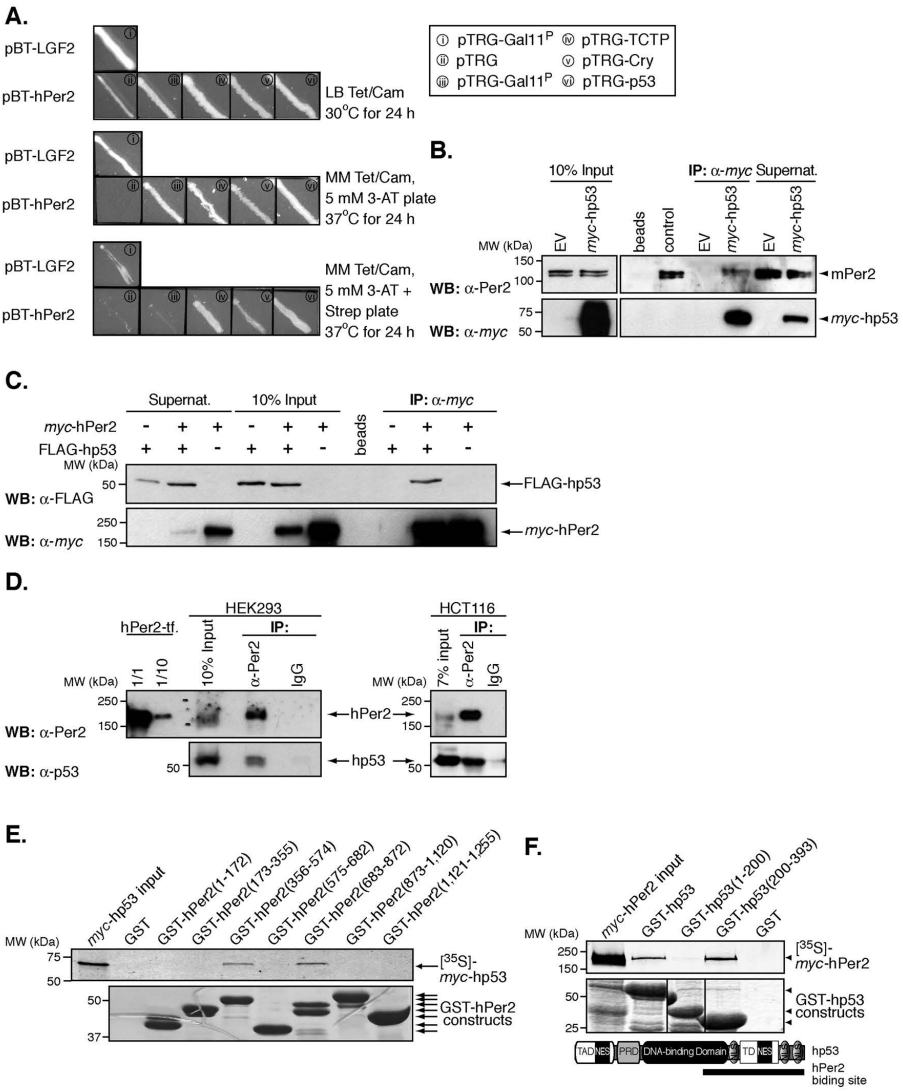

FIGURE 1: The circadian factor hPer2 interacts with hp53. (A) Two-hybrid protein–protein interaction between hPer2, hp53, TCTP, and Cry. Each pair of plasmids—i) pBT-LGF2 + pTRG-Gal11^{P}, ii) pBT-hPer2 + pTRG, iii) pBT-hPer2 + pTRG-Gal11^{P}, iv) pBT-hPer2 + pTRG-TCTP, v) pBT-hPer2 + pTRG-Cry, and vi) pTRG-hp53—was grown on nonselective medium plus antibiotics (LB tetracycline [Tet]/chloramphenicol [Cam]) and later patched on both selective screening minimum medium (MM Tet/Cam/5 mM 3-AT) and dual-selective minimum medium containing MM Tet/Cam/5 mM 3-AT/streptomycin (Strep). Positive controls were i and v, whereas ii and iii were negative. (B) Pellets from CHO cells transfected with pCS2+*myc*-hp53 were lysed in 25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, and 1% Triton X-100, and extracts (~300 μg) were incubated with α-*myc* beads. Endogenous mPer2 and recombinantly expressed hp53 were detected by using either α-Per2 (top) or -*myc* antibodies (bottom). Control indicates 20 μg of total extract. (C) Samples from CHO cells cotransfected with pCS2+*myc*-hPer2 and pCS2+FLAG-hp53 were immunoprecipitated using α-*myc* beads and immunoblotted using α-FLAG (top) and -*myc* antibodies (bottom). (D) One milligram of HEK293 and HCT116 extracts was incubated with either α-Per2 or IgG. Complexes were immunoprecipitated using protein A beads and immunoblotted for endogenous proteins using α-Per2 (top) and -p53 antibodies (bottom). For the positive control, HEK293 cells were transfected with pCS2+hPer2 (hPer2-tf), and total cell extracts (20 μg [1/1] and 2 μg [1/10]) were loaded. (E) Recombinant GST-tagged fragments of hPer2 were purified using affinity chromatography, and bound beads were incubated with ^{35}S-labeled *myc*-hp53 and assayed for binding as described in the Supplemental Material. Bound

used as a positive control (Supplemental Figure S2B; Ohsaki *et al.*, 2008). Results indicate that the hPer2/Mdm2 complex exists even in an hp53-null background, providing evidence for the physical association of hPer2 to Mdm2 in a p53-independent manner. Further studies established that the formation of the hp53/hPer2/Mdm2 complex is independent of the order of association of its components, as shown in Supplemental Figure S2C. In vitro binding of transcribed and translated recombinant hp53, hPer2, and Mdm2 into the trimeric complex occurred when hp53 was first associated with either Mdm2 or hPer2. Of note is the level of hPer2 associated with the preformed hp53/Mdm2 complex versus the one detected associated with hp53. This level seems to be lower, suggesting that binding of hPer2 precedes Mdm2 association with hp53 (Supplemental Figure S2C, lane 1 vs. lane 3). Overall these results suggest a dynamic scenario in which hPer2 might influence the activity and/or function of the Mdm2/hp53 complex or any of its components in a model in which Mdm2 acts as an E3-ubiquitin ligase for hp53, further linking components of the checkpoint machinery to circadian factors.

Binding of hPer2 modulates the extent of Mdm2-mediated hp53 ubiquitination

Next we asked whether binding of hPer2 to the C-terminus of hp53 modulates the extent of its ubiquitination when complexed to Mdm2. To test this possibility, we used a modified in vitro ubiquitination assay in which hp53, Mdm2, and hPer2 proteins were incorporated stepwise into a cell-free system. First, we allowed the hp53/hPer2 complex to form, followed by addition of Mdm2 in the presence of inhibitors of both proteasome and ubiquitin deconjugating enzymes, thus favoring the accumulation of ubiquitinated hp53 complexes (FLAG-hp53$(Ub)_n$) before immunoprecipitation (Figure 2C and Supplemental Figure S4A). Results show that hPer2 efficiently prevented Mdm2-mediated hp53 ubiquitination when prebound. This is in contrast to what was observed when hp53 was solely incubated with Mdm2 (Figure 2C, lane 2 vs. lane 3, bottom, and Supplemental Figure S4A). A similar result was obtained using proteasome inhibitor–treated HEK293 cells cotransfected with hp53 and Mdm2 in the presence or absence of hPer2-expressing plasmid (Figure 2D, lane 3 vs. lane 4, top, and Supplemental Figure S4B). Comparative ratios of all proteins were detected in cells (Supplemental Figure S2D), complexes were efficiently formed (Figure 2D), and hp53 ubiquitinated forms were detected by specific immunoprecipitation (Figure 2D, top) and quantified (Supplemental Figure S4B). Although the data presented here suggest a role for hPer2 in controlling hp53 modifications mediated by Mdm2, they do not rule out the possibility of a deubiquitination activity associated with hPer2 that might counteract Mdm2 action. However, in vitro preliminary studies using hp53$(Ub)_n$ as a substrate suggest that this might not be the case (unpublished data). Consequently, the evidence suggests that binding of hPer2 to hp53 prevents Mdm2-mediated ubiquitination, allowing for the formation of a stable trimeric complex.

Binding of hPer2 influences hp53 stability

Rhythmic expression of p53 protein, but not its mRNA, has been observed in human tissues and correlates with those of the circadian oscillators Per and Bmal1 (Bjarnason *et al.*, 1999, 2001). Remarkably, hp53 oscillations are not due to rhythmic expression of Mdm2, since its mRNA and protein levels exhibit a very modest oscillation throughout a 24-h cycle (Fu *et al.*, 2002; Panda *et al.*, 2002). Consequently, we hypothesized that, when available, hPer2 binds to the C-terminal half of hp53 and inhibits its Mdm2-mediated ubiquitination, thus stabilizing hp53. To test this possibility, we knocked down the endogenous expression of hPer2 using small interfering RNA (siRNA) in HCT116 cells and monitored hp53 stability in the presence of cycloheximide (CHX), an inhibitor of protein translation (Figure 3A). Effective down-regulation of hPer2 was achieved 48 h after siRNA transfection (Figure 3A, top, lane 1 vs. lane 7) and was sustained over the time course analyzed (Figure 3A, top, lanes 7–12). Expression of hp53 was then evaluated at different times after CHX addition ($t = 0$, Figure 3A, middle). Our findings show that endogenous hp53 levels dramatically dropped 1 h after CHX treatment (from 100 to 70 and 40% in mock- vs. siRNA-treated samples, respectively (Figure 3A, middle, lane 3 vs. lane 9) and decreased thereafter (Figure 3B, top). Thus hp53 half-life ($t_{1/2}$) decreased ~60% in the absence of hPer2 expression, supporting a role for hPer2 in hp53 stability. Accordingly, we observed a direct correlation between the down-regulation of hPer2 after CHX addition in mock samples and the progressive decrease of hp53 (Figure 3A, lanes 1–6) to the point in which hPer2 was undetectable and only trace levels of hp53 were identified (Figure 3, A, lane 6, and B). It is worth noting both the role of de novo transcription/translation in keeping hp53 levels detectable in the cell even in the absence of hPer2 as evidenced in Figure 3A (lane 7) and the relevance of hPer2 presence in sustaining hp53 levels (Figure 3A, middle). A clearer picture of the effect of hPer2 in hp53 stability arises from the quantitative analysis of the experiments shown in Figure 3A and summarized in Figure 3B. Here we represent the remaining levels of hp53 (Figure 3B, top) and hPer2 (Figure 3B, bottom) detected in both mock- and siRNA hPer2 CHX-treated cells. The drop in hp53 levels is evident within the first 2 h post CHX treatment (Figure 3B, top, shaded box) and is concomitant with a decrease in hPer2 levels in mock samples (Figure 3B, bottom, shaded box). Further support for our observations came from transfection studies in which hPer2 was overexpressed in HCT116 and endogenous hp53 levels were monitored after CHX addition (Supplemental Figure S3). As a result, we observed a significant increase (~60%) in endogenous hp53 levels in samples transfected with hPer2 ($t = 0$; Supplemental Figure S3). As expected, this effect was sustained while hPer2 was overexpressed in cells but dropped dramatically once CHX affected hPer2 translation, and its own stability was compromised by 4 h (Supplemental Figure S3A, middle, and graph, shaded box).

Although our results establish a role for hPer2 in promoting hp53 stability, its influence on total hp53 cellular levels includes an additional transcriptional component when hPer2 is overexpressed. This

complexes were visualized by Coomassie staining (bottom) and the radiolabeled protein detected by autoradiography (top). (F) Mapping of hPer2-binding regions in hp53. Schematic representation of hp53 architecture (393 residues), including the transactivation (TAD; residues 1–42), proline-rich (PRD; residues 61–92), DNA-binding (residues 101–300), and tetramerization domains (TD; residues 326–356). The binding site in hPer2 is indicated with a solid line. Bead-bound GST-hp53 and recombinant proteins were incubated with [^{35}S]*myc*-hPer2 and analyzed for complex formation as described. In all cases, GST beads were used as a negative control; beads, matrix sample with no antibody bound; EV, empty vector; Supernat, supernatant fraction after immunoprecipitation (IP); transf, transfected cells; B–F show immunoblot data from a single experiment that was repeated three times with similar results.

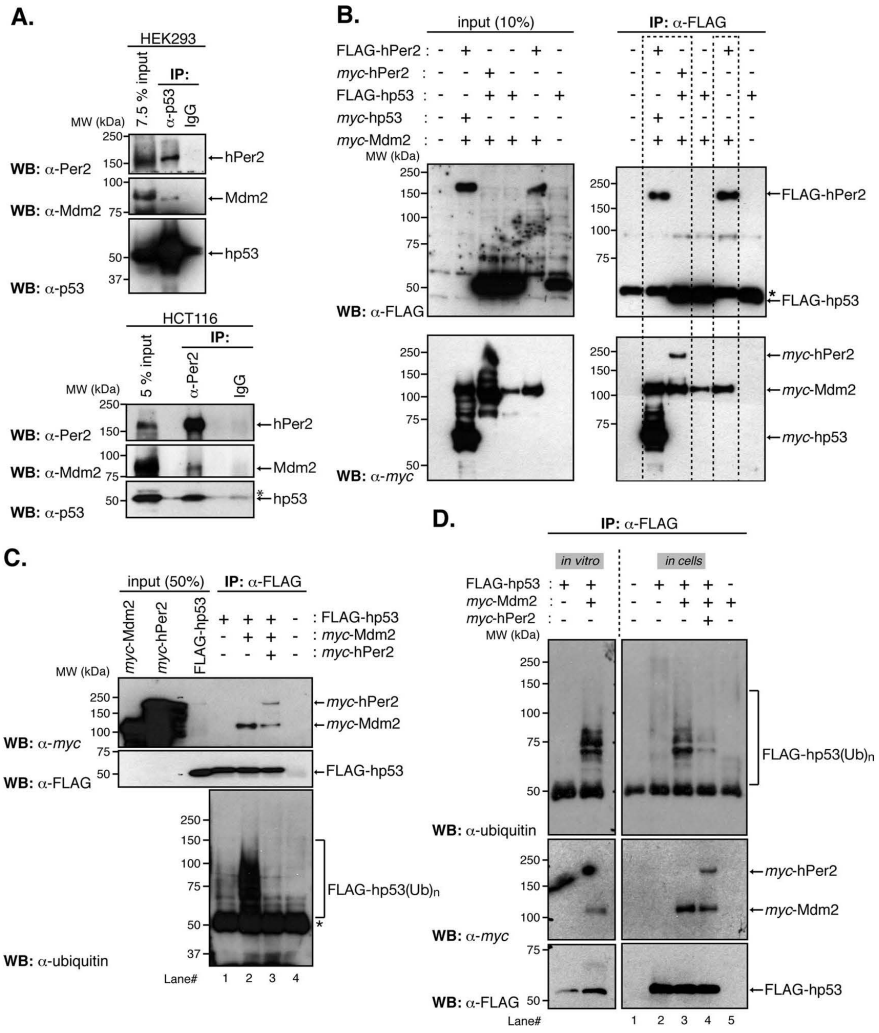

FIGURE 2: The hPer2 protein forms a ternary complex with hp53 and Mdm2, controlling the extent of hp53 ubiquitination. (A) HEK293 and HCT116 protein extracts (1 and 2 mg, respectively) were incubated with either α-p53 or α-Per2, respectively, and protein A beads. Rabbit IgG was used as a negative control. Immunoprecipitated complexes were analyzed for the presence of hPer2, hp53, and Mdm2 using specific antibodies (top for HEK293 and bottom for HCT116). Asterisk indicates a nonspecific signal. (B) HEK293 cells were transfected with pCS2+*myc*-Mdm2, pCS2+*myc*-hp53, pCS2+FLAG-hp53, pCS2+FLAG-hPer2, pCS2+*myc*-hPer2, or a combination of plasmids and complexes immunoprecipitated using α-FLAG–coupled beads. Complex components were identified by immunoblotting using α-FLAG and α-*myc* antibodies (right top and bottom). Input amounts were monitored in cell lysates (20 μg) and shown in the left top and bottom. Results similar to those presented were observed in two independent experiments. (C) In vitro–synthesized *myc*-hPer2 and FLAG-hp53 proteins were preincubated before the addition of *myc*-Mdm2 (ratio 1:2:5: FLAG-hp53:*myc*-Mdm2:*myc*-hPer2). Samples were then subjected to in vitro ubiquitination, followed by immunoprecipitation of hp53-bound proteins using α-FLAG antibody and protein A beads. Bound proteins were detected by immunoblotting and are indicated with arrows (top and middle). FLAG-hp53$(Ub)_n$ forms of hp53 were detected using α-ubiquitin antibody (bottom). Asterisk indicates IgG heavy chain. (D) HEK293 cells were transfected with pCS2+*myc*-Mdm2, pCS2+*myc*-hPer2, pCS2+FLAG-hp53, or a combination of plasmids and collected 12 h after treatment with 10 μM MG132. Cell lysates (100 μg) were incubated with α-FLAG and protein A beads and hp53-ubiquitinated complexes (FLAG-hp53$(Ub)_n$) detected using α-ubiquitin antibody (top right). Bound proteins were visualized by immunoblotting using α-*myc* and α-FLAG antibodies (middle and lower right). An in vitro ubiquitination reaction was performed as described in C and is shown as control and for comparison purposes with the "in cells" result (left). Brackets denote ubiquitinated hp53. A, C, and D show immunoblot data from a single experiment that was repeated three times with similar results.

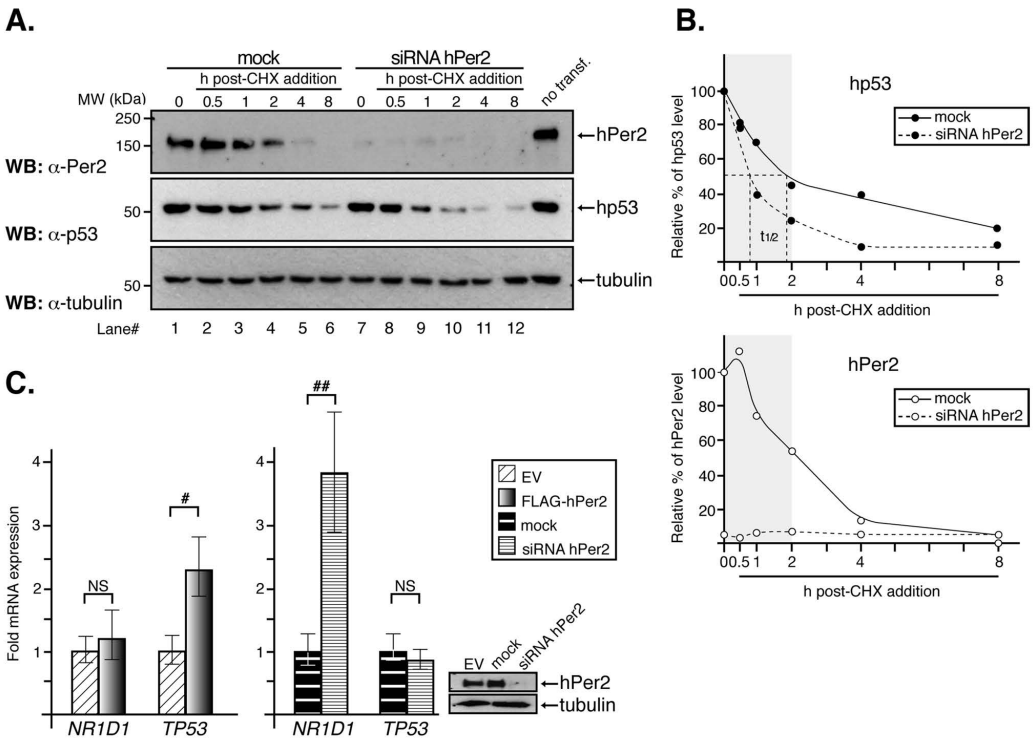

FIGURE 3: Binding of hPer2 to hp53 modulates its stability. (A) HCT116 cells were transfected with siRNA hPer2 (25 nM) or not (mock), and a sample equivalent to $t = 0$ h was collected 72 h later. Samples ($t = 0.5$–8 h) were harvested from cells maintained in complete medium plus cycloheximide (CHX, 100 μg/ml). Extracts were analyzed by immunoblotting using specific antibodies. A nontransfected control sample was loaded onto lane 12 and indicates the position of endogenous proteins. Immunoblot data from a single experiment that was repeated three times with similar results. (B) Protein levels (hPer2 and hp53) from mock- and siRNA hPer2-treated samples were quantified using ImageJ, version 1.45 (Schneider *et al.*, 2012), and values were normalized to tubulin levels. Bar graphs indicate the percentage of protein remaining plotted as a function of time. The curve was fitted and hp53 half-life calculated using GraphPad Prism software (top). The gray box indicates the window of time while endogenous hPer2 was readily detected in mock samples. Data from a single experiment that was repeated three times with similar results. In all cases, mock and siRNA are represented by solid and dashed lines, respectively, and hp53 and hPer2 proteins are symbolized by ● and ○, respectively. (C) HCT116 cells were transfected with either pCS2+FLAG-hPer2 (left bar graph) or siRNA hPer2 (right bar graph) and samples subjected to qRT-PCR as described in *Materials and Methods*. Data are presented as mean ± SEM from three independent experiments performed in triplicate. Statistical comparisons were done by two-tailed unpaired *t* test and analyses performed using SPSS. NS, not significant; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$. In all samples, endogenous hPer2 levels were monitored by immunoblotting and before performing quantitative RNA analyses. Tubulin was a loading control (right).

scenario became clear from experiments performed in HCT116 cells transfected with either FLAG-hPer2 or hPer2 siRNA and where hp53 mRNA levels were measured in real time (Figure 3C). It also became clear from analyses of lung cancer cells, in which overexpression of hPer2 led to an increase in p53 mRNA and p53-mediated apoptosis (Hua *et al.*, 2006). As shown in Figure 3C (left), overexpression of hPer2 resulted in a significant increase in *TP53* transcription (encodes p53), an effect that was overturned in cells transfected with siRNA hPer2 (Figure 3C, right). Transcription of *NR1D1* (encodes orphan nuclear receptor Rev-erbα) was used as an internal control, as its level is influenced by the presence of hPer2 in the cell, with repression taking place when hPer2 is at its highest (Ko and Takahashi, 2006). In summary, our data place hPer2 as a modulator of hp53 cellular levels by acting through different mechanisms; a canonical signaling pathway via *TP53* gene transcription and, independently, a noncanonical pathway that involves hPer2 binding to hp53, modulation of Mdm2-mediated hp53 ubiquitination and therefore hp53 stability.

The hPer2 factor influences the expression of hp53-target genes

We then asked whether increased stability of hp53 as a result of hPer2 transfection (Supplemental Figure S3) affects activation of hp53-mediated gene transcription, thus functionally linking hPer2 to the hp53 pathway. First, we monitored the expression of p53-targeted genes in HCT116 cells that were either overexpressing

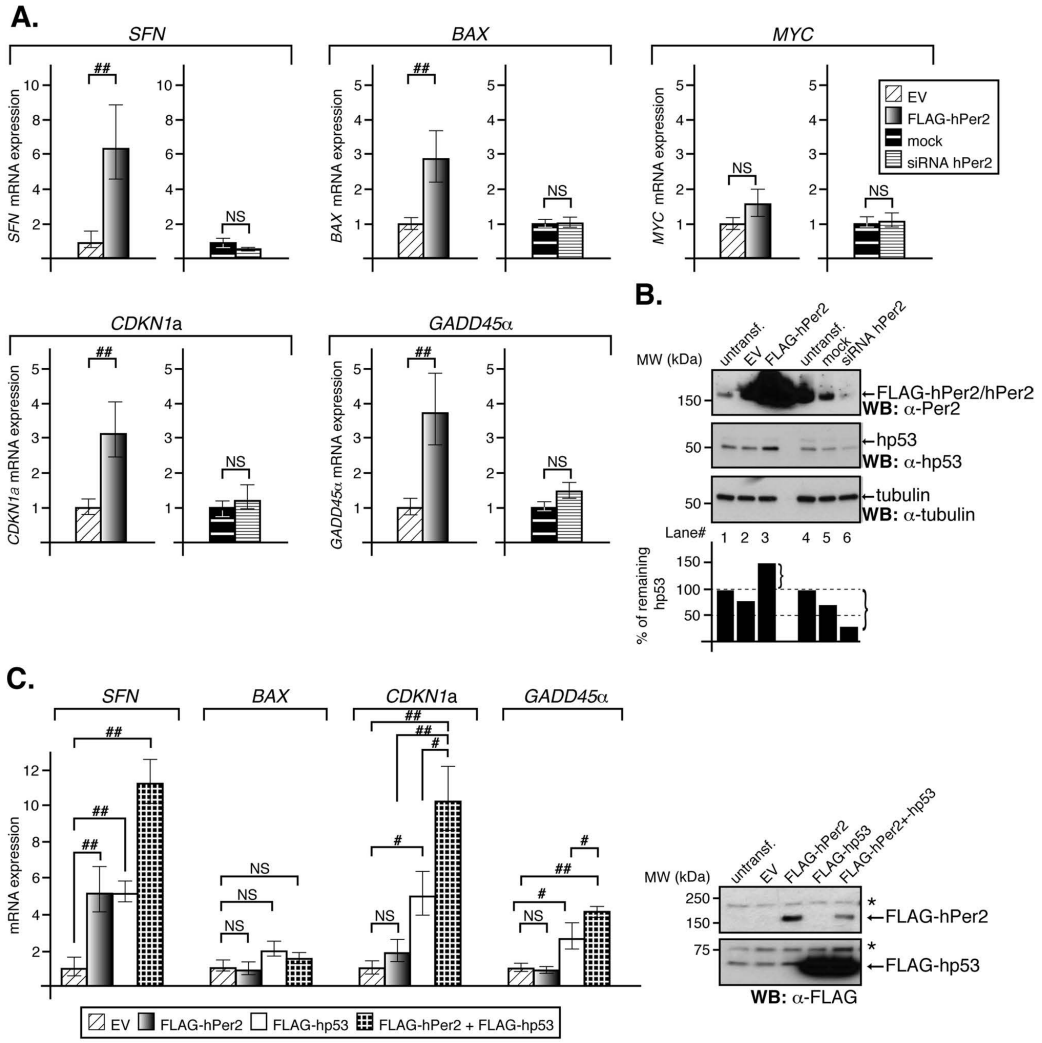

FIGURE 4: The hPer2 protein influences the expression of hp53 target genes. (A) HCT116 cells were transfected with either FLAG-hPer2 or siRNA hPer2 and collected at 24 and 48 h posttransfection, respectively. Empty vector (EV) and mock samples were controls. qRT-PCR data are presented as the mean ± SEM from three independent experiments performed in triplicate. Statistical comparisons were done by two-tailed unpaired *t* test and analyses performed using SPSS. NS, not significant; $^{\#\#}p \leq 0.01$. (B) HCT116 cell extracts (20 μg) from the various samples in A were analyzed for hPer2 (top), endogenous hp53 (middle), and tubulin (bottom) by immunoblotting. Bands were quantified using ImageJ, version 1.45, and values normalized to tubulin levels (loading control). Bar graphs indicate the percentage of the remaining hp53 protein. Immunoblot data from a single experiment that was repeated three times with similar results. (C) H1299 cells were transfected with pCS2+FLAG-hPer2, pCS2+FLAG-hp53, empty vector (EV), or a combination of plasmids. Cells were harvested 24 h after transfection and samples prepared for qRT-PCR. Aliquots of cell extracts were analyzed by immunoblotting (right). Asterisks indicate nonspecific signal. Data are presented as the mean ± SEM from three independent experiments performed in triplicate. Statistical comparisons were evaluated by ANOVA using Bonferroni or Games–Howell post hoc analyses when needed (SPSS). NS, not significant; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$. *SFN* encodes 14-3-3σ; *BAX* encodes the proapoptotic factor Bax; *MYC* encodes the oncogene c-myc; *CDKN1a* encodes hp21$^{WAF1/CIP1}$; *GADD45α* encodes the gadd45α protein.

hPer2 (Figure 4A, left, for each set of genes) or silenced for hPer2 expression (Figure 4A, right, for each set of genes). We chose to analyze the expression of those genes because they represent the diversity of cellular pathways controlled by p53 and the various forms of regulation, ranging from transcriptional repression (i.e., *MYC*) to activation (i.e., *SFN* [encodes 14-3-3σ], *BAX*

[encodes Bcl-2–associated X protein (Bax)], *CDKN1a* [encodes cyclin-dependent kinase inhibitor human p21 (hp21$^{WAF1/CIP1}$)], and *GADD45α* [encodes the growth arrest and DNA-damage-inducible protein 45 α (Gadd45α)]). Accordingly, hPer2-transfected cells showed a significant increase in expression of *CDKN1a*, *SFN*, *GADD45α*, and the proapoptotic component *BAX*, whereas the expression of the *MYC* oncogene remained invariable (Figure 4A, left, for each set of genes). Specificity of response toward hPer2 was assessed by effectively abrogating its expression using siRNA in HCT116 and monitoring gene transcription in real time (Figure 4A, right, for each set of genes). As shown, down-regulation of hPer2 counteracts the effect of the transcription of p53-target genes triggered by hPer2 overexpression, supporting a role for this circadian modulator in their transcriptional control. Consistent with our previous observations, increased hp53 levels were solely observed in hPer2-transfected samples (Figure 4B), supporting a model in which hPer2 action on hp53-mediated transcription might be the result of its stabilization.

Next we wanted to ascertain hp53 as the chief mediator of the hPer2 transcriptional effect shown in Figure 4A. We reasoned that if hPer2 induces *CDKN1a*, *SFN*, *GADD45α*, and *BAX* transcription via a p53-dependent pathway, transfection with hPer2 should not restore their expression in an hp53-null background unless either a p53-independent mechanism exists or hp53 is cotransfected along with hPer2 and the p53-mediated signaling pathway is restored. To test this possibility, H1299 cells (p53-null) were transfected with FLAG-tagged forms of hp53, hPer2, or both plasmids together (Figure 4C, inset) and analyzed for transcription of specific genes in real time (Figure 4C). Results show FLAG-hPer2 by itself was unable to significantly induce the expression of *CDKN1a*, *GADD45α*, or *BAX* in a p53-null background. Although the contribution of other mediators and additional signaling mechanisms cannot be completely ruled out, hPer2 seems to act primarily through hp53 and not vice versa, as hp53 does not seem to have a direct effect on hPer2 mRNA and protein expression in HCT116 cells (Supplemental Figure S5). Of interest, *SFN* expression seemed to be enhanced by just hPer2 transfection, suggesting the existence of at least an hp53-independent but hPer2-dependent mechanism involved in *SFN* expression. As expected, FLAG-hp53 transfection led to increased transcriptional expression of *SFN*, *CDKN1a*, and *GADD45α*, whereas *BAX* levels remained largely unchanged. Of note, cotransfection of FLAG-hp53 and -hPer2 resulted in a synergistic effect that led to overexpression of *SFN*, *CDKN1a*, and *GADD45α* but not *BAX*.

Overall these results establish that 1) hp53 is a mediator of the hPer2 transcriptional response, 2) hp53 target genes such as *SFN* and *BAX* might have at least an additional mode of regulation by hPer2 that is independent of hp53, and 3) hPer2 most likely influences the ability of hp53 to function as a sequence-specific transcription factor indirectly by influencing hp53 levels. Thus our data substantiate the biological relevance of hPer2 in modulating hp53 target gene expression, and we speculate that the hPer2-hp53 axis might act on multiple hp53 canonical and noncanonical response elements to varying extents.

DISCUSSION

The emerging view of the role of core circadian clock components is that their function is no longer restricted to the generation of circadian rhythms but that they also intervene in other cellular pathways believed to be under circadian regulation. Examples are many and growing and include signaling pathways involved in cell growth, division, death regulation, metabolism, behavioral disorders, immunity, and xenobiotic responses (for review, see Takahashi *et al.*, 2008, and references therein). These findings highlight the complexity of the circadian-controlled network and emphasize its physiological relevance for human health and new therapeutic interventions.

Our findings provide the first evidence of direct protein–protein interaction between the clock factor hPer2 and the tumor suppressor hp53 (Figures 1 and 2). They are further validated by converse studies performed in human cells in search of new modulators of p53-mediated proliferation arrest using large-scale RNAi screening (Berns *et al.*, 2004). Although it is unclear how Per2 is involved in p53-mediated cell cycle arrest from the high-throughput studies, those results support Per2 as a tumor suppressor relevant to the p53 pathway. Other forms of validation of the p53–clock connection result from the identification of modifiers of the clock's amplitude and period. These include molecules that physically interact with p53 (CDK9, NCL, ABL1, BRCA2; Zhang *et al.*, 2009), down-regulation of which causes either short- or long-period changes, as well as high-amplitude phenotypes in mammalian cells (Zhang *et al.*, 2009). Furthermore, knockdown of hp53 itself generates low-amplitude circadian rhythms in U2OS cells, further linking the tumor suppressor to the clock (Zhang *et al.*, 2009).

Binding of the E3 ligase Mdm2 to the N-terminus domain of hp53 allows for the formation of a trimeric complex in which hPer2 binding to p53's C-terminus prevents its ubiquitination and promotes hp53 stabilization (Figures 2, C and D, 3, and 5). The stability of p53 has been the subject of numerous studies and is known to be largely influenced by posttranslational modifications, intracellular distribution, and binding to other interacting proteins (for review, see Lavin and Gueven, 2006). As a result, different scenarios should be considered when evaluating the mechanism by which hPer2 leads to hp53 stabilization, including 1) intracellular localization of the hPer2/hp53 complex (unpublished data), 2) inhibition of Mdm2 E3 ligase activity when in contact with hPer2 in the trimeric complex (Supplemental Figure S2C, lane 6), and, alternatively, 3) blockage of Mdm2 access to its substrate region within hp53. In support of these scenarios is the existence of multiple proteins that influence the stabilization of p53, although the major control mechanism remains its interaction with and ubiquitination by Mdm2 (Harris and Levine, 2005). However, proteins that reverse this modification and others that either enhance translation of p53 mRNA or alter its subcellular localization influence the total level of p53 present in the cell at a given time. The herpes virus–associated ubiquitin-specific protease (HAUSP) is a p53-interacting protein that stabilizes p53 by deubiquitination even in the presence of an excess of Mdm2 (Li *et al.*, 2002). More recently, the ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (Otub1) was found to directly suppress Mdm2-mediated p53 ubiquitination and drastically stabilize p53 in response to DNA damage (Sun *et al.*, 2012). An additional ubiquitin-independent but proteasome-dependent p53 degradation process is linked to its association to NAD(P)H quinone oxidoreductase (NQO1; Asher and Shaul, 2005). Both p53 and p73 interact with NQO1, which is largely associated with the 20S proteasome, unless an excess of NADH is present and competes off their interaction, preventing p53 degradation. Unlike in unstressed cells, in which Mdm2 prevents p300 from associating with p53, both CBP/p300 transcriptional coactivators and Strap, a partner protein of p300, associate with p53 in response to DNA damage, increasing its level and half-life through a mechanism that involves p53 acetylation (Lambert *et al.*, 1998; Barlev *et al.*, 2001). Methylation of p53 by overexpression of various methyltransferases has also been linked to its hyperstabilization (Chuikov *et al.*, 2004). Finally, protein–protein interaction has been proven effective in increasing the stability of various forms of mutant p53. For example, heat shock protein 90

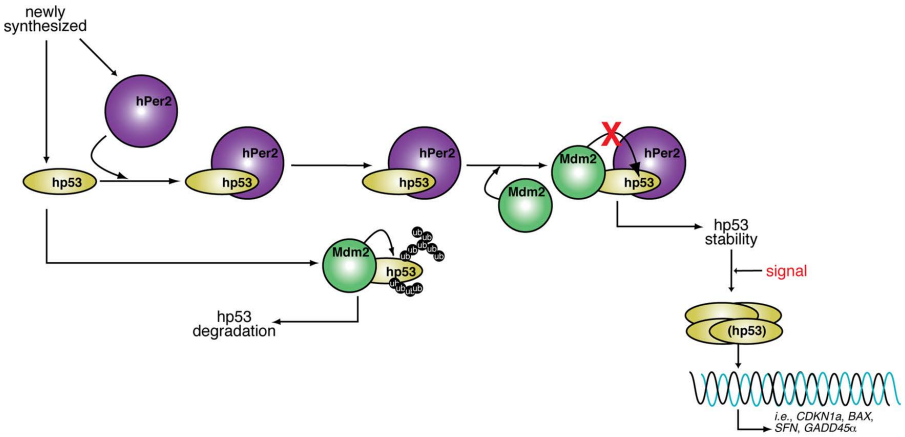

FIGURE 5: Proposed model of hPer2 and hp53 interaction and function. The hPer2 protein associates with cytosolic hp53, forming a stable complex that keeps hp53 in a stable state and ensuring that basal levels of hp53 exist ("priming state"). This heterodimer eventually incorporates Mdm2, forming a trimeric and stable Mdm2/hp53/hPer2 complex. In this scenario, Mdm2 is not able to ubiquitinate hp53 unless binding between these two proteins occurs in the absence of hPer2. We hypothesize that existence of the trimeric complex is compromised under, for example, stress signals, leading to release and activation of the hp53 downstream pathway.

(hsp90) binds to the C-terminus of mutant p53 when in complex with Mdm2; however, in this case, hsp90 acts by inhibiting the ubiquitin ligase activity of Mdm2, blocking the ubiquitination of both Mdm2 itself and mutant p53 (Peng *et al.*, 2001). An additional mode of regulation results from association of the central and C-terminus regions of p53 to poly(ADP-ribose) polymerase-1 (PARP-1; Wesierska-Gadek *et al.*, 2003). In this case, inactivation of PARP-1 resulted in decreased levels of p53, whereas inhibition of nuclear export by leptomycin B prevented accelerated degradation of p53 in PARP-1–knockout cells, favoring p53 accumulation. As a result, p53 stability results from its nuclear accumulation, as PARP-1 blocks p53's nuclear export signal located in its carboxy-terminal area (Wesierska-Gadek *et al.*, 2003). Overall the mechanisms for controlling p53 stabilization are many and varied, but they all serve the purpose of monitoring different aspects of cellular homeostasis.

What makes hPer2's interaction with hp53 particularly attractive is the possibility of building a new level of regulation by understanding how cell homeostasis harmonizes with its environment. Accordingly, we asked about the relevance of the hp53/hPer2 regulatory mechanism in a physiological context. Here we embrace the concept of "biological anticipation" first introduced to explain the function of the cardiomyocyte circadian clock in both myocardial health and disease (for review, see Durgan and Young, 2010). Basically, the concept proposes that a biological system is able to prepare for an event before it happens, thus ensuring rapid response in a temporally appropriate manner. Consequently, we hypothesize that the presence of hPer2 allows for the formation of the hp53/hPer2 complex and hp53 stabilization. Thus the existence of basal levels of the hp53 tumor suppressor helps "prime" the signaling pathway to rapidly respond to a stress condition (metabolic, genotoxic). In this scenario, induction of *TP53* transcription via hPer2 (Figure 3C) or by any additional factor is a secondary step and would most likely help to sustain the response. Of interest, Miki *et al.* (2013) showed that transcriptional regulation may be reciprocal and that p53 targets the Clock/Bmal1 complex, inhibiting the transcriptional expression of Per2 in the suprachiasmatic nuclei, all of which result in $p53^{-/-}$ animals that exhibit altered circadian behavior, further supporting various levels of regulation in the Per2-p53 axis.

The role of hPer2 as mediator of the hp53 transcriptional response was established in a p53-knockout background (H1299, p53-null cells) in which the hPer2-hp53 axis was reconstituted in a stepwise manner (Figure 4). First, we showed that manipulation of hPer2 levels influences both the transcription of a broad range of hp53 target genes and the stability of endogenous hp53 (Figure 4, A and B). In fact, knocking down hPer2 expression counteracts any effect in transcription of hp53 target genes caused by hPer2 overexpression (Figure 4A). Whereas this result establishes a correlation between the presence of hPer2 and the expression of genes whose transcription is mediated by hp53, a direct connection is further established when coexpression of hPer2 and hp53 synergistically enhances the transcription of *SFN*, *CDKN1a*, and *GADD45α* in an hp53-null background (Figures 4C and 5). Overall our results pose more relevant questions that refer to how both selectivity in terms of gene expression and diversity in terms of regulation are accomplished in the hPer2/hp53 axis and whether posttranslational modifications play a role in fine-tuning the strength of their interaction for a wider range of control.

MATERIALS AND METHODS

Bacterial two-hybrid screening

To assay for two-hybrid interactions between a specific bait (pBT-hPer2) and target plasmid pair from a library (pTRG cDNA library), we performed a two-hybrid screening analysis using the BacterioMatch II system (Stratagene, La Jolla, CA) following the manufacturer's instructions. Briefly, BacterioMatch II validation reporter–competent cells were cotransformed with ~200 ng each of the bait vector plus pTRG target vector. Aliquots of each of the cotransformation mixtures were plated in both nonselective screening medium (no 3-AT; Sigma-Aldrich, St. Louis, MO) and selective screening medium (5 mM 3-AT). Strong interactors were isolated from

selective media within the first 24 h of incubation, and positive clones were maintained in lysogeny broth (LB) tetracycline/chloramphenicol (Tet/Cam) agar plates. Activation of a second reporter gene, *aadA*, encoding streptomycin resistance, was used to verify the specificity of the interaction between bait and target proteins. In this case, putative positive colonies were patched from selective screening medium onto a dual-selective screening medium plate containing both 3-AT and streptomycin. The pBT-LFG2/pTRG-Gal11^{P} cotransformant was used as a positive control in the experiment and taken from a selective screening medium plate. Negative controls were the recombinant pBT-hPer2 cotransformed with either empty pTRG or pTRG-Gal11^{P} vectors taken from a nonselective screening medium plate. Purification of plasmid DNA from the 3-AT–resistant colonies isolated during library screening was performed from cultures grown in LB supplemented with tetracycline. All cDNAs were sequenced to confirm the identity of the clones.

RNA extraction and quantitative reverse-transcription PCR

Total RNA was extracted from cell pellets using the TRIzol reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. RNA was quantified by spectrophotometric reading at 260 nm and analyzed for quality assurance using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) at the Virginia Bioinformatic Institute Proteomics Core Facility. Quantitative reverse-transcription PCR (qRT-PCR) was conducted essentially as previously described (Yang *et al.*, 2008). Briefly, total RNA was pretreated with DNaseI (Promega, Madison, WI) at 37°C for 30 min, and a 1-µg sample was used as a template for first-strand cDNA synthesis using the iScript cDNA Synthesis system (Bio-Rad, Hercules, CA). qRT-PCR assay was performed using IQ SYBR Green Supermix (Bio-Rad) as follows: 10 ng of cDNA (50 ng for the 14-3-3σ gene) was added to a 20-µl reaction volume containing the indicated primers for amplification (see Supplemental Materials and Methods and Supplemental Table S1). Real-time assays were performed in triplicate on a MyIQ single color Real-Time PCR Detection instrument (Bio-Rad). Data were collected and analyzed with Optical System software, version 1.0. The glyceraldehyde-3-phosphate dehydrogenase and β-actin genes were used as internal controls to compute the relative expression level (ΔC_t) for each sample. The fold change of gene expression in each sample was calculated as $2^{-\Delta\Delta Ct}$.

Immunoprecipitation and immunoblot assays

For (co)immunoprecipitation experiments, transfected cells were harvested in lysis buffer, and extracts (~100 µg) were incubated with either α-FLAG M2 agarose beads (Sigma-Aldrich) or α-*myc* (9E10) beads (Santa Cruz Biotechnology, Dallas, TX) either for 2 h or overnight at 4°C with rotation before washing. Where indicated, immunoprecipitations were carried out in a two-step procedure, with extracts being incubated with the antibody (α-*myc*, α-FLAG, or α-p53) overnight at 4°C before the addition of protein A beads (50% slurry; Sigma-Aldrich). Sample beads were then washed four times with lysis buffer, resolved by SDS–PAGE, and analyzed by immunoblotting using specific primary antibodies (α-FLAG [Sigma-Aldrich], α-*myc* [Santa Cruz Biotechnology], α-Per2 [Sigma-Aldrich, Farmingdale, NY], α-p53 [Santa Cruz Biotechnology], α-ubiquitin [Enzo Biomol]). For (co)immunoprecipitation experiments of endogenous proteins, cells were harvested in lysis buffer, and extracts (~1 mg) were incubated with α-Per2 (Santa Cruz Biotechnology) or α-p53 (Santa Cruz Biotechnology) overnight at 4°C before the addition of protein A beads (50% slurry; Sigma-Aldrich). Samples were then kept for an additional 2 h at 4°C with rotation before washing and then processed as described, with the exception that in some of the experiments, blots were incubated with either α-Mdm2 (Santa Cruz Biotechnology and Calbiochem, Billerica, MA) primary antibody. In all cases, horseradish peroxidase–conjugated α-rabbit or α-mouse IgG secondary antibodies (GE Healthcare Life Sciences, Buckinghamshire, UK; Cell Signaling, Danvers, MA) were used for immunoblotting following standard procedures. Chemiluminescence reactions were performed using the SuperSignal West Pico substrate (Pierce, Rockford, IL).

In vitro binding assays

In vitro transcription and translation of pCS2+ *myc*-hPer2, *myc*-Mdm2, FLAG-hPer2, and FLAG-hp53 were carried out using the SP6 high-yield TNT system (Promega) following the manufacturer's instructions, although, unlike the standard procedure, the reaction was cold. Aliquots (1-4 µl) of indicated recombinant proteins were preincubated for 20 min at room temperature to allow the complex to form before adding NP40 lysis buffer. Immunoprecipitation of the various complexes was carried out essentially as described.

Protein pull-down assays

GST fusion proteins were expressed in *Escherichia coli* strain *Rosetta* (Novagen) and purified by glutathione-Sepharose chromatography based on the manufacturer's instructions (GE Healthcare Life Sciences). For pull-down assays, a total of 5 µg of GST-hp53-bound beads, its recombinant fragments, or an equivalent amount of glutathione beads (GST control) were washed in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100) and incubated with 2 µl of in vitro–transcribed and translated [^{35}S]*myc*-hPer2 at 4°C for 1 h. After washing of the beads with low- and high-salt binding buffer (100 mM and 1 M NaCl, respectively), bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by SDS–PAGE and autoradiography. A similar procedure was followed to evaluate binding of [^{35}S]*myc*-hp53 to various tagged recombinant fragments of hPer2. For competition assays, untagged hp53 was generated by digestion of the fusion protein with thrombin, followed by concentration and buffer exchange (10 mM Tris-HCl, pH 8.0). Reactions were set using 5 µg of GST-hp53 and increasing amounts of untagged p53 (0–20 µg) in the presence of a constant radiolabeled hPer2. Reactions were incubated at 4°C for 1 h, beads were washed, and samples analyzed by SDS–PAGE as described. Densitometric quantitation was carried out using a FluoChem digital imaging system (Alpha Innotech, Santa Clara, CA).

Ubiquitination assays

For in vitro assays, pCS2+ constructs of *myc*-hPer2, *myc*-Mdm2, and FLAG-hp53 were transcribed and translated in vitro as described. Aliquots of each tagged protein (1–4 µl), or a combination of them, were incubated at room temperature for 30 min to allow complex formation to happen before adding the reaction buffer containing 1× ubiquitination buffer (Enzo Biomol), 2 mM dithiothreitol, 20 µg/ml ubiquitin-aldehyde, 100 µg/ml ubiquitin, 1xATP-energy regeneration system (5 mM ATP/Mg^{2+}; Enzo Biomol), 40 µM MG132, and 1 mg/ml HeLa S100 lysate fraction (Enzo Biomol) to a final volume of 10 µl. Reactions were further incubated at 37°C for 30 min before being terminated by the addition of lysis buffer. Diluted samples were incubated twice at 4°C for 1 h, first after addition of 3 µg of α-FLAG antibody (Sigma-Aldrich) and then after the addition of 8 µl of protein A–Sepharose 4B (50% slurry; Sigma-Aldrich). Bound complex were washed four times with NP40 lysis buffer, resuspended in Laemmli buffer, and resolved by SDS–PAGE and immunoblotting.

For detection of in-cell ubiquitination, HCT116 cells were transfected with pCS2+*myc*-hp53 and either pCS2+FLAG-hPer2 or empty vector and maintained in complete medium for 20 h before the addition or not (–MG132) of 50 μM MG132 and ubiquitin aldehyde (5 nM). Cells were harvested 4 h later and subjected to subcellular fractionation as previously described (Schreiber *et al.*, 1989). Lysates were immunoprecipitated with α-FLAG or α-*myc* antibodies as described, and the complexes were resolved in SDS–PAGE. Ubiquitinated complexes were detected by immunoblotting using an α-ubiquitin antibody.

Analysis of hp53 half-life

Extracts were from HCT116 cells treated with 100 μg/ml cycloheximide. Protein levels were quantitated by immunoblot analysis using ImageJ, version 1.45 (National Institutes of Health software package; Schneider *et al.*, 2012), and values normalized to tubulin levels. The percentage of protein remaining was calculated based on $t = 0$, and the data were fitted using Prism software (GraphPad Software, La Jolla, CA).

Immunofluorescence microscopy

HCT116 was used for detection of endogenous hp53 and hPer2 proteins. Cells were fixed (3.7% formaldehyde/phosphate-buffered saline [PBS]/0.5% Triton X-100) 2 h after exposure at room temperature, washed with PBS/0.5% Triton X-100 and then 0.1% Triton X-100, and blocked with goat serum at room temperature for 30 min. Subcellular localization was detected using commercially available antibodies. Nuclei were visualized by incubating fixed cells with SYTO 60 (Life Technologies). Fluorescence was visualized using a Nikon Eclipse TE2000-E microscope (Nikon, Melville, NY) equipped with a Cascade II E2V CCD97 camera (Photometrics, Tucson, AZ) at 488, 568, and 647 nm. Images were processed using the NIS-Elements AR 3.0 Nikon software.

Gene reporter activity

H1299 cells were seeded onto 12-well plates and cotransfected with ~200 ng of $hp21^{WAF1/CIP1}$-luciferase (Luc), pCS2+FLAG-hp53, -hp53(ch)GST, -hp53(ch)hPer2, or empty vector (pCS2+FLAG) each. The pCMV-β-gal (~200 ng) plasmid was included as an internal control and harvested 2 h later. Reporter activity was measured using the Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Readings were recorded from a Glomax Luminometer, and results were normalized for expression of β-gal, which was determined separately using the Galacto-Light Plus System (Bio-Rad). Experiments were done in triplicate and repeated at least twice.

Statistical analyses

To assess the overall significance of our results, data were processed using either a two-tailed unpaired Student's *t* test or analysis of variance (ANOVA), followed by either Bonferroni or, when necessary, Games–Howell post hoc test (SPSS statistical software; IBM, Armonk, NY). Levene's test was used to determine homogeneity of variances, whereas data normality was examined using the Shapiro–Wilk *W* test and the Box–Cox Y transformation (JMP 9 statistical software; SAS, Cary, NC) and applied when necessary. Values of $p \leq 0.05$ were considered statistically significant.

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Association of the circadian factor Period 2 to p53 influences p53's function in DNA-damage signaling

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ABSTRACT Circadian period proteins influence cell division and death by associating with checkpoint components, although their mode of regulation has not been firmly established. hPer2 forms a trimeric complex with hp53 and its negative regulator Mdm2. In unstressed cells, this association leads to increased hp53 stability by blocking Mdm2-dependent ubiquitination and transcription of hp53 target genes. Because of the relevance of hp53 in checkpoint signaling, we hypothesize that hPer2 association with hp53 acts as a regulatory module that influences hp53's downstream response to genotoxic stress. Unlike the trimeric complex, whose distribution was confined to the nuclear compartment, hPer2/hp53 was identified in both cytosol and nucleus. At the transcriptional level, a reporter containing the $hp21^{WAF1/CIP1}$ promoter, a target of hp53, remained inactive in cells expressing a stable form of the hPer2/hp53 complex even when treated with γ-radiation. Finally, we established that hPer2 directly acts on the hp53 node, as checkpoint components upstream of hp53 remained active in response to DNA damage. Quantitative transcriptional analyses of hp53 target genes demonstrated that unbound hp53 was absolutely required for activation of the DNA-damage response. Our results provide evidence of the mode by which the circadian tumor suppressor hPer2 modulates hp53 signaling in response to genotoxic stress.

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INTRODUCTION

Transcription of *period* genes oscillates in a circadian manner and is essential for maintaining a functional clock that is driven by interacting transcription-translation–based autoregulatory feedback loops (for review, see Takahashi *et al.*, 2008). Three homologues (*period* 1–3) have been identified in mammals, whose levels oscillate in the suprachiasmatic nuclei, where the master clock is located, and in peripheral tissues (Albrecht *et al.*, 2007). Period (Per), cryptochrome (Cry), casein kinase Iε/δ (CKIε/δ), circadian locomoter output cycles kaput (Clock), and brain and muscle Arnt-like protein 1 (Bmal1) are the main players responsible for driving the primary negative-feedback loop of the clock, where transcriptional activation, heterodimerization, and translocation maintain

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T.G. provided data in Figures 1, 2, 4, and 5D and Supplemental Figures S1, S2, S4, S8F, S9, B and C, and S10 and contributed with reagents in Figures 3 and 5 and Supplemental Figures S3, S5, and S8. M.V.-C. performed the experiments shown in Figures 3 and 5, B and C, and Supplemental Figures S3, S8, A–E, and S9A, contributed in the execution of the experiments shown in Figure 5D and Supplemental Figures S1, S8F, and S9C, and performed the statistical analyses except for those mentioned otherwise. J.L. performed the experiments shown in Supplemental Figures S6 and S7 (top and middle) and S9C. S.S. provided Supplemental Figures S5 and S7 (bottom) and the statistical analyses shown in Supplemental Figures S5, S7, and S9. C.V.F., T.G., and M.V.-C. analyzed the overall data, refined the hypothesis, and proposed the model. C.V.F. supervised and coordinated all investigators for the project and wrote the manuscript.

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Abbreviations used: *BAX*, encodes the Bcl-2-associated X protein (Bax); *CDKN1a*, encodes cyclin-dependent kinase inhibitor p21 ($p21^{CIP1/WAF1}$); *GADD45α*, encodes growth arrest and DNA damage–inducible protein 45α (Gadd45α); H1299, human non–small cell lung carcinoma cells; HCT116, human colorectal carcinoma-116 cells; hp53, human p53 transcription factor; hPer2, human Period 2; Mdm2, murine [human] double minute-2; *SFN*, encodes 14-3-3σ.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2014/11/17/mbc.E14-05-0994v1.DC1.html

the dynamics of the process (for review, see Takahashi *et al.*, 2008).

In recent years, it has been determined that clock component roles have expanded beyond their strict function as modulators of the organism's adaptive response to environmental cues (i.e., light/dark cycles) to include regulating sleep–wake cycles and release of hormones, maintaining the body's thermoregulation, and having a role in photoperiodism. Extensive work in various organismal systems has identified clock factors as obligatory intermediates that control numerous physiological processes directly relevant to human diseases and behavioral disorders (for review, see Takahashi *et al.*, 2008). For example, a *Bmal1/Mop3* (*Arnt1*)–null mutant mouse exhibits infertility, decreased body weight usually associated with abnormal gluconeogenesis and lipogenesis, premature aging, and sleep fragmentation (Bunger *et al.*, 2000; Rudic *et al.*, 2004; Laposky *et al.*, 2005; Shimba *et al.*, 2005; Kondratov *et al.*, 2006), whereas a *ClockΔ19* (antimorph) mouse mutant is hyperphagic and obese, hypersensitive to chemotherapeutic agents, and exhibits a manic phenotype (Naylor *et al.*, 2000; Rudic *et al.*, 2004; Gorbacheva *et al.*, 2005; Turek *et al.*, 2005). Others, such as the *Cry1* and *Cry2* double-null mutant mouse, exhibit a delay in tissue regeneration as monitored in the liver (Matsuo *et al.*, 2003). *Csnk1e* ($CK1\varepsilon^{tau}$ mutant) mutant animals have an enhanced metabolic but reduced growth rate (Oklejewicz *et al.*, 1997; Lucas *et al.*, 2000), whereas both *Csnk1d* (CK1δ)- and *timeless*-null mutations result in a lethal phenotype (Gotter *et al.*, 2000; Xu *et al.*, 2005). Additional physiological phenotypes, including diminished pupillary light reflex, impaired temporal regulation of metabolism and feeding, age-related autoimmune diseases, and defects in skeletal muscle regeneration, have also been described in mice exhibiting mutations in other clock-related genes, such as *Rora, b,* and *c, Dec 1* and *2, Opn4, Vip, Vipr2,* and *Nocturnin* (for review, see Takahashi *et al.*, 2008). Moreover, deletion and mutations of mouse *Period* genes result in numerous changes in an animal's phenotype, including shortening or loss of the circadian period (in the case of Per1 and Per2 double-null mutant mice), sensitization of animals to drugs, improper alcohol intake, altered glucose metabolism, and abnormal cellular proliferation (Zheng *et al.*, 1999, 2001; Shearman *et al.*, 2000; Bae *et al.*, 2001; Cermakian *et al.*, 2001; Fu *et al.*, 2002). Of interest, neither *Per1*- nor *Per3*-null mutant mice exhibit a phenotype that is reminiscent of that observed in animals in which the expression of checkpoint proteins is compromised (Fu *et al.*, 2002); however, *Per2*-null mutant mice do. Accordingly, *Per2*-null mice show increased hyperplastic growth, tumor development, and severe morbidity, a phenotype that is accompanied by hair graying and hair loss, which is exacerbated in the presence of genotoxic stress (Fu *et al.*, 2002). Although there have been attempts to identify molecular signatures responsible for the *Per2*-null phenotype (Fu *et al.*, 2002), the mechanistic foundation that further supports the observed phenotype is lacking.

Connections between clock molecules and the cellular DNA damage response have been identified. In *Neurospora crassa*, the clock gene *period 4* was identified as an orthologue of the mammalian checkpoint kinase 2 (Chk2) gene (Pregueiro *et al.*, 2006). In colon cancer cell lines, overexpression of Per1 sensitizes cells to DNA damage–induced apoptosis by a yet-unknown mechanism that involves interaction with Chk2 (Gery *et al.*, 2006). However, unlike Per2, Per1 does not seem to act as a tumor suppressor, since homozygous Per1 mutant mice display only a shorter circadian period with reduced precision and stability, and ablation of the *per1* gene does not affect cell proliferation (Zheng *et al.*, 2001). Finally, the human Timeless (hTim) protein seems to be required for the phosphorylation and activation of Chk1 by the ataxia telangiectasia and Rad3-related protein (ATR), whereas Per3 appears to physically bind to both ATM and Chk2. However, the role of hTim in circadian regulation of mammalian cells is controversial (Unsal-Kacmaz *et al.*, 2005), and the *per3* gene product is not necessary to sustain circadian rhythmicity in mice (Shearman *et al.*, 2000). Overall these studies suggest a scenario in which multiple circadian players converge on a multilevel cellular clock that links environmental conditions to the biochemical and genetic machinery of the cell to influence cell cycle progression and the response to genotoxic stress.

More recently, we performed interaction studies using human Period 2 (hPer2) as bait to map and identify protein–protein interactions and endogenous protein partner complexes (Gotoh *et al.*, 2014). The transcription factor and checkpoint-component p53 (human p53 transcription factor [hp53]) is among the novel hPer2 interactors. Association of hPer2 to the C-terminus region of hp53 results in formation of a trimeric complex in which the oncogenic protein murine [human] double minute-2 (Mdm2) is bound to the N-terminus of hp53. As a result, hPer2 promotes hp53 stability by a mechanism that involves inhibition of hp53 ubiquitination by Mdm2 (Gotoh *et al.*, 2014). The end result is the intersection of circadian and checkpoint components at the key hp53 node and the modulation of the hp53 transcriptional response. Our findings are in agreement with the observation that endogenous p53 is largely undetectable in thymocytes from *Per2*-null mice and that de novo accumulation of p53 seems to occur several hours after the insult is applied in *Per2*-null animals (Fu *et al.*, 2002), an observation that goes along with our findings of hPer2 acting as a transcriptional regulator of the *TP53* gene (Gotoh *et al.*, 2014).

A number of additional findings indirectly point toward cross-talk between Per2 function and the p53-mediated DNA damage response; however, it remains unclear how Per2 relates to that process mechanistically. For example, it is known that overexpression of Per2 results in reduced cellular proliferation and increased apoptosis in lung and mammary carcinoma, but not in embryonic fibroblast NIH3T3 cells, by a transcriptional mechanism that involves the up-regulation of proapoptotic components (i.e., *TP53* and *BAX* [encodes the Bcl-2-associated X protein, Bax]) and the simultaneous attenuation of antiapoptotic transcripts, including *MYC, BCL2L1,* and *BCL2* (Hua *et al.*, 2006). Sun *et al.* (2010) expanded these findings to leukemia cells by showing that Per2 overexpression promotes p53-dependent G2/M arrest by down-regulation of *CCNB1* and *MYC* expression followed by apoptosis. In line with these observations is the finding that overexpression of Per2 in hematopoietic cancer cell lines results in a phenotype that includes growth inhibition, cell cycle arrest, apoptosis, and loss of clonogenic ability (Gery and Koeffler, 2009). More recently, the known $Ser^{662}Gly$ ($S^{662}G$) mutation in Per2, responsible for familial advanced sleep phase syndrome, has been linked to enhanced resistance to x-ray–induced apoptosis and increased E1A- and RAS-mediated oncogenic transformation (Gu *et al.*, 2012). Accordingly, animals bearing the $Per2^{S662G}$ mutation show accelerated tumorigenesis in a $p53^{R172H/+}$ background. This effect is independent of the length of the circadian cycle but influences the relative phases of expression of p53-regulated, clock-controlled cell cycle genes (i.e., *CDKN1a* [encoding cyclin-dependent kinase inhibitor p21, $p21^{CIP1/WAF1}$] and *CCND1*; Gu *et al.*, 2012). More recently, we showed that hPer2 acts on hp53 by controlling its stability and activity in unstressed conditions (Gotoh *et al.*, 2014) and hypothesized that exposure to genotoxic stress triggers a rapid, hp53-mediated transcriptional checkpoint response by releasing hp53 from a preformed, nucleus-localized, hPer2/hp53 stable endogenous complex. Our findings establish the spatial distribution of

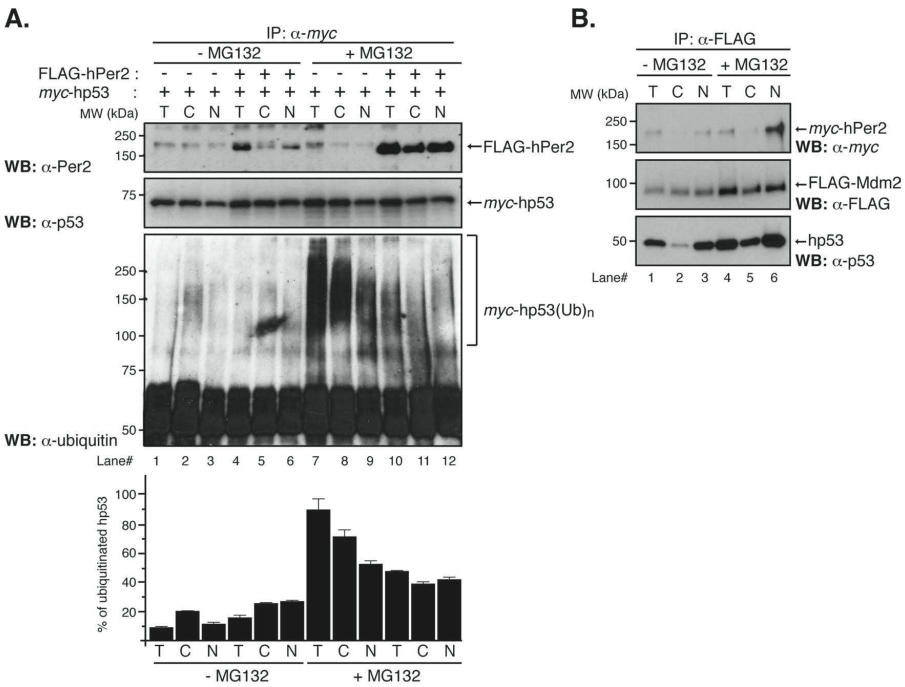

FIGURE 1: Distribution of the hPer2/hp53 complex among cellular compartments. (A) HCT116 cells were transfected with pCS2+*myc*-hp53 and either pCS2+FLAG-hPer2 (+) or empty vector (–) and maintained in complete medium for 20 h before adding or not (control; –MG132) MG132 (50 μM) and ubiquitin aldehyde (5 nM). Cells were maintained an additional 4 h before harvesting. Lysates (6.4×10^5 cells) were used to prepare the cytosolic (C) and total (T) fractions, whereas 32×10^5 cells were used for nuclear (N) preparation. Total, cytosolic, and nuclear extracts were incubated with α-*myc* antibody and protein A beads in NP40 lysis buffer containing MG132 and ubiquitin aldehyde. Washed samples were analyzed by immunoblotting using specific antibodies. Ubiquitinated *myc*-hp53 complexes (*myc*-hp53$(Ub)_n$) are indicated between brackets. Immunoblot data from a single experiment repeated three times with similar results. Quantification of the sample's ubiquitinated signal was performed using ImageJ, version 1.45 (National Institutes of Health software package; Schneider *et al.*, 2012; bar graph). (B) HCT116 lysates (2.8×10^5 cells for T/C; 14×10^5 cells for N) from pCS2+FLAG-Mdm2– and pCS2+*myc*-hPer2– cotransfected cells treated or not (–MG132) with 50 μM MG132 and 5 nM ubiquitin aldehyde were immunoprecipitated using α-FLAG and protein A beads and analyzed by immunoblotting for endogenous hp53 (bottom) and *myc*- and FLAG-expressed proteins (top and middle, respectively).

the various hPer2, hp53, and Mdm2 complexes, the need for hPer2 association with hp53 to maintain basal levels of this protein, and the relevance of hPer2/hp53 dissociation for an effective hp53-mediated DNA damage checkpoint response.

RESULTS

Spatial organization and shuttling of circadian molecules are common themes when it comes to understanding how oscillations are generated and sustained in biological clock systems. They are also common subjects when considering how sensor components segregate signals in response to stress conditions at various points in the cell cycle. In light of our previous findings in which the circadian factor hPer2 directly binds the tumor suppressor and checkpoint component hp53 in unstressed cells, we now ask whether the complex's spatial organization, as well as association, is critical for an effective hp53-mediated transcriptional response under stress conditions.

Subcellular distribution of hPer2/hp53 complexes

To assess whether functional compartmentalization of the hp53/hPer2 complex occurs, we performed cell fractionation experiments and monitored the formation of polyubiquitinated hp53 complexes and hPer2 binding in extracts from cells treated with proteasome inhibitors (Figure 1, A and B). Human colon carcinoma HCT-116 (HCT116) cells ($p53^{+/+}$) were cotransfected with *myc*-hp53 and FLAG-hPer2 or empty vector (–) and treated with MG132 (+MG132) or vehicle (–MG132). Total, cytosolic, and nuclear fractions were analyzed for hp53/hPer2 complex formation and the presence of polyubiquitinated hp53. Input levels of *myc*-hp53 and FLAG-hPer2, as well as of endogenous hp53 and hPer2 levels, were monitored in each fraction (Supplemental Figure S1, A and B) and normalized to those of *myc*-hp53 for the experiment shown in Figure 1A. Unlike nontreated MG132 cells, immunoprecipitation of *myc*-hp53 showed the presence of stable *myc*-hp53$(Ub)_n$ forms in total (T) and cytosolic (C) fractions and, to a lesser extent, in the nuclear (N) fraction of samples treated with MG132 (Figure 1A, bottom, lanes 1–3 vs. lanes 7–9). These results most likely represent the effect of proteasome inhibitors in preserving the *myc*-hp53 ubiquitinated complexes and accumulation in the cytosol as the preferred site for their ubiquitin-mediated degradation.

Significantly, overexpression of FLAG-hPer2 followed by *myc*-hp53 binding abrogated the formation of hp53$(Ub)_n$ in both nuclear and cytosolic fractions (Figure 1A, bottom, lanes 7–9 vs. lanes 10–12), in agreement with the subcellular distribution of hPer2 in those compartments (Figure 1A, top, lanes 10–12, and Supplemental Figure S1B) and the proposed role of hPer2 in modulating hp53 polyubiquitination. These results establish a physical and functional presence of hp53/hPer2 complex in the cytosol and nucleus. Further support comes from results shown in Figure 1A (lanes 1–6), in which studies similar to the ones described earlier were conducted in the absence of MG132 (–MG132), allowing the proteasomal machinery to be fully functional. As a result, hp53$(Ub)_n$ forms were undetected (lanes 1–6), and only trace amounts of hPer2 were associated with *myc*-hp53 in immunoprecipitated nuclear samples (Figure 1A, top and bottom, lane 6) even when input amounts were comparable to those used in the +MG132 experiment (Supplemental Figure S1B, top, lanes 4–6 vs. lanes 10–12). As previously shown, Per2 is degraded by the proteasomal pathway unless associated (Yagita *et al.*, 2002), and thus the level of FLAG-hPer2 was expected to be low in both the cytosolic and nuclear compartments in the absence of MG132 but detectable if associated to a protein counterpart. In sum, our data establish that 1) the hPer2/hp53 complex can be found in both the nuclear and cytosolic subcellular compartments and 2) physical interaction between hp53 and hPer2 might influence hPer2 degradation as well.

Next we asked whether Mdm2 associates with the hPer2/hp53 once in the nucleus. To answer this question, we cotransfected HCT116 cells with FLAG-Mdm2 and *myc*-hPer2 and looked for formation of the trimeric complex with endogenous hp53 (Figure 1B). Transfected cells were incubated in the absence (–) or presence (+) of MG132 and subjected to cellular fractionation. Consistent with their endogenous distribution, input *myc*-hPer2 was found in both cytosolic and nuclear fractions (Supplemental Figure S1C, lanes 2 and 3; Gotoh *et al.*, 2014), and FLAG-Mdm2 preferably distributed in the nuclear fraction (Supplemental Figure S1C, lane 2), although some signal was detected in the cytosol, most likely as a result of MG132 addition and inhibition of self-ubiquitination (Supplemental Figure S1C, lane 5). Consequently, endogenous hp53 levels were also increased in MG132-treated samples (Supplemental Figure S1C, lanes 2 and 3 vs. lanes 5 and 6).

Analysis of α-FLAG–immunoprecipitated samples shows that FLAG-Mdm2 associated with *myc*-hPer2 and hp53 in the nuclear fraction of cells treated with MG132, whereas the trimeric complex was less conspicuous but still detectable in the absence of the proteasome inhibitor (Figure 1B, lane 3 vs. lane 6). Of interest, *myc*-hPer2 did not seem to be associated with the Mdm2/hp53 complex in the cytosolic fraction (Figure 1B, lanes 2 and 5) but likely formed a two-component complex with hp53 in the absence of ubiquitination (Figure 1A, lanes 11 and 12). Because the input levels of *myc*-hPer2 were comparatively similar in –MG132 and +MG132 samples (Supplemental Figure S1C, top), one might speculate that the trace levels of hPer2 associated with the FLAG-Mdm2/hp53 complex within the nuclear fraction in MG132-untreated cells (Figure 1B, lane 3) were the result of either hPer2 being a substrate of Mdm2-mediated ubiquitination once the complex was in place or the action of an additional nuclear E3-ligase for hPer2 (i.e., β-TRCP; Ohsaki *et al.*, 2008). Further experiments need to be done to test both possibilities. As expected, FLAG-Mdm2 was detected in association with hp53 in the cytosolic compartment, in agreement with previous findings (Freedman and Levine, 1998), and relative amounts of FLAG-Mdm2/hp53 complex increased in MG132-treated samples (Figure 1B, lane 2 vs. lane 5). In summary, our data establish that hPer2/hp53/Mdm2 exists only in the nucleus and that the hPer2/hp53 and hp53/Mdm2 complexes can be readily detected in both nuclear and cytosolic fractions.

Functional insights into the hPer2/hp53 complex

We previously identified the hp53/hPer2 complex within the nuclear compartment (Figure 1). Thus we next asked whether hp53 transcriptional activity was compromised when bound to hPer2. To answer this question, we first needed to generate a form of the hp53/hPer2 complex that would be constitutively bound and not dissociate once formed in cells. For this task, we generated a chimera set in which either hPer2 full-length or glutathione *S*-transferase was cloned downstream of hp53 and immediately after a flexible linker (called hp53(ch)hPer2 and hp53(ch)GST hereafter, respectively; Figure 2A). The rationale behind this design is that hPer2 would interact with hp53 by flipping over and forming a stable, covalently bound complex through their domain interactions, which would be nondissociable as seen using biofluorescence complementation assays (unpublished data). As a result, we expect to find the ubiquitination of hp53 compromised without altering its compartmentalization. Therefore hp53(ch)hPer2 would be an adequate tool to evaluate the effect of a nondissociable hp53/hPer2 complex in hp53 downstream gene activation.

To further functionally validate the hp53(ch)hPer2 chimera, we evaluated the ubiquitination status of hp53 in the complex (Figure 2B). In vitro ubiquitination assays were performed using recombinantly expressed proteins (FLAG-hp53, FLAG-hp53(ch)GST, FLAG-hp53(ch)hPer2(356-574/683-872), and FLAG-hp53(ch)hPer2) preincubated, or not (–), with *myc*-hPer2, followed by *myc*-Mdm2 addition. Once the ubiquitination reaction took place, hp53 and its chimera proteins were immunoprecipitated and analyzed for ubiquitin incorporation by immunoblotting (Figure 2B and Supplemental Figure S2). Results show that FLAG-hp53 and FLAG-hp53(ch)GST behave similarly with respect to overall ubiquitination status when prebound to hPer2 and compared with their controls in the absence of hPer2 addition (Figure 2B, bottom, lane 7 vs. lane 8 and lane 2 vs. lane 3). Consistent with its role as a stable complex, ubiquitination of FLAG-hp53(ch)hPer2(356-574/683-872) and FLAG-hp53(ch)hPer2 closely resembled the basal signal obtained with just hp53 (or hp53(ch)GST) when preincubated with hPer2 (Figure 2B, bottom, lane 5 vs. lanes 3 and 8; Supplemental Figure S2, lane 3 vs. lane 5), further validating the chimera as an nondissociable hp53/hPer2 complex mimetic. In all cases, binding components were confirmed by immunoblotting and are shown in Figure 2B (top and middle).

Next we compared the localization of hp53(ch)hPer2 with that of *myc*-tagged hp53 and its chimera form (hp53(ch)GST) in human non–small cell lung carcinoma-1299 (H1299) cells (p53-null). Accordingly, we transfected H1299 cells with the various constructs and monitored their subcellular localization by fluorescence microscopy. Representative pictures are shown in Figure 3 and Supplemental Figure S3. In most normal cells, p53 is cytoplasmic; however, it is primarily located in the nucleus in rapidly growing normal cells, transformed cells, and various tumor cells, including those from breast and colon (Liang and Clarke, 2001). In agreement, *myc*-hp53 and *myc*-hp53(ch)GST were largely detected in the nuclear compartment (Figure 3 and Supplemental Figure S3, i and ii). The hp53(ch)hPer2 chimera was detected in both cytosolic and nuclear compartments (Figure 3 and Supplemental Figure S3, vi), in agreement with hPer2/hp53 distribution as in Figure 1A. Moreover, hp53(ch)hPer2 was recognized by α-p53 and α-Per2 antibodies targeting conformational native epitopes in both proteins, suggesting that the integrity of the folding in the complex was not compromised. Comparable results were also obtained with tagged forms of the chimera complex

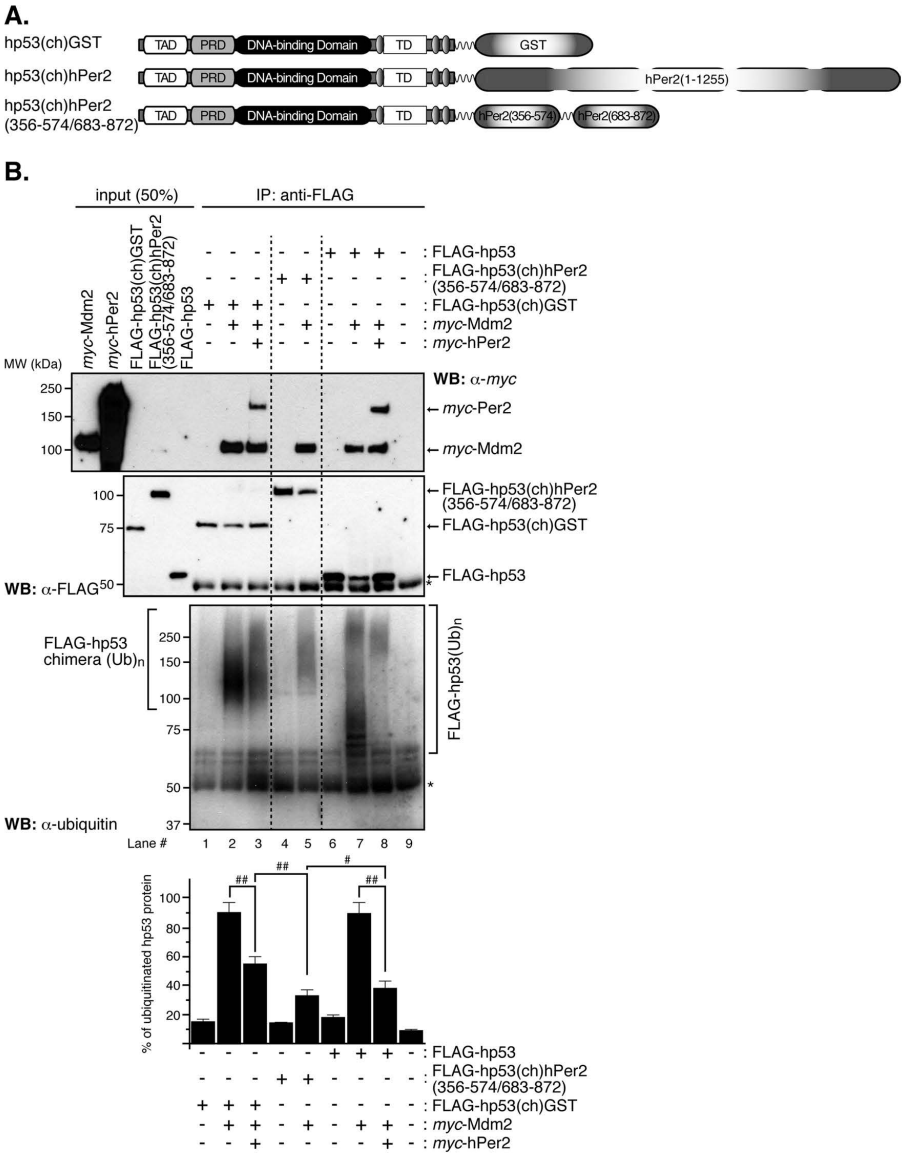

FIGURE 2: In vitro ubiquitination of hp53 is compromised when stably bound to hPer2. (A) Schematic representation of all chimeras designed for this study. Constructs were 5′ FLAG-tagged, *myc*-tagged, or untagged upstream of hp53, followed by downstream cloning of GST, hPer2, or hPer2(356-574/683-872) open reading frames. A linker encoding for six Gly was inserted between both the hp53 and hPer2 genes and the two hPer2-coding fragments. (B) In vitro transcribed and translated FLAG-hp53, FLAG-hp53(ch)hPer2(356-574/683-872), FLAG-hp53(ch)GST, *myc*-Mdm2, and *myc*-hPer2 proteins were used for ubiquitination experiments. When indicated, *myc*-hPer2 and either FLAG-hp53 or FLAG-hp53(ch)GST were preincubated; thus the complex was formed before adding *myc*-Mdm2. For the FLAG-hp53(ch)hPer2(356-574/683-872) chimera, the translated protein and *myc*-Mdm2 were incubated together before the ubiquitination reaction took place. Ubiquitination was carried out as described in *Materials and Methods*. FLAG-tagged proteins were immunoprecipitated with α-FLAG/protein A beads and blotted using α-ubiquitin antibody. Membranes were then stripped and reprobed with α-FLAG and -*myc* antibodies to detect complex bound proteins. Asterisk indicates IgG heavy chain. Immunoblot data from a single experiment repeated three times with similar results. Quantification of the sample's ubiquitinated signal was performed using ImageJ, version 1.45 (bar graph). Statistical comparisons were evaluated by *t* test. $^{\#}p \leq 0.05$; $^{\#\#}p < 0.005$.

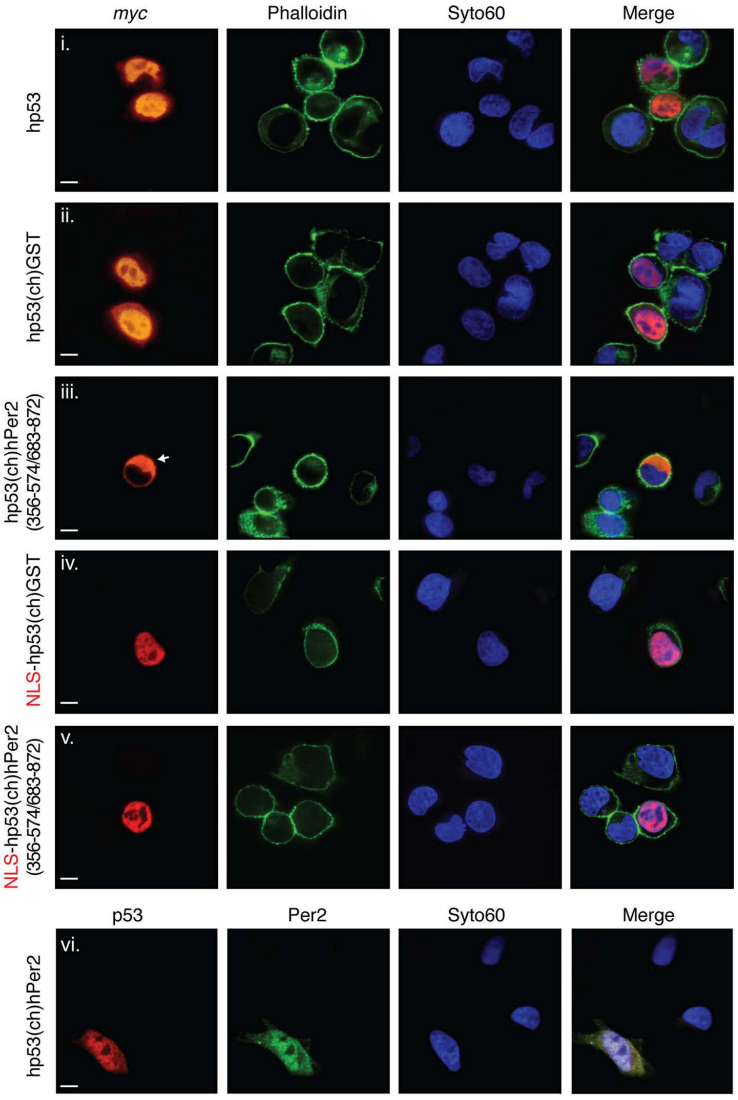

FIGURE 3: Relevance of hPer2-interacting domains for hp53 localization. H1299 cells were transfected with *myc*-tagged forms of hp53, hp53(ch)GST, hp53(ch)hPer2(356-574/683-872), NLS-hp53(ch)GST, and NLS-hp53(ch)hPer2(356-574/683-872) (i–v) or the untagged form of hp53(ch)hPer2 (vi). Proteins were visualized by confocal microscopy using α-*myc*-Cy3–conjugated primary antibody (i–v) or α-p53 and -Per2 primary antibodies and α-mouse Cy3– and α-rabbit Alexa Fluor 488 (Life Technologies)–conjugated secondary antibodies, respectively (vi). Actin fibers and DNA were stained with phalloidin Alexa Fluor 488 and Syto60 (Life Technologies), respectively. Merge images (right) were from protein staining, phalloidin, and DNA (i–v) and protein and DNA (vi). A Nikon ECLIPSE TE2000-E microscope and NIS-Elements AR 3.0 software were used to record images. Scale bars, 10 μm.

(unpublished data). Profile plotting of signal intensity along cross sections of cells transfected with the various constructs unambiguously determined their distribution and levels of expression (Supplemental Figure S3).

Localization of p53 in the nucleus is the result of the presence of three monopartite nuclear localization signals (NLSs) located within the C-terminus of p53, with NLS1 (^{316}PQPKKKP^{322}) being the most active (Shaulsky *et al.*, 1990). Remarkably, it is the C-terminus of hp53 that interacts with hPer2, and, thus, it seems reasonable that hp53 NLSs would be occluded at the interface of the two interacting molecules. Moreover, the strongest NLS described in hPer2 so far is a bipartite sequence located between residues 778 and 794 (Yagita *et al.*, 2002) that in hPer2 directly interacts with hp53 and most likely would not be exposed. Therefore we ask what element(s) actually drive the hp53(ch)hPer2 complex to the nucleus. To answer this question, we generated additional chimeras of hp53 in which the hPer2-interacting fragments 356–574 and 683–872 (which contain the hPer2 NLS) were cloned downstream of hp53 (contains all NLSs) and were separated by flexible linkers (called hp53(ch)hPer2(356-574/683-872) hereafter; Figure 2A). As shown in Figure 3 and Supplemental Figure S3iii, hp53(ch)hPer2(356-574/683-872) failed to localize in the nucleus of H1299 cells and readily accumulated in the cytoplasmic compartment, suggesting that neither NLS was functional and that hp53(ch)hPer2 translocation had most likely been driven by a yet-to-be identified NLS in hPer2 or that there is an additional cargo protein associated with the complex involved in translocation. To evaluate this possibility, we engineered an NLS sequence, in this case from SV40 (Kalderon *et al.*, 1984), inserted it upstream of hp53(ch)hPer2(356-574/683-872), and monitored its localization in H1299 cells. Of interest, the sole addition of this NLS resulted in hp53(ch)hPer2(356-574/683-872) shuttling to the nucleus, supporting the existence of a yet-to-be identified component related to hPer2 that provides a signal for transportation (Figure 3 and Supplemental Figure S3, iii vs. v). Finally, we showed that hCry1 was able to bind hPer2/hp53 when tested in vitro, a result that points to the existence of multiple regulators of diverse biochemical nature. Binding of hCry1 could be explained as the result of its interaction with the C-terminus of hPer2 (Yagita *et al.*, 2002), a region that does not overlap with the binding site mapped for hp53 (Supplemental Figure S4A; Gotoh *et al.*, 2014). Despite this result, this last finding needs to be taken with caution, as we failed to find compelling evidence to prove the existence of an endogenous hPer2/hCry1/hp53 complex in cells, a result that might reflect its transient nature or simply expose the low efficiency of the immunoprecipitating antibodies that were used.

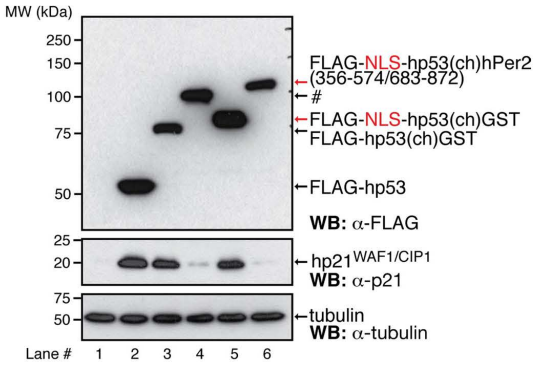

FIGURE 4: Binding of hPer2 to hp53 prevents hp21 from being expressed. H1299 cells were transfected with pCS2+FLAG-hp53, -hp53(ch)GST, -NLS-hp53(ch)GST, -hp53(ch)hPer2(356-574/683-872) (labeled #), or -NLS-hp53(ch)hPer2(356-574/683-872) and harvested 24 h later. Cell lysates (~40 µg) were resolved by SDS–PAGE and recombinant (top left and right) and endogenous proteins (middle and bottom left and right) detected by immunoblotting using α-p21, -FLAG, and -tubulin antibodies.

hp53 transcriptional activity is compromised when bound to hPer2

First, we investigated whether shuttling of the hPer2/hp53 complex from the cytosol to the nucleus was sufficient to trigger hp53-mediated downstream signaling (Figure 4 and Supplemental Figure S4B). Accordingly, we transfected H1299 cells (p53-null) with various chimera constructs, as well as with wild-type hp53, and monitored the expression of the cyclin-dependent inhibitor human p21$^{WAF1/CIP1}$ (hp21$^{WAF1/CIP1}$), a transcriptional target of p53, by immunoblotting. Transfection of cells with hp53, FLAG-hp53(ch)GST, and FLAG-NLS-hp53(ch)GST resulted in localization of all three proteins in the nucleus (Figure 3, i, ii, and iv) and hp21$^{WAF1/CIP1}$ expression (Figure 4, lanes 2, 3, and 5, and Supplemental Figure S4B, lanes 3 and 4). As expected, transfection of H1299 with FLAG-hp53(ch)hPer2(356-574/683-872) did not lead to hp21$^{WAF1/CIP1}$ expression, as the complex remained sequestered in the cytosol (Figures 3iii and 4, lane 4). Of interest, and despite the addition of an NLS to favor shuttling, transfection of cells with either FLAG-NLS-hp53(ch)hPer2(356-574/683-872) (Figure 4, lane 6) or FLAG-hp53(ch)hPer2 (Supplemental Figure S4B, lane 6) did not result in hp21$^{WAF1/CIP1}$ expression. These findings show that other events besides translocation are involved in triggering hPer2-mediated hp53 downstream signaling.

To further investigate the contribution of hPer2 in p53-mediated signaling, we tested the ability of hPer2 to modulate the reporter activity of a p53-responsive promoter *CDKN1a* (called *hp21$^{WAF1/CIP1}$*-luc hereafter) in the context of H1299 cells cotransfected with various hp53 and hPer2 chimeras when exposed, or not, to genotoxic stimuli (Figure 5, A and B). Chimeric transfections did not alter cell viability (Supplemental Figure S5). Unlike cells transfected with FLAG-hp53(ch)hPer2, H1299 cells show a roughly fourfold increase in *hp21$^{WAF1/CIP1}$*-luc activity compared with empty vector–transfected cells when expressing comparative levels of either FLAG-hp53 or hp53(ch)GST in the absence of radiation (–γ-IR; Figure 5B and Supplemental Figure S6A). As expected, H1299 cells transfected with either FLAG-hp53 or hp53(ch)GST and exposed to genotoxic stress (+γ-IR) showed a greater enhancement of *hp21$^{WAF1/CIP1}$* activity (~50% more than similar untreated cells) that is down-regulated to basal levels when cells were transfected with FLAG-hp53(ch)hPer2 instead (Figure 5B and Supplemental Figure S6A). Moreover, this result seems to be independent of the radiation dose, as shown in Supplemental Figure S7. In accordance, *hp21$^{WAF1/CIP1}$* activity remained low in FLAG-hp53(ch)hPer2(356-574/683-872)–transfected cells despite overexpression of the recombinant proteins and relocalization of the FLAG-NLS-hp53(ch)hPer2(356-574/683-872) chimera to the nucleus (Figure 3 and Supplemental Figures S4B, S6B, and S8E). Collectively these results suggest that when bound to hPer2, hp53 is unable to perform its transcriptional function despite the chimera being localized in the same cellular compartment (Figures 3 and 5B).

We then expanded our studies to examine the transcriptional effect of hp53(ch)hPer2 chimera on other hp53 target genes (i.e., *SFN* [encodes 14-3-3σ], *BAX*, *MYC*, *CDKN1a*, *GADD45α* [encodes growth arrest and DNA damage–inducible protein 45α, Gadd45α]) by measuring mRNA levels using quantitative reverse-transcription PCR (qRT-PCR). The rationale behind this experiment is that hp53(ch)hPer2 would counteract the effect of hp53 (or hp53(ch)GST) in gene expression due to its incapability of dissociating hPer2 from hp53, thus acting as a dominant-negative complex. Moreover, we expect that, whereas genotoxic stress (+γ-IR) would exacerbate specific p53-mediated gene expression, hp53(ch)hPer2 would continue to maintain a negative effect despite the stimuli.

As shown in Figure 5C, untreated H1299 cells (–γ-IR) transfected with hp53(ch)hPer2 showed down-regulation of both *SFN* and *CDKN1a* expression relative to the values obtained for hp53 transfection. Conversely, *MYC* exhibited positive regulation, whereas *BAX* and *GADD45α* remained largely unchanged. These results are in agreement with the positive role of hp53 in transcription of *SFN* and *CDKN1a* and its repressor role toward *MYC*. Three independent controls were simultaneously tested for all analyzed genes and incorporated as supplementary material (Supplemental Figure S8, A–C). There were no significant differences in gene expression when 1) empty vector (EV)–transfected cells were exposed, or not, to γ-IR, ruling out off-target effects (Supplemental Figure S8A), 2) cells transfected with either FLAG-hp53(ch)GST or FLAG-hp53 showed comparative levels of expression for all transcripts in the absence of radiation (Supplemental Figure S8B), and 3) under genotoxic stress (Supplemental Figure S8C). In all cases, gene expression levels were relative to those obtained by transfecting FLAG-hp53.

Next H1299 cells were exposed to genotoxic stress (+γ-IR) and transcripts amplified and compared relative to that of FLAG-hp53 (–γ-IR) (Figure 5C). As expected, both *SFN* and *CDKN1a* transcripts increased in response to radiation, confirming activation of the hp53 pathway (Figure 5C, white vs. black bars). The relevance of hPer2 dissociation for hp53 activity was evident when similar transcript levels were measured in extracts from hp53(ch)hPer2-transfected cells and were found significantly down-regulated (Figure 5C, black vs. black dashed bars). In other cases, neither hp53 nor hp53(ch)hPer2 transfections affected the expression of hp53 target genes (i.e., *GADD45α* and *BAX*) independently of whether cells were exposed or not and collected shortly after genotoxic stress (–/+ γ-IR). Finally, *MYC* expression was upregulated, approximately threefold, in hp53(ch)hPer2-transfected H1299 cells relative to FLAG-hp53, opposing its repressor role on *MYC* expression. Similar experiments were performed using FLAG-hp53(ch)hPer2(356-574/683-872), a chimera construct that effectively blocks hp53 ubiquitination (Supplemental Figure S8D) but remains secluded in the cytosolic compartment unless an NLS is added (Figure 3). As expected, transcript analyses of FLAG-hp53(ch)hPer2(356-574/683-872)–H1299 transfected cells showed a pattern

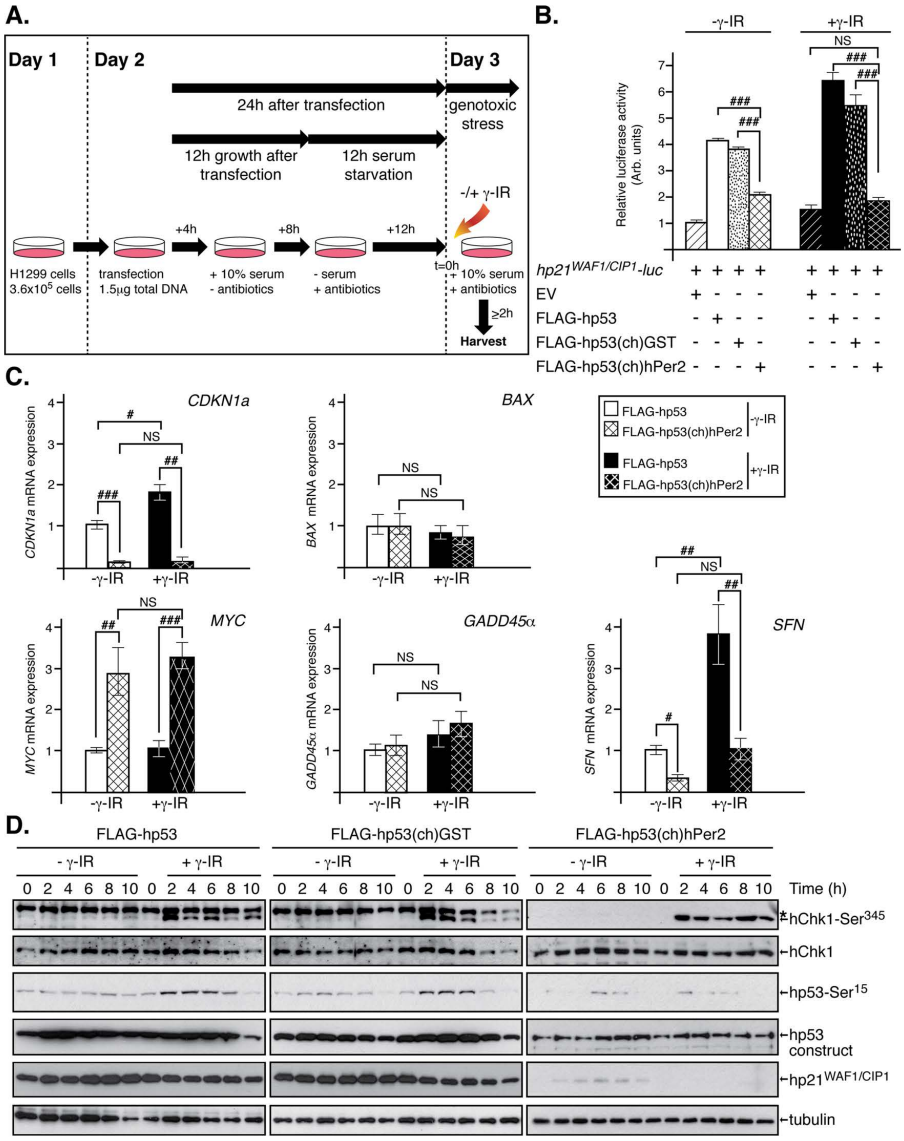

FIGURE 5: The hPer2 protein maintains hp53 transcriptionally inactive when complexed. (A) Schematic representation of the approach followed. In all cases, H1299 cells were harvested after transfection and before irradiation (γ-IR, 5 Gy; $t = 0$). (B, C) Cells harvested 2 h after irradiation. (D) Time points taken at 2-h intervals after irradiation. (B) H1299 cells were cotransfected with the reporter *hp21*$^{WAF1/CIP1}$-luc construct, pCS2+FLAG-hp53, pCS2+FLAG-hp53(ch)GST, pCS2+FLAG-hp53(ch)hPer2, or EV, with pCMV-β-gal as internal control. Extracts from cells treated (+γ-IR) or not (–γ-IR) with radiation were assayed for luciferase and β-galactosidase activities. The experiment was replicated thrice; error bars indicate SEM, and data were evaluated by ANOVA using Bonferroni posthoc test (SPSS). $^{###}p \leq 0.001$. (C) H1299 cells were transfected with either pCS2+FLAG-hp53 or pCS2+FLAG-hp53(ch)hPer2 and treated (+γ-IR) or not (–γ-IR) with radiation. The qRT-PCR data were normalized to the levels of expression in untreated FLAG-hp53–transfected cells (–γ-IR). Data are presented as the mean ± SEM from three independent experiments performed in triplicate. Statistical comparisons were evaluated by ANOVA using Bonferroni or Games-Howell posthoc analyses when needed (SPSS). NS, not significant; $^{#}p \leq 0.05$; $^{##}p \leq 0.01$; $^{###}p \leq 0.001$. (D) H1299 cells were transfected with pCS2+FLAG-hp53, pCS2+FLAG-hp53(ch)GST, or pCS2+FLAG-hp53(ch)hPer2 and treated (+γ-IR) or not (–γ-IR) with radiation as indicated in A and *Materials and Methods*. Aliquots of lysates taken at different times (20 μg) were blotted using specific antibodies

of expression that resembles the one observed when cells were transfected with hp53(ch)hPer2 and opposite to that resulting from hp53 transfection (Supplemental Figure S8D). Taken together, our data support a model in which hp53 binding to hPer2 is required for maintaining basal levels of hp53 in the nucleus in an inactive transcriptional state by forming a stable complex. Moreover, our findings imply that unbound hp53 from hPer2/hp53 is an absolute requirement to signaling downstream in response to DNA damage.

The hPer2 protein directly influences checkpoint signaling

In an attempt to gain better understanding of the role of hPer2 in hp53-mediated checkpoint signaling, we investigated the integrity of the checkpoint pathway upstream of hp53 and for hp21$^{WAF1/CIP1}$ expression in various transfected scenarios in irradiated H1299 cells (Figure 5D and Supplemental Figure S8F). Phosphorylation of downstream ATM/ATR kinase (i.e., Ser^{345} in Chk1) precedes p53 stabilization and is a marker of checkpoint activation (for review, see Meek, 2009). Therefore H1299 cells were transfected with EV, FLAG-hp53, FLAG-hp53(ch)GST, FLAG-hp53(ch)hPer2, or FLAG-hp53(ch)hPer2(356-574/683-872) and exposed, or not, to genotoxic stress. Extracts were analyzed for endogenous active forms of hChk1 and hp53 and levels of hp21$^{WAF/CIP1}$ (Figure 5D and Supplemental Figure S8F). First, we monitored hChk1 protein in all samples to find steady levels of expression throughout the time course analyzed in both exposed and transfected samples (Figure 5D and Supplemental Figure S8F). In addition, activation of Chk1 by phosphorylation in Ser^{345} was detected as early as 2 h postirradiation in exposed samples from transfected cells (+γ-IR), thus confirming both checkpoint activation and the integrity of the p53 upstream signal cascade in all cases (Figure 5D and Supplemental Figure S8F). On the other hand, phosphorylation of Ser^{15} of hp53 was detected exclusively in extracts from FLAG-hp53 and FLAG-hp53(ch)GST cells and in response to radiation (Figure 5D), suggesting that, if present, hp53 would get activated. Of interest, neither FLAG-hp53(ch)hPer2 nor FLAG-hp53(ch)hPer2(356-574/683-872) was phosphorylated on Ser^{15} of the hp53 portion of the chimera, implying that this site might not be accessible when the complex with hPer2 is formed (Figure 5D and Supplemental Figure S8F). Together our present data and previous studies support a model in which binding of hPer2 to hp53 modulates its stability and compromises hp53 function (Figure 6; Gotoh *et al.*, 2014).

DISCUSSION

The connection of circadian components to various aspects of cell division promises a better understanding of how cells sense and respond to changes in environmental conditions. At the transcriptional level, the integration of clock core components to multiple signaling networks is evident from studies carried out using genome-wide RNA interference screening analysis and high-throughput microarray technologies (Duffield *et al.*, 2002; Panda *et al.*, 2002; Mullenders *et al.*, 2009; Zhang *et al.*, 2009). Indeed, these studies have thoroughly identified genes whose knockdown directly modulates the circadian clock and others whose expression is controlled by clock core components (Duffield *et al.*, 2002; Panda *et al.*, 2002; Mullenders *et al.*, 2009; Zhang *et al.*, 2009). Regardless of the analysis platform, it is clear that cell cycle and circadian components are interlocked through genetic, protein interaction, and physiological mechanisms and that perturbation of the circadian system affects cell growth and proliferation (Fu *et al.*, 2002; Matsuo *et al.*, 2003; Miller *et al.*, 2007). Accordingly, circadian-regulated cell cycle genes such as *CCND1*, *WEE1*, *MYC*, *GADD45*α, and *CDKN1a* are known to show periodic patterns of expression in a 24-h cycle, a finding further strengthened by the identification of Per2 and Bmal1 as direct modulators of *WEE1*, *MYC*, and *CDKN1a* expression (Grundschober *et al.*, 2001; Fu *et al.*, 2002; Matsuo *et al.*, 2003; Grechez-Cassiau *et al.*, 2008). Further theoretical analyses of protein interaction networks helped place gene products that are directly or indirectly associated to known clock components, including various cell cycle modulators, in a global interactome map, which has provided clues about the many aspects of cell physiology directly regulated by the clock (for review, see Zhang and Kay, 2010).

An initial question relates to the subcellular localization of hp53, hPer2, and Mdm2 and their respective complexes. Our studies establish the presence of the hp53/hPer2 complex in both cytosolic and nuclear compartments, whereas the presence of the trimeric complex with Mdm2 seems to be restricted to the nucleus (Figure 1 and Supplemental Figure S1). Our chimera experiments provide evidence of distinct roles of hPer2 in both subcellular compartments. We believe that, when in the cytosol, hPer2 provides the signal needed for the hp53/hPer2 complex to translocate to the nucleus, whereas in the nucleus, hPer2 helps to maintain hp53 in a stable, inactive form until a stimulus (either external or internal) is applied to the biological system (Figure 6). This model is largely supported by our previous work (Gotoh *et al.*, 2014) and experiments showing that 1) blockage of the hp53 C-terminus NLSs by binding to hPer2 domains (hp53(ch)hPer2(356-574/683-872)), but not full-length hPer2 (hp53(ch)hPer2), prevents hp53 from nuclear localization (Figure 3, iii and vi, and Supplemental Figure S3), 2) addition of NLS to the same construct relocalizes the chimera protein in the nucleus (Figure 3v and Supplemental Figure S3), 3) only a constitutive hp53/hPer2-bound chimera (hp53(ch)hPer2) localizes in both compartments (Figure 3vi and Supplemental Figure S3), suggesting a role for hPer2 in hp53 translocation, and, finally, 4) constitutive association of hPer2 to hp53 in the form of [hp53(ch)hPer2] prevents hp53 from exerting its transcriptional activity in cells even in the presence of genotoxic stress (Figure 5 and Supplemental Figures S7 and S8). At present, it cannot be formally excluded that hPer2, hCry1, and hp53 or a fraction of these molecules could, a priori, form a trimeric complex in the cell. This is largely due to our finding that in vitro–expressed proteins were able to form multiple stable complexes (Supplemental Figure S4A). In addition, rhythmic hPer2 heterodimerizes with hCry1 through feedback to the nucleus and sustains oscillations. Thus, it is plausible that two pools of hPer2 might exist—one bound to hp53 and a separate pool that associates with its circadian counterpart. Nevertheless, the two pools of hPer2 might not be exclusive and might serve to assign distinct functions to hPer2 in each of these pools.

Once the hp53/hPer2 complex is in the nucleus, Mdm2 binds to the N-terminus domain of hp53, forming a trimeric complex in which hp53 ubiquitination does not take place (Figures 1B and 6;

(α-Chk1 and α-p21 for endogenous Chk1 kinase and hp21$^{WAF1/CIP1}$, respectively; α-FLAG for FLAG-hp53, FLAG-hp53(ch)GST, and hp53(ch)hPer2; α-Chk1-Ser^{345} for phosphorylation in Ser^{345} of endogenous Chk1; and α-p53-Ser^{15} for phosphorylation in Ser^{15} in FLAG-hp53, FLAG-hp53(ch)GST, and hp53(ch)hPer2). Tubulin was used as a loading control (bottom). Asterisk indicates a nonspecific signal.

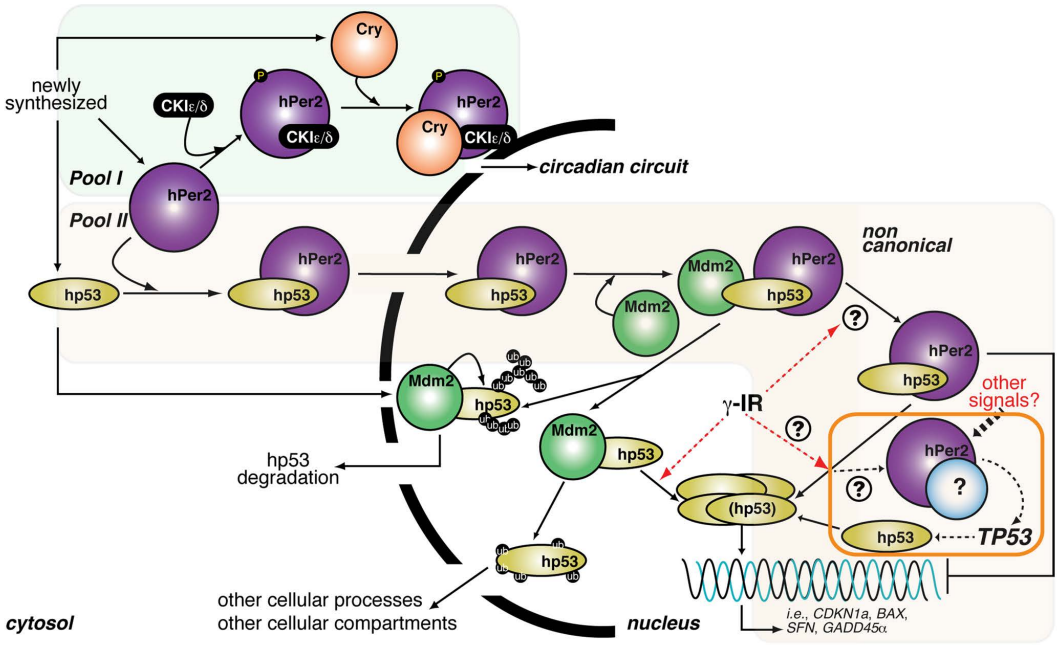

FIGURE 6: Proposed model of hPer2 and hp53 interaction and function. Newly synthesized hp53, hPer2, and Cry localize in the cytosolic compartment where the Cry/hPer2/CKIε/δ (pool I) is formed, and hPer2 is incorporated in one or more complexes that constitutes pool II. Pool I: As extensively reviewed, cytosolic hPer2 is phosphorylated by casein kinase I ε/δ (CKIε/δ) and initially degraded by the ubiquitin proteasome pathway (Ko and Takahashi, 2006). Later, Cry accumulates and associates with hPer2/CKIε/δ, and this complex translocates to the nucleus, where Cry disrupts the Clock/Bmal1-associated transcriptional complex, resulting in inhibition of *CRY*, *PER*, and *REB-ERVα* and derepression of *BMAL1* transcription and modulation of the expression of other clock-controlled genes (shown as "circadian circuit" for simplicity). Pool II: The hPer2 protein associates with cytosolic hp53, forming a stable complex (Gotoh *et al.*, 2014) that translocates to the nuclear compartment and keeps hp53 in a functionally inactive but stable state, ensuring that basal levels of hp53 exist ("priming state"). This heterodimer eventually incorporates Mdm2, forming a trimeric and stable Mdm2/hp53/hPer2 complex. In response to, for example, a genotoxic stress (labeled as γ-IR in the cartoon), the trimeric complex disassembles by a yet-unknown mechanism, which leads to release of hp53 and downstream activation of genes involved in cell cycle arrest and DNA repair. An amplification loop exists in which hPer2, alone or in association with an unidentified partner, transcriptionally activates *TP53*, further sustaining the hp53-mediated response (boxed in orange). Alternatively, cytosolic hp53 enters the nucleus, where it is targeted by Mdm2 and either polyubiquitinylated and degraded back into the cytosol or monoubiquitinated and translocated to a different compartment, as has been described (for review, see Kruse and Gu, 2009).

Gotoh *et al.*, 2014). This is in addition to the already established canonical p53 pathway, in which nuclear trafficking of hp53 is followed by Mdm2 binding and either monoubiquitination and nuclear export or p53 polyubiquitination and proteasomal degradation (incorporated in Figure 6; Honda *et al.*, 1997; Li *et al.*, 2003). We speculate that, under physiological conditions, hp53 remains an inactive component of the hp53/hPer2/Mdm2 and hp53/hPer2 complexes and is therefore unable to modulate the expression of downstream target genes (Figures 5 and 6 and Supplemental Figure S4).

Regulation of stress-mediated p53 activation has largely relied on a well-established model in which p53 stabilization precedes activation. Stabilization is primarily accomplished via the release of p53 from its interaction with Mdm2 by a mechanism that involves checkpoint activation, p53 phosphorylation, and inhibition of p53's interaction with Mdm2 (for review, see Meek, 2009), thus preventing p53 ubiquitination and proteasomal degradation (for review, see Kruse and Gu, 2009). Once stabilized, p53 is further posttranslationally modified, and its tetrameric form binds to DNA at specific high- and low-affinity p53 response elements, which regulates the expression of target genes via the recruitment of coactivators and corepressors. Despite being capable of providing an explanation for the many modes by which p53 controls gene expression, the canonical model is not sufficient to explain more recent genetic studies. Therefore further refinement has been introduced. As a result, the most recently updated model takes into consideration the many stress types that converge in p53, the various posttranslational modifications to which p53 is subjected (i.e., acetylation, sumoylation), and the tissue-specific function of p53. The end result is a model in which regulatory redundancy among posttranslational modifications is a common theme and serves to permit p53 to sense different signals and intensities, which allows for a signaling response that is properly modulated and tissue specific (for review, see Vousden, 2009). As expected, there is also a myriad of p53 binding partners, whose interactions exert selective influences on p53 target genes,

ranging from Mdm2 and MdmX negative regulators to orphan receptors, and for which a wealth of studies already exists (for review, see Vousden, 2009). Thus some common steps exist in the regulation of p53 activity (stabilization, modification, protein interaction, and promoter-specific activation), but many players differentially intervene in each step to influence the outcome of the response.

Questions arise about how free hp53 accumulates in response to radiation and what the role of hPer2 is in this event. Although we cannot provide a definitive mechanism for how the transition from hPer2 bound to hPer2 free leads to hp53 accumulation and transcriptional response (Figure 6, dashed arrow), it does not seem to be globally associated with hPer2 dissociation and shuttling, as is the case for HCT116 cells (Supplemental Figure S10A), but, more likely, to be specific for certain cell types, as is for HEK293 cells (Supplemental Figure S10B). In addition, it also seems linked to posttranslational events, as we found that hPer2 is associated with checkpoint kinases in response to radiation (unpublished data). Finally, we cannot rule out the existence of additional players in this response, including other circadian factors that may act by sequestering hPer2 away from (or "pulling hPer2 out of") the complex with hp53 when cells are exposed to a genotoxic stimulus. As a result, our model stresses the need for the existence of an hp53 tetramer that is free of hPer2 in order for its transcriptional activity to take place (Figures 5 and 6 and Supplemental Figure S4); nonetheless, more in-depth studies need to be performed to uncover the exact mechanism.

An additional component in the model refers to the regulation of *TP53* (encodes p53) transcription by hPer2, a process we propose that acts as a form of amplification loop that helps sustain the initial hPer2/hp53-mediated response (Supplemental Figure S9; Gotoh *et al.*, 2014). The relevance of hPer2-mediated *TP53* transcription is exposed when the expression of hp53 downstream target genes in the context of hPer2 overexpression is analyzed (Supplemental Figure S9A; Gotoh *et al.*, 2014). Based on our proposed model, overexpression of hPer2 should lead to stabilization of hp53 and inactivation of its transcriptional activity as a result of hPer2/hp53 complex formation. However, Gotoh *et al.* (2014) reported that this is not the case and that whereas hp53 is stabilized by hPer2 overexpression, transcription of the hp53 downstream target genes remains largely unaltered. A way to reconcile these data is to monitor *TP53* transcription in the context of hPer2 overexpression (Supplemental Figure S9, A and B; Gotoh *et al.*, 2014). Accordingly, our data show that whereas cells expressing hPer2 (HCT116 cell, $p53^{+/+}$) up-regulate *TP53* (Supplemental Figure S9A), the pool of hp53 complexed to hPer2 is only a fraction of the total level of hp53 in the cell, and thus "free" hp53 still exists and is able to transcriptionally activate downstream target genes (Supplemental Figure S9, A and B). This concept is further validated in H1299 cells (p53-null), in which hPer2 overexpression does not result in *CDKN1a* expression but FLAG-hp53 and -hPer2 cotransfection does when the level of hp53 is greater than that of hPer2 (Supplemental Figure S9C). Finally, an interesting aspect of our work is the level within the checkpoint hierarchy at which hPer2 intersects this pathway, the hp53 node. Because cancer development largely relies on sustained inactivation of the p53 pathway, the existence of factors that respond to environmental signals and influence hp53 stability encourages the search for unconventional drug targets and would certainly provide a new direction as to when and how to treat various cancers.

Our results, which establish a connection between the circadian regulatory system and the DNA-damage response mechanism at the level of hPer2–hp53 interaction, have important implications for our understanding of the etiology of cancer and the development of novel treatment modalities. Because cancer initiation and progression largely rely on a variety of DNA-damage repair mechanisms going awry in normal cells, thus facilitating tumor growth, the identification of hPer2 as a factor that responds to environmental signals and stabilizes hp53 provides a unique opportunity to evaluate the influence of the environment in the development of, for example, sporadic forms of cancers. Furthermore, our findings encourage a search for unconventional drug targets and treatment regimens based on the dynamics of the circadian control system.

MATERIALS AND METHODS

Plasmid constructs

The FLAG-tagged, *myc*-tagged, and untagged chimera constructs of hp53 and hPer2 were generated as follows. The hp53 cDNA clone was amplified by PCR and the stop codon removed before cloning into the LIC site of either pCS2+FLAG or *myc* tag vectors. For the GST chimera construct of hp53, the GST sequence was cloned downstream of hp53 into the *SalI*/*Xho*I sites in either vector. A glycine linker of six residues was introduced between hp53 and GST to favor flexibility. These chimera constructs are called FLAG-hp53(ch)GST and *myc*-hp53(ch)GST throughout the text. For the hPer2 chimera of hp53, the two hPer2-interacting fragments (residues 356–574 and 683–872) were subcloned downstream of hp53 following essentially the approach described except that hPer2(356-574) and hPer2(683-872) fragments were sequentially cloned into the *SalI*/*Xho*I and *SalI*/*SalI* sites, respectively. As was the case with the GST chimera construct, six glycine residues were added between hp53 and hPer2(356-574) and between the hPer2 fragments. These chimera constructs are referred to as FLAG-hp53(ch)hPer2(356-574/683-872) and *myc*-hp53(ch)hPer2(356-574/683-872). Chimeras containing an NLS were generated by cloning the NLS sequence encoding the TPPKKKRKVEDP residues from SV40 (Rexach and Blobel, 1995) upstream of hp53 using the appropriate pCS2+ as the template. The constructs are called FLAG-NLS-hp53(ch)GST, *myc*-NLS-hp53(ch)GST, FLAG-NLS-hp53(ch)hPer2(356-574/683-872), and *myc*-NLS-hp53(ch)hPer2(356-574/683-872). Full-length Per2-containing chimeras were generated as follows. The hPer2 cDNA was cloned downstream of hp53 into the *SalI*/*Xba*I sites. A six-glycine linker was genetically engineered between hp53 and hPer2 sequences to allow for flexible rotations. The full-length chimeras are referred to as FLAG-hp53(ch)hPer2, *myc*-hp53(ch)hPer2, and hp53(ch)hPer2. The $hp21^{WAF1/CIP1}$-Luc reporter plasmid was a kind gift from Daiqing Liao (University of Florida, Gainesville, FL) and is described in Zhao *et al.* (2003).

Cell culture and transfections

The HCT116 and H1299 cell lines were purchased from the American Type Culture Collection and maintained according to the manufacturer's recommendations. For transfection experiments, cells were seeded in six- or 12-well plates until they reached 50–80% confluence. Transfections were optimized using Lipofectamine LTX (Life Technologies, Grand Island, NY) for HCT116 and H1299 following the manufacturer's instructions. Otherwise, transfections in all cell lines were in HyClone HyQ-RS reduced serum medium (Thermo Scientific, Waltham, MA) for 4 h for H1299 and HCT116. Proteins were then allowed to express at 37°C/5% CO_2 in the appropriate medium containing 10% fetal bovine serum without antibiotics, after which they were either collected or further synchronized. Extracts for protein analysis were prepared in NP-40 lysis buffer containing 10 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM EDTA, 10% glycerol,

0.5% NP-40, 80 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, and protease inhibitors (10 μM leupeptin, 1 μM aprotinin A, and 0.4 μM pepstatin).

In vitro binding assays

In vitro transcription and translation of pCS2+*myc*-hPer2, *myc*-Mdm2, *myc*-hCry1, FLAG-hPer2, and FLAG-hp53 were carried out using the SP6 high-yield TNT system (Promega, Madison, WI) following the manufacturer's instructions, although, unlike the standard procedure, the reaction was cold. Aliquots (1–4 μl) of indicated recombinant proteins were preincubated for 20 min at room temperature to allow the complex to form before adding NP40 lysis buffer. Immunoprecipitation of the various complexes was carried out essentially as described.

Immunoprecipitation and immunoblot assays

For (co)immunoprecipitation experiments, transfected cells were harvested in lysis buffer, and extracts (~100 μg) were incubated with either α-FLAG M2 agarose beads (Sigma-Aldrich, St. Louis, MO) or α-*myc* (9E10) beads (Santa Cruz Biotechnology, Dallas, TX) for either 2 h or overnight at 4°C with rotation before washing. Where indicated, immunoprecipitations were carried out in a two-step procedure, with extracts being incubated with the antibody (α-*myc*, α-FLAG, or α-p53) overnight at 4°C before the addition of protein A beads (50% slurry; Sigma-Aldrich). Sample beads were then washed with lysis buffer, resolved by SDS–PAGE, and analyzed by immunoblotting using specific primary antibodies (α-FLAG [Sigma-Aldrich], α-*myc* [Santa Cruz Biotechnology], α-Per2 [Sigma-Aldrich]), α-p53 [Santa Cruz Biotechnology], and α-ubiquitin [Enzo Biomol, Farmingdale, NY]). When indicated, the purity of the various cellular fractions was monitored by immunoblotting using α-tubulin (Sigma-Aldrich) and α-lamin A/C (Santa Cruz Biotechnology) antibodies for the cytosolic and nuclear fractions, respectively. In all cases, horseradish peroxidase–conjugated α-rabbit or α-mouse immunoglobulin G (IgG) secondary antibodies (GE Healthcare Life Sciences, Buckinghamshire, UK; Cell Signaling, Danvers, MA) were used for immunoblotting following standard procedures. Chemiluminescence reactions were performed using the SuperSignal West Pico substrate (Pierce, Rockford, IL).

In other experiments, aliquots of total cell extracts were analyzed for expression of endogenous proteins. Circadian-synchronized HCT116 cells were collected at different times after synchronization and extracts (20–100 μg) analyzed for endogenous expression of hPer2, hp53, Mdm2, hCry1, and tubulin using specific antibodies. In addition, H1299 cells transfected with FLAG-hp53, FLAG-hp53(ch)GST, FLAG-hp53(ch)hPer2(356-574/683-872), FLAG-hp53(ch)hPer2, or EV were treated or not (control; –γ-IR) with ionizing radiation (5 Gy; +γ-IR; Di Leonardo *et al.*, 1994) and collected at different times after irradiation. Extracts (20 μg) were analyzed for expression of endogenous and recombinant proteins as well as posttranslational modifications by immunoblotting using α-FLAG, -Chk1 (Santa Cruz Biotechnology), -Chk1-Ser^{345} (Cell Signaling), -p53-Ser^{15} (Cell Signaling), -p21 (Cell Signaling), and -tubulin (Sigma-Aldrich) antibodies.

Finally, endogenous hp21 expression was monitored in extracts (30–40 μg) from H1299 cells transfected with FLAG-hp53, FLAG-hp53(ch)GST, FLAG-NLS-hp53(ch)GST, FLAG-hp53(ch)hPer2(356-574/683-872), NLS-FLAG-hp53(ch)hPer2(356-574/683-872), FLAG-hp53(ch)hPer2, FLAG-hp53(ch)hPer2, or EV using an α-p21 antibody (Cell Signaling). Fusion proteins were detected using α-FLAG antibody as described. In all cases, tubulin levels were monitored by immunoblotting and used as a loading control.

RNA extraction and qRT-PCR

Cell samples were obtained from cultures and treated or not (control; –γ-IR) with ionizing radiation (5 Gy; +γ-IR) 2 h before collection. In other experiments, cells were transfected and/or circadian synchronized before radiation treatment. Total RNA was extracted from cell pellets using the Trizol Reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified by spectrophotometric reading at 260 nm and analyzed for quality assurance using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) at the Virginia Bioinformatics Institute Proteomics Core Facility (Blacksburg, VA). qRT-PCR was conducted essentially as previously described (Yang *et al.*, 2008). Briefly, total RNA was pretreated with DNaseI (Promega) at 37°C for 30 min, and a 1-μg sample was used as a template for first-strand cDNA synthesis using the iScript cDNA Synthesis system (Bio-Rad, Hercules, CA). qRT-PCR assay was performed using IQ SYBR Green Supermix (Bio-Rad) as follows: 10 ng of cDNA (50 ng for the 14-3-3σ gene) was added to a 20-μl reaction volume containing the indicated primers for amplification (see Supplemental Methods and Supplemental Table S1). Real-time assays were performed in triplicate on a MyIQ single-color Real-Time PCR Detection instrument (Bio-Rad). Data were collected and analyzed with Optical System Software, version 1.0. The glyceraldehyde-3-phosphate dehydrogenase and β-actin genes were used as internal controls to compute the relative expression level (ΔC_T) for each sample. The fold change of gene expression in each sample was calculated as $2^{-\Delta\Delta Ct}$.

Immunofluorescence microscopy

H1299 cells were cultured on coverslips for 24 h and then transfected with pCS2+*myc*-hp53, -hp53(ch)GST, -hp53(ch)hPer2(356-574/683-872), -NLS-hp53(ch)GST, or -NLS-hp53(ch)hPer2(356-574/683-872) or untagged hp53(ch)hPer2 using Lipofectamine LTX (Life Technologies). After transfection, cells were fixed (3.7% formaldehyde/phosphate-buffered saline [PBS]/0.5% Triton X-100), washed with PBS/0.5% Triton X-100 and then 0.1% Triton X-100, and blocked with goat serum at room temperature for 30 min. Subcellular localization of *myc* fusion proteins was detected using an anti-*myc*-Cy-3–conjugated antibody (Sigma–Aldrich). Localization of untagged chimera recombinant protein (hp53(ch)hPer2) was visualized using α-p53 (Sigma-Aldrich) and -Per2 antibodies (Santa Cruz Biotechnology). Actin and nuclei were visualized by incubating fixed cells with phalloidin-Alexa 488 (Life Technologies) and SYTO 60 (Life Technologies). Fluorescence was visualized using a Nikon Eclipse TE2000-E microscope equipped with a Cascade II E2V CCD97 camera (Photometrics, Tucson, AZ) at 488, 568, and 647 nm. Images were processed using the NIS-Elements AR 3.0 Nikon software.

Gene reporter activity

H1299 cells were seeded onto 12-well plates and cotransfected with ~200 ng of $hp21^{WAF1/CIP1}$-luciferase (Luc) and pCS2+FLAG-hp53, -hp53(ch)GST, -hp53(ch)hPer2, or -hp53(ch)hPer2(356-574/683-872) or empty vector (pCS2+FLAG) each. The pCMV-β-gal (~200 ng) plasmid was included as an internal control. Cells were treated or not (controls; –γ-IR) with ionizing radiation (5 Gy; +γ-IR) and harvested 2 h later. Reporter activity was measured using the Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Readings were recorded from a Glomax Luminometer, and results were normalized for expression of β-gal, which was determined separately using the Galacto-Light Plus System (Bio-Rad). Experiments were done in triplicate and repeated at least twice.

Statistical analyses

Data were processed using either a two-tailed unpaired Student's *t* test or an analysis of variance (ANOVA) followed by either Bonferroni or, when necessary, Games-Howell post hoc test (SPSS; IBM, Armonk, NY). Levene's test was used to determine homogeneity of variances, whereas data normality was examined using Shapiro–Wilk *W* test and Box–Cox *Y* transformation (JMP 9; SAS) and applied when necessary. Values of $p \leq 0.05$ were considered statistically significant.

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