DOCTOR OF PHILOSOPHY

Modulation of Human ATM-Chk2 and ATR-Chk1 Kinases by Period-1 in the Response to DNA Damage and the Role of the Single Nucleotide Polymorphism Per1-A962P in the Response to Genotoxic Stress

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Modulation of Human ATM-Chk2 and ATR-Chk1 Kinases by Period-1 in the Response to DNA Damage and the Role of the Single Nucleotide Polymorphism Per1-A962P in the Response to Genotoxic Stress

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Summary

Human Period 1 (Per1) is a circadian clock regulator with two N-terminal PAS domains that belongs to a group of three highly related Period proteins (Per1, Per2, Per3).

The main aims of this thesis are to explore the novel link between Per1 and the DNA damage signalling pathways ATM-Chk2 and ATR-Chk1, and to investigate the functional significance of the single nucleotide polymorphism (rs2585405) which replaces alanine-962 by a proline residue in the C-terminal section of human Per1. While the alanine and proline alleles have an almost 50:50 ratio in East Asia, a strong selection against the proline residue exists in Europe. In line with the previous finding that human Per1 associates with ATM and Chk2 kinases, this thesis finds a strong correlation between elevated Per1 protein levels and increased ATM-Chk2 activity in human colon carcinoma cells (HCT116) compared to embryonic kidney cells (HEK293). Isoelectric focusing experiments reveal four Per1 splice variants in HEK293 cells. Each of the two larger variants exists as a mixture of at least two forms with distinct post-translational modifications. The abundance of both positively charged, large forms increase in the response to DNA damage. Endogenous Per1 appears to be mainly cytoplasmic in untreated and UV irradiated HEK cells. Using two stable HEK cell lines, which either express EGFP-PER1-A962 or EGFP-PER1-P962 in addition to the endogenous Per1 protein, the thesis provides evidence that a proline at position 962 reduces Chk1 phosphorylation at serine-345 in the response to UV-induced DNA damage and when DNA replication forks break in the presence of camptothecin.

Abbreviations

A	
A	Alanine
Amp	Ampicillin
APC	Adenomatous polyposis coli protein
APS	Ammonium Persulfate
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR interacting protein
B	
β	Beta
BGH	Bovine growth hormone
bp	base pair
BMAL1	Brain and Muscle Aryl hydrocarbon receptor nuclear translocator - Like1
BSA	Bovine Serum Albumin
C	
°C	Degrees Centigrade
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA

ChK1	Checkpoint kinase 1
ChK1S345ph	Checkpoint kinase 1-phosph Serin345
Chk2	Checkpoint kinase 2
CK1-ε	Casein kinase 1
Clock	Circadian Locomotor Output Cycles Kaput
CMV	Cytomegalovirus
CNS	Central nervous system
CPT	Camptothecin
Cry	Cryptochrome
D	
DAPI	4′, 6-Diamidino-2-phenylindole dihydrochloride
DEPC	Diethylpyrocarbonate
dH2O	Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Four deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate-buffered saline
DSBs	DNA Double -strand breaks
DTim	Drosophila Timeless

E	
E-box	Enhancer box
E.coli	Escherichia coli
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
F	
FBS	Fetal Bovine Serum
FRT	Flippase recognition target
G	
GAPDH	Glyceralaldehydephosphate-dehydrogenase
GFP	Green Fluorescent Protein
H	
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HCT116	Human male diagnosed with colon cancer
HEK293	Human Embryonic Kidney 293 cells
HRP	Horseradish peroxidase
HS	Heat Stress
hrs	Hours
I	

IF	Immunofluorescent staining
K	
kb	Kilobase
KDa	KiloDalton
L	
L	Litter
LB	Luria Bertani
LBA	Luria Bertani Agar
M	
MAPKs	MAP Kinase Signaling
MCM	Minichromosome maintenance protein complex
mg	Milligram
Mdm-2	Mouse double minute 2 homolog
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Milliliter
mM	Millimolar
MRN complex	MRE11-RAD50-NBS1
N	
Na_2EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate

Na_2HPO4	Sodium phosphate dibasic heptahydrate
NaCl	Sodium Chloride
NaH_2PO4	Sodium phosphate monobasic
NaOH	Sodium Hydroxide
NEB	New England Biolabs
ng	Nanogram
nM	Nanomolar
$NPAS_2$	Neuronal PAS domain-containing protein 2
N-terminal	Amino-terminal domain
O	
ORF	Open Reading Frame
P	
PAS	**Per**- period circadian protein **Arnt**- aryl hydrocarbon receptor nuclear translocator protein **Sim**- single-minded protein
Per-ARNT-Sim Domains	PAS Domains
P21WAF1/CIP1	Cyclin-dependent kinase inhibitor 1
PBS	Phosphate- buffered saline
P.Chk2	Checkpoint kinase 2-phosph (Thr68)

PCR	Polymerase Chain Reaction
PER1	Period circadian protein homolog 1
PER2	Period circadian protein homolog 2
PER3	Period circadian protein homolog 3
PFA	Paraformaldehyde
pH	Power of hydrogen
P	Proline
PVDF	Polyvinylidene difluoride
R	
RAD50	DNA repair protein RAD50
RFP	Red Fluorescent Protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPA	Replication protein A
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription-PCR
S	
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

shRNA	Short hairpin RNA or small hairpin RNA
siRNA	Small interfering RNA
SNPs	Single nucleotide polymorphisms
sORF	Short Open Reading Frame
SSB	Single strand break
ssDNA	Single-stranded Deoxyribonucleic acid
T	
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Tet	Tetracyclin
TIM	Timeless
Tipin	Timeless-interacting Protein
TopBP1	DNA topoisomerase 2-binding protein 1
T-REx	Tetracycline regulated expression
Tris-HCl	Tris-Hydrochloride
Trp	Tryptophan
TSAP	Thermosensitive Alkaline Phosphatase
U	
UV	Ultraviolet radiation

V	
V	Volt
W	
WB	Western blot
w/v	Weight/ Volume
Y	
YEA	Yeast Extract Agar

2D	Two dimension
3’	Three prime end of DNA
5’	Five prime end of DNA
µg	Microgram
µl	Microliter

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Chapter 1: Introduction

1.1 Circadian Clocks

Circadian rhythms are endogenous ~24h cycles in physiology and behavior, which are generated by a master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus of the brain and located in most other tissues (Stojkovic, Wing & Cermakian, 2014). This master clock is comprised of approximately 20,000 neurons, which receive photic information from the environment via neurons transcending from the retina via the retino-hypothalamic tract. The circadian system organizes the different biological functions in 24h, such as sleep, activity, temperature (Serón-Ferré et al., 2013), heart rate, glucose level, and cortisol production (Valenzuela et al., 2008).

The molecular clock is organized by a transcriptional/translational feedback loop of clock genes, named *Clock*, *Bmal1*, *Per1–3*, and *Cry1-2*. Historically, *Drosophila* has been at the forefront of circadian rhythm research due to its ease of use and its advanced molecular and genetic toolkit (Evans & Davidson, 2013). The period gene was originally discovered in the fruit fly, as a transcript from the period gene locus that alternated through the light and dark cycle (Reddy et al., 1984). Importantly, the molecular circadian clock is highly conserved from *Drosophila* to humans, and consists of interlocked transcriptional negative feedback loops. In mammals, the transcription factors CLOCK and BMAL1 dimerize and activate *Period* (*Per*1&2) and *Cryptochrome* (*Cry*1&2) genes. The PER1/2 and CRY1/2 proteins then enter the nucleus and inhibit the activity of CLOCK/BMAL1, thereby repressing their own transcription. This feedback loop is regulated by Casein kinase 1-epsilon.

The importance of circadian clocks is illustrated by the impacts of circadian disruption in humans. For example, shift work increases the risk of developing various illnesses, such as mental disorders, metabolic syndrome, and cancer (Evans & Davidson, 2013).

1.2 Structure of the circadian clock proteins PER-1, PER-2 and PER-3

The circadian clock is an oscillating system dependent on the light and dark patterns of day and night respectively, which is responsible for diurnal changes in different aspects of body functions in an organism such as behavior, physiology, and biochemical pathways. The circadian clock is present in all tissues of the body and is strongly influenced by both internal and external stimuli, with the most important being light. A number of genes have been found to belong to the circadian clock system, such as Period, Cryptochrome, TIMELESS, CLOCK (Circadian Locomotor Output Cycles Kaput), Bmal1 (Brain and Muscle Aryl hydrocarbon receptor nuclear translocator Like1) and NPAS-2. These genes product interact among themselves and regulate their own expression, as well as the expression of other genes, through positive and negative feedback loops.

Ron Konopka and Seymour Benzer were the first to discover the basic genes of circadian rhythms found in higher eukaryotes. In 1971, they found that the period locus existed in *Drosophila melanogaster*, from the genes they screened (Konopka & Benzer, 1971). They later proposed a model that encompassed both negatively and positively autoregulated feedback loops of translation and transcription. Interestingly, the vital components for the generation and regulation of circadian rhythms are not conserved across the different kingdoms of life. Although it was long thought that transcriptional feedback loops were required for a functional clock, recent studies have found the cyclic oxidation and reduction of peroxiredoxins to be common features of circadian rhythms. They are found in human red blood cells and act independently of a transcriptional loop. This led to the conclusion that the origins of circadian clocks are ancient oxidation-reduction systems (Ray & Reddy, 2016). In line with this conclusion, simple eukaryotic cells like budding yeast have a metabolic cycle that links the oxidation status of the cell to DNA replication, limiting the S phase to phases of low oxidative damage (Chen et al., 2007).

The Period proteins own their name to their cyclic expression as key regulatory elements of the circadian clock. Period proteins inhibit their own transcription by blocking the master transcription factor the BMAL-CLOCK heterodimer which is bound

to the E box in the *per* gene promoter regions. Essential to this regulation are the two PAS domains in the N-terminal section of the Per proteins: PAS-A and PAS-B (Figure 1-1A). PAS stands for PER-ARNT-SIM related domain (Kucera et al., 2012). This domain is conserved across all life forms, and is being discovered in a number of proteins. The PAS domains consist primarily of 5 anti- parallel β- sheets (βA- βE), one side of which is covered by a number of α-helices (αA- αC). These domains are primarily responsible for interactions among themselves in a heterodimeric or homodimeric fashion and with other circadian proteins. In the case of the circadian clock, the association of the *Drosophila* Per proteins with Timeless or of the human Per proteins with Cryptochrome, via the PAS domains of both binding partners protects the Period proteins from degradation and allows the heterodimers to enter the nucleus to down-regulate the BMAL-CLOCK complex, in a manner initiated by phosphorylation of the Period proteins by Casein Kinase 1 (Price et al., 1998, Meyer, Saez & Young, 2006, Langmesser et al., 2008). Formation of the Per homodimeric protein via the PAS domains initiates the degradation of the Period proteins (King et al., 2011). Figure 1-1B shows the structure of the *Drosophila* Period PAS domains in a homodimeric complex. The molecular structure of the PAS domain dimers is supported by interactions between the beta-sheets (PAS-B domains) and conserved tryptophan residues that vary between the three human homologues (Trp448 for Period-1, Trp419 for Period-2 and Trp359 for Period-3) (Kucera et al., 2012). Stabilisation of the dimers in the human Period proteins differs slightly for the different homologues. For the PER-1 protein, stabilisation occurs due to PAS domain interactions between the dimers and for the PER-3, protein stabilisation results from a unique N-terminal part consisting of a specific motif of the helix-loop-helix type. Even though the overall structure of the three human Period proteins is similar, they differ in length (Per1: 1290aa, Per2: 1255aa, Per3: 1210aa), with differences in the N and C-terminal regions. Appendix 1 shows a full alignment of the three human Period proteins. Figure 1-2A shows the domain organization of human Period-1 (Per1). Interestingly, the amino acid sequence of the PAS-A domain is much less conserved between the three proteins, when compared to the PAS-B domain (Figure 1-2B). The domain required for the phosphorylation by Caseine Kinase-1 (CK1-ε) is located between amino acids 596 and 815, and the last 141aa (1149-1290aa) are required for the association with Cryptochrome.

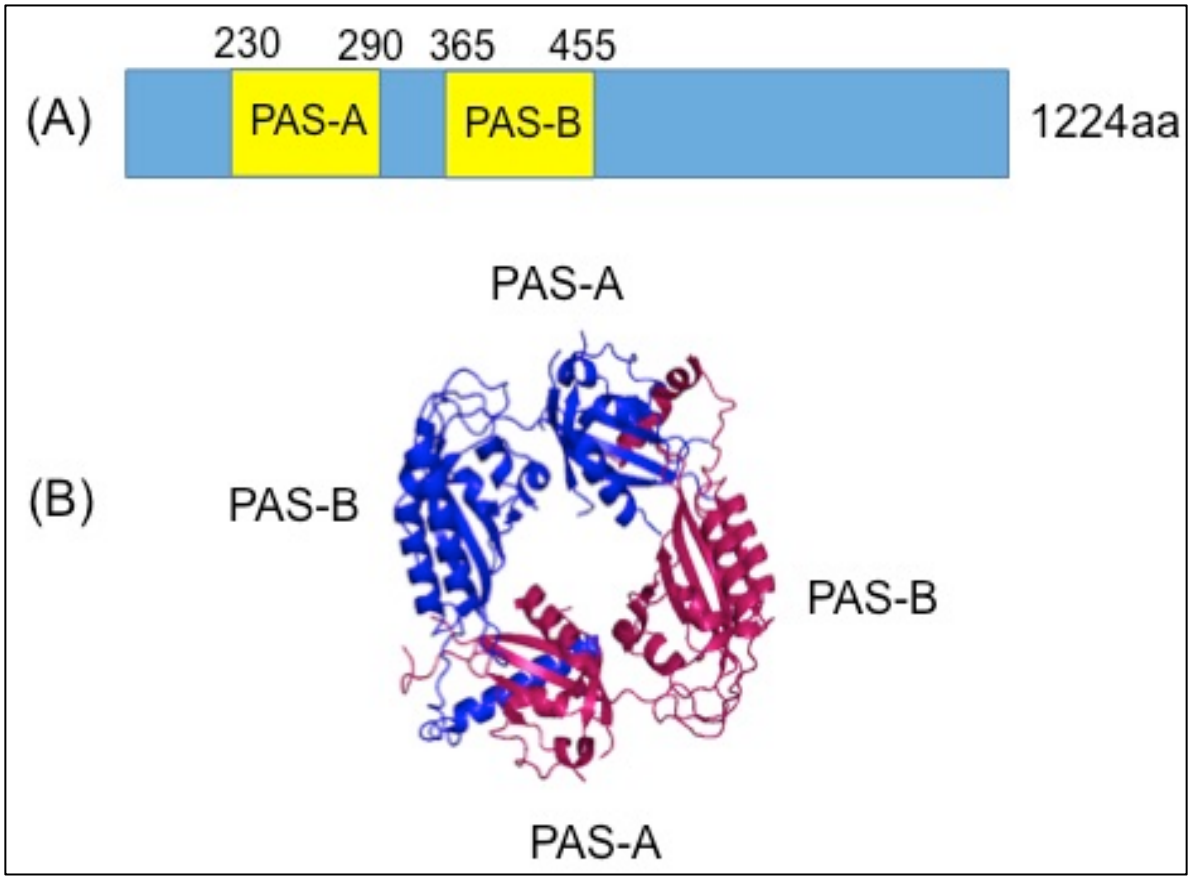


Figure 1–1: Schematic representation of the general structure of a dimeric Period protein complex in *Drosophila* (King et al., 2011). A: domain organization of *Drosophila* Period protein. The two PAS domains are indicated. B: the structure of the homodimeric complex of PAS-A and PAS-B (Protein ID: 3RTY; the image was generated with Polyview 3D). Please note that an extra alpha helix of the PAS-B domain interacts with the PAS-A domain in the opposite protein. The crystalized fragment encompasses amino acids 229- 575.

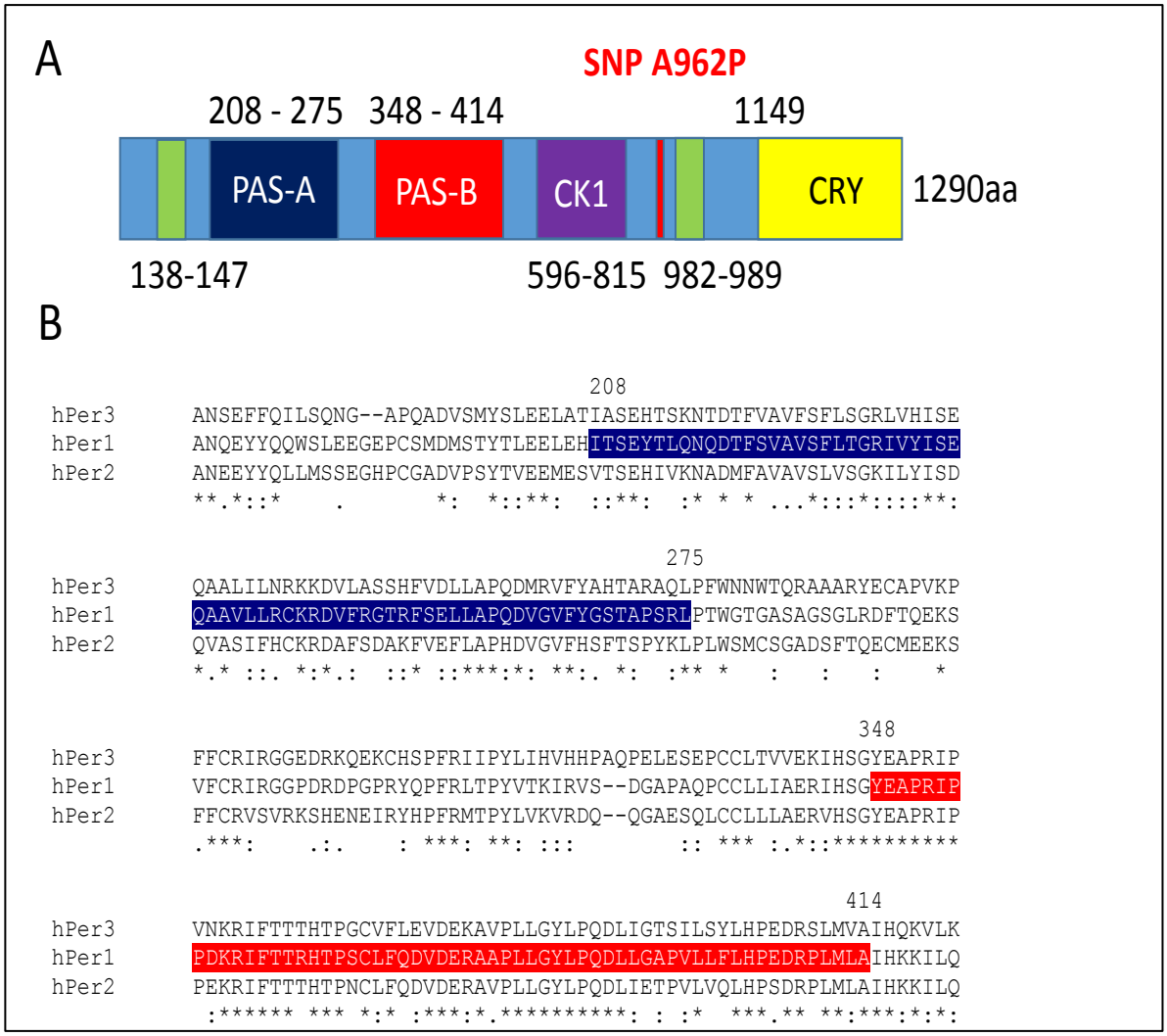


Figure 1–2: Structure of human Per1. A: Schematic representation of the domain organization of human Period-1 (Per1). The PAS-A, PAS-B domains are indicated. The domains required for Caseine kinase1 (CK1) phosphorylation and the domain required for the association with Cryptochrome (CRY) are shown. The position of the SNP A962P is indicated. The two nuclear export sequences are shown in green. B: Alignment of the PAS-A (blue) and PAS-B (red) domains of human Per1, Per2 and Per3 (Uniprot numbers: Per1: O15534; Per2: O15055; Per3: P56645). The full alignment is shown in Appendix 1.

1.3 Description of the Phenotypes of Mouse Knockouts of the *Period* Genes

Three main genes homologous of the *Drosophila* period (*Per1, Per2,* and *Per3*) are known to exist in mice (Tataroglu & Emery, 2014). Rhythmic locomotor activity is the measure of output pertaining to the biological clock (Tataroglu & Emery, 2014).

Per genes have been shown to oscillate in various non- neuronal peripheral tissues (Oishi et al., 1998, Zylka et al., 1998). As reported before, the expression of Per genes in kidney, heart and skeletal muscle is delayed compared with that in the SCN (Zylka et al., 1998).

Anatomical, physiological and behavioural basis of circadian system regulation is located in suprachiasmatic nucleus of the brain. Positive and negative feedback loops exist that regulate protein synthesis in the nucleus. Some of the genes involved in the negative feedback loop are the three Period genes (Per 1-3). The function of these genes has been characterised by phenotypic studies in mouse knock-out studies (Bae et al., 2001). The recent studies on *Per* knockout mice have shown that *mPer* genes have functionally different roles (Bae et al., 2001). PER1 gene knock-out mice lost synchronicity or rhythmic activity after transfer to constant darkness phases. However majority of the PER1 deficient mice still maintained rhythmicity in the first 10-14 days of being transferred to constant darkness and started to become severely arrhythmic in the second ten day interval and totally arrhythmic at third ten day intervals. Some animals showed slight rhythmicity in the eye in later stages but it was not sustained. The loss of rhythmicity was noted to be gradual. These results are corroborated by other studies that reported decreased stability and precision in addition to decreased duration of circadian period (Zheng et al., 2001). Similar to PER1 knock-out mice, PER2 knock-out mice also displayed loss of rhythmicity after being moved to constant darkness; however, considerable variability was observed in the time and length of persistence of circadian rhythm. In general, some PER2 knock-out mice lost rhythmicity immediately being shifted to constant darkness while another set of these mice maintained rhythmicity up to three weeks in constant darkness and lost it only after that time-point. A key finding was that irrespective of the genotype, locomotor activity was no different in both PER1 and PER2 knock-out mice. Thus, mutation or silencing of

these genes leads to instability of circadian system leading to a loss of rhythmic responses. In contrast to PER1 and Per 2 knock-out mice, PER3 knock-out mice were noted to not exhibit a severe response and displayed only a slight effect on rhythmicity of circadian clock as noted by a decrease in length of cycle. One common finding across all studies is that both the genes have unique patters of upstream control but they do complement each other in the maintenance of normal circadian rhythm.

Also, it is found that the deletion of both Per1 and Per2 clock genes in mice leads to multiple discrete changes in retina (Ait-Hmyed et al., 2013). Furthermore, Per1 and Per3 knockout mice have a milder phenotype (free-running period) of activity rhythms, which is slightly shorter than normal mice (Shearman et al., 2000, Cermakian et al., 2001). Also, free- running period of Per1- knockout mice is moderate than Per2 mutants mice, but more impairing in running than Per3 mutants, thereby the three Per genes may have different function (Cermakian et al., 2001).

1.4 Period Proteins and Cancer Suppression

While the Period proteins are one of the major components of the circadian system, emerging evidence suggests a role in the DNA damage response. The three main isoforms of the Period proteins PER-1, PER-2, and PER-3 (Savvidis & Koutsilieris, 2012), complement each other in their function towards circadian regulation. The PER proteins have been shown to heterodimerize with the TIM protein encoded by the two *TIMELESS* genes in *Drosophila* (DTim, DTim2). This is of interest, as the yeast orthologues of Timeless (ScTof1, SpSwi1) travel with the DNA replication fork and sense DNA replication stress (Dalgaard & Klar, 2000). A similar function has also been reported for the human Timeless protein. Human Timeless associates with its partner Tipin (ScCsm3, SpSwi3) to respond to UV light and oxidative stress (Gotter, Suppa & Emanuel, 2007). Human cells have only one *Timeless* gene, which encodes two splice variants. Only the longer variant associates with Cryptchrome1 (Cry1) and the DNA damage checkpoint kinase Chk1. This Timeless-Cry1 complex is regulated by the expression of Period-2 as it competes with Timeless for binding to Cry1 (Engelen et al., 2013). Interestingly, Period proteins have also been shown to play an important role in cell cycle regulation and responses of the cells to DNA damage, and disruption of the

circadian expression pattern in human and animals have been found to be associated with increased cancer risk (Gery et al., 2006). Period proteins have been shown to modulate the expression of a number of genes whose dysregulation has been implicated in cancer, such as the p53-regulator Mdm-2, Cyclin D1, Cyclin A, the transcription factor c-MYC, Wee1 kinase and the DNA-inducible nuclear GADD45α protein (Savvidis & Koutsilieris, 2012). The correlation between decreased *Period* gene expression and tumorigenesis has been demonstrated in animal studies, where it was shown that knockout animals for the *PER-2* gene displayed an increased susceptibility to tumour development and sensitivity to DNA breaks caused by ionizing radiation. Human epidemiological studies have revealed that the Period proteins are down-regulated in a number of human cancers (Fu et al., 2002). Work by Gery and colleagues has shown that Per-1 associates with the ATM and Chk2 kinases of the DNA break response pathway (Gery et al., 2006), and Unsal-Kaçmaz and colleagues have demonstrated the association of Timeless with the ATR and Chk1 kinases of the response to DNA replication stress (Unsal-Kacmaz et al., 2005)(Figure 1-3). The latter report identified also Cryptochrome2 as an interaction partner of Timeless, which indicates that Timeless may be able to interact with Cry1 and Cry2. The separation between the ATR-Chk1 and ATM-Chk2 pathways may not be so clear-cut, as Timeless depletion also affects ATM-Chk2 signaling in the presence of broken chromosomes (Yang, Wood & Hrushesky, 2010).

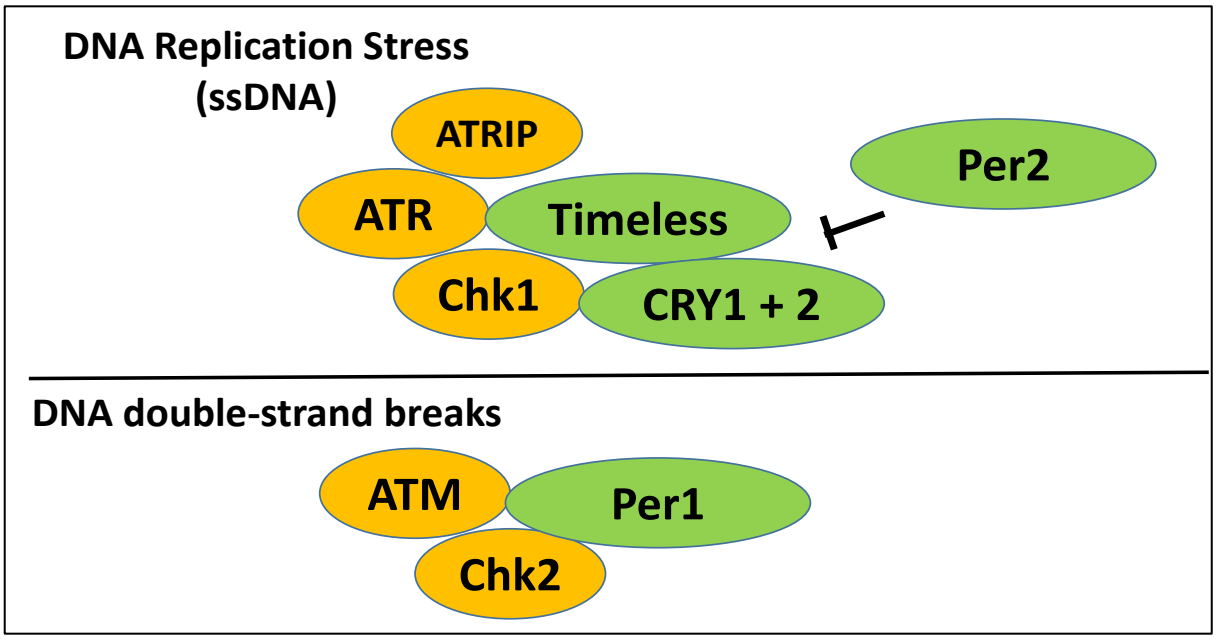


Figure 1–3: Regulation of the DNA damage response by some circadian proteins. Timeless may regulate the response to single-stranded DNA (ssDNA) exposed at stalled DNA replication forks, through the ATR-Chk1 checkpoint kinases, in association with Cryptochrome-1 and Cryptochrome-2. This may be negatively regulated by Period-2. Period-1 may modulate the response to DNA breaks by the ATM-Chk2 kinases.

The human ATR-Chk1 kinase pathway is activated when ATR kinase is recruited to single-stranded DNA exposed at stalled DNA replication forks, or at DNA breaks that have been converted into single-stranded DNA tails by their partner ATRIP (Awasthi, Foiani & Kumar, 2015). In contrast, the ATM-Chk2 kinase cascade is activated when ATM kinase is recruited to a double-stranded DNA break by the Mre11-Rad50-Nbs1 (MRN) complex. One recent report indicates that the Cryptochrome proteins Cry1 and Cry1 are not essential for the ATR-Chk1 response to UV-induced DNA damage, but provide a circadian oscillation pattern to the DNA damage signal (Kang & Leem, 2014).

1.5 Down-regulation of Period1 in Human Tumors

An epidemiological analysis of the incidence of female breast and colon cancer has shown that there is a definite correlation with an increased incidence and long periods of night shifts (Hansen, 2001, Schernhammer et al., 2003). This has led to the hypothesis that the circadian system may have tumour suppressor activity and that, conversely, its reduced activity may result in the enhancement of certain tumors, especially those related to the hormonal systems. Tissue culture studies have demonstrated that there is a definite involvement of human PER-1 in the DNA damage response and overall cellular growth (Gery et al., 2006). Real-time RT-PCR analysis of the expression of PER-1 in normal and breast cancer cell lines, and also in non-small cell lung cancer cell lines has demonstrated that there is a considerable down-regulation of PER-1 in the cancerous cell lines (Yang et al., 2006). In the lung cancer cell lines, *PER-1* transcription was found to be down-regulated by 70%, while in the breast cancer cell lines a 46% decrease in the levels of PER-1 was observed (Figure 1-4). These data therefore suggest that the *PER-1* gene may have tumour suppressing activity, and that its suppression could result in an increased incidence of tumors that are linked closely to a loss of cellular control and thereby to unchecked cellular growth.

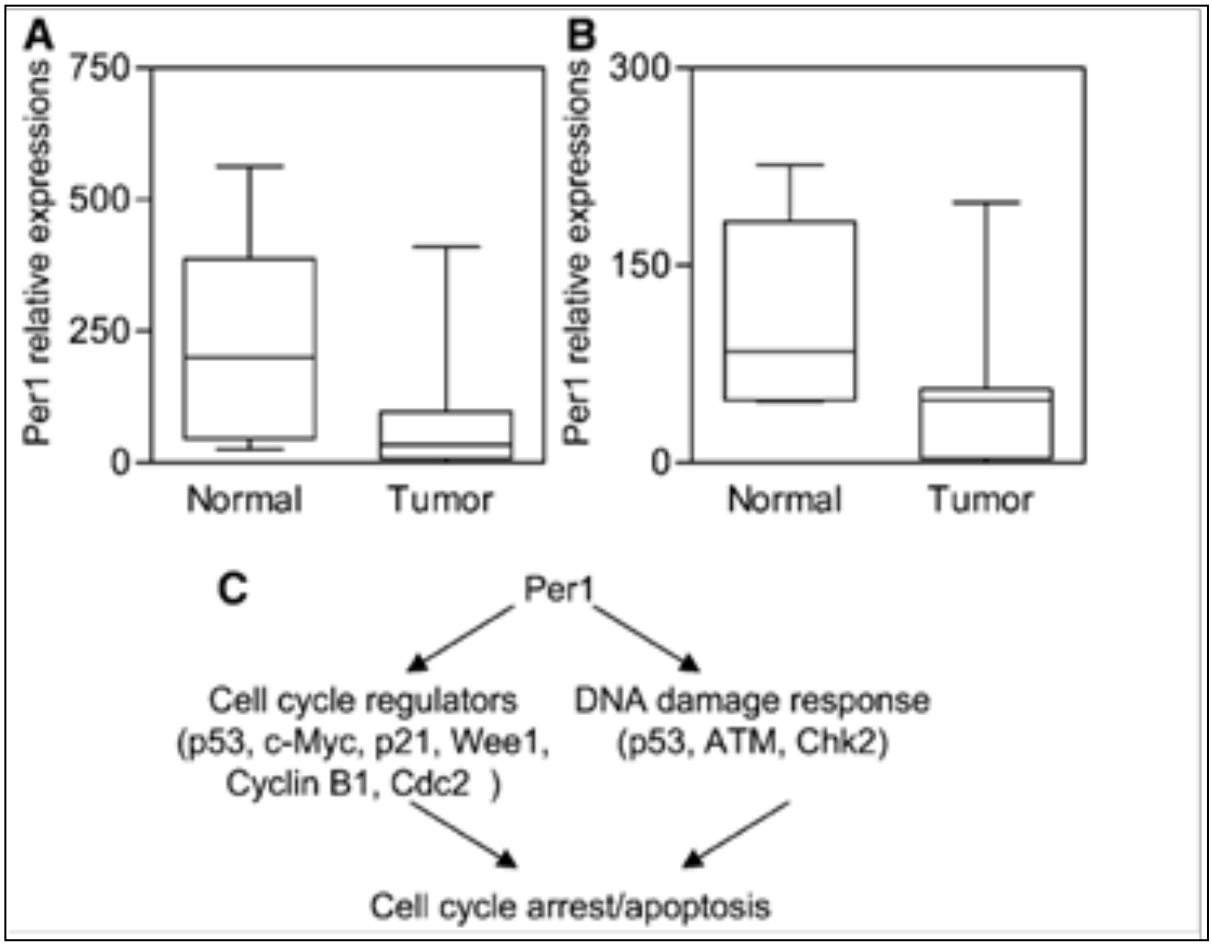


Figure 1–4: Down-regulation of PER-1 in human cancers from (Gery et al., 2006).

(A&B) Real-time RT-PCR analysis of Per1 expression. A: Thirty-three non-small cell lung cancer and control lung samples. B: Twenty-four breast cancer samples and six normal breast tissues. C: A model of how Per1 affects cell growth by regulating key factors in the cell cycle and the DNA damage pathways.

Recent evidence suggests the down-regulation of other genes in the Period family, including PER-1 in pancreatic and endometrial cancers (Pogue-Geile, Lyons-Weiler & Whitcomb, 2006, Yeh et al., 2005). Although other *Period* genes are also affected in human cancers, their level of down-regulation is not as severe as PER-1. The reduced expression of PER-2, for example, varies significantly among patients suffering from Acute Myeloid lymphoma (Gery et al., 2005). This suggests that either PER-2 down-regulation is only a consequence of the malignant transformation, or that already small

reductions in expression levels can cause cancer.

A study by (Chen et al., 2005), demonstrated significant down-regulation of not only PER-1 and PER-2 proteins but also PER-3 protein, in 56 out of a total of 59 breast cancer specimens, when compared with normal control tissues. Another study indicated that human endometrial carcinoma was closely associated with a significant decrease of the PER-1 protein (Yeh et al., 2005). These results suggest that Period proteins play a very important role in tumorigenesis in mouse and humans. Despite the higher association of the *Period* genes with breast and colon cancer, their disruption has also been linked to other cancer types, including prostate cancer, gliomas, chronic myeloid leukaemia, and also liver metastasis. PER-2 mutation has also been found to be strongly associated with a number of different lymphomas and salivary gland hyperplasia (Fu et al., 2002).

Down-regulation of PER-1 and PER-2 could lead to changes in the response to how cells monitor DNA damage, given that PER-1 interacts closely with the ATM-Chk2 pathway and PER-2 associates with the ATR-Chk1 kinases (Figure 1-3). Other oncogenic proteins, which are under control of the Period proteins, are p53, Wee1 kinase and the transcription factor c-Myc (Gery et al., 2006).

Recent research implicated PER-3 in the aetiology of breast cancer. It was reported that dysregulation in the expression level of PER-3, as a result of polymorphisms in the *PER-3* gene, promotes breast cancer (Zhu et al., 2005). These findings have also been confirmed with animal studies, where mice lacking PER-3 were shown to develop breast cancer when exposed to carcinogens (Climent et al., 2010). These studies demonstrate that PER-3 might also be an important candidate for the evaluation of circadian gene-mediated tumorigenesis and might also contribute to cancers in humans.

Although the exact mechanisms associated with the down-regulation of the *Period* genes and increased cancer risk are not known, there is enough data to support that a cascade of downstream molecular events happen when the level of expression of the *Period* genes falls below a certain threshold. It is known that different levels of DNA methylation in the *Per* genes have the ability to reduce their expression (Weber et al., 2005). Similar mechanisms have been shown to be responsible for the reduced expression of *Period* genes through different degrees of methylation in the promoter sequences, which results in varying levels of expression, from down-regulation to

silencing (Yeh et al., 2005). This suggests that hyper-methylation of promoter sequences could result in the silencing of *Period* genes, which could in turn increase the incidence of cancers. Both *PER-1* and *PER-2* genes have a direct modulatory effect on the levels of the transcription factor beta-catenin, which is linked with colon cancer (Wood, Yang & Hrushesky, 2009). PER-2 mutations have been shown to result in an increase in the levels of beta-catenin and, subsequently, in the formation of polyps in the colon. PER-2 mutation has also been shown to result in increased levels of the APC scaffold protein, which regulates the stability of beta-catenin (Wood, Yang & Hrushesky, 2009). As mentioned before, a possibility of feedback inhibition has also been shown to exist in circadian system-associated tumorigenesis, by which changes in the levels of beta-catenin and APC result in a further decrease in the expression of PER-2. This, in turn, affects the levels of beta-catenin and APC, thus forming a vicious cycle and ultimately leading to cancer (Wood et al., 2008).

1.6 Activation of ATM and ATR kinases

DNA surveillance mechanisms in eukaryotes have evolved into complex pathways, both for the proper maintenance of genomic integrity and for the accurate transmission of hereditary information from one generation to another. The cellular machinery in eukaryotes has various mechanisms available in response to DNA damage and heat stress to preserve the integrity of the genome (Figure 1-5).

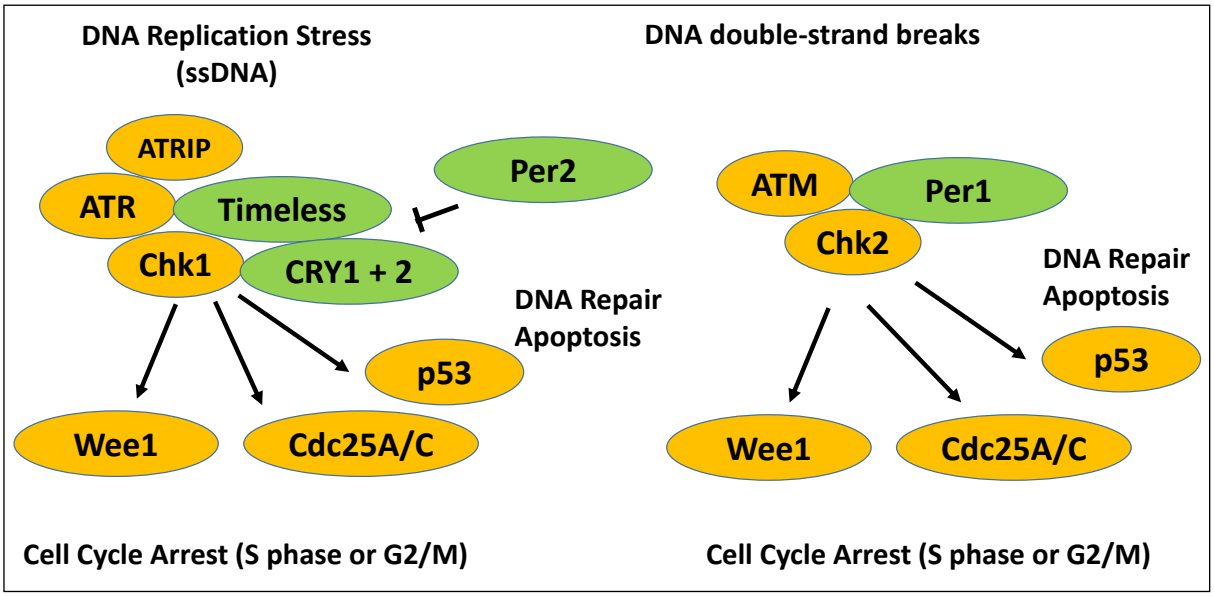


Figure 1–5: ATM-Chk2 and ATR-Chk1 checkpoint pathways in relation to the circadian proteins. While the ATR-Chk1 pathway is activated by single-stranded DNA (ssDNA) at stalled DNA replication forks or converted DNA breaks, the ATM-Chk2 pathway responds to broken chromosomes. The cell cycle is arrested by activating the CDK inhibitor Wee1 kinase, and by either degrading the CDK activator Cdc25A in G1/S phase or removing Cdc25C from the nucleus in G2. Activation of the transcription factor p53 leads to DNA repair or cell death, depending on the severity of the damage.

The conserved mechanism of a DNA damage checkpoint is to stop the progression of the cell cycle through complex signaling networks involving kinases (Velichko et al., 2012). An additional function is the initiation of pathways that lead to DNA repair or cell death, depending on the extent of the damage. Two major kinase pathways are associated with cellular response to DNA damage and heat stress: ATM-and Rad-3-

related (ATR)-Chk1 and ataxia telangiectasia mutated (ATM)-Chk2 kinase cascades (Yang et al., 2003). The ATR-Chk1 kinase pathway is activated by single-stranded DNA, either at processed DNA breaks or at stalled replication forks, while the ATM-Chk2 pathway is stimulated by double-strand DNA breaks. ATM is recruited to a broken chromosome by the Mre11-Rad50-Nbs1 (MRN complex), where it phosphorylates a large number of proteins, including histone H2AX at S139 and Chk2 at T68 (Giunta, Belotserkovskaya & Jackson, 2010). ATR binds to single-stranded DNA via its subunit ATRIP, where it phosphorylates Chk1 at S317 and S345 with the help of Clasplin and the Rad9-Rad1-Hus1 ring complex (Reinhardt & Yaffe, 2009).

When DNA replication stops abruptly in S-phase because of DNA damage or the lack of nucleotides, the MCM helicases continue to uncoil the DNA strands in front of the stalled fork, thereby generating large sections of the single-stranded DNA. This vulnerable ssDNA is then rapidly covered by RPA, a single-strand DNA binding protein. This binding of RPA to ss-DNA results in the recruitment of the ATR-ATRIP kinase complex. The latter then binds strongly to RPA through its ATRIP subunit (Zou & Elledge, 2003, Byun et al., 2005). Further interactions that lead to the activation of ATR are dependent on the scaffold protein TopBP1 and the Rad9-Rad1-Hus1 (9-1-1) ring complex (Cimprich & Cortez, 2008). While TopBP1 associates with chromatin during every normal S phase (Jeon et al., 2007), the 9-1-1 ring is loaded by Rad17-RFC2-5 at junctions between single-stranded and double-stranded DNA (Lee & Dunphy, 2010).

The upstream activation of ATM kinases is different from ATR. Upon initiation of DSBs or bulky DNA lesions, two major events occur: First is the binding of a mediator complex (MRN) comprised of Nbs1, Rad50, and Mre11 to the break, an event that recruits ATM (via Nbs1); and second is the phosphorylation of H2AX at S139 (a variant of the H2A histone)(Fernandez-Capetillo et al., 2004, Burma et al., 2001). The H2AX modification occurs in close proximity to the region of DNA damage, and extends several megabases upstream and downstream of the damaged region. The phosphorylated H2AX and MRN in the mediator complex, together result in the full activation of ATM (Falck, Coates & Jackson, 2005).

As discussed earlier, Timeless (TIM) and Period-1 (PER-1) associate with ATR-Chk1 and ATM-Chk2 kinases, respectively (Kondratov & Antoch, 2007). The Timeless-Tipin

complex associates with the DNA replication fork and is involved in the full activation of the Chk1 kinase in human cells and the Cds1 kinase in fission yeast (Aria et al., 2013, Sommariva et al., 2005). Interestingly, the *S cerevisiae* Timeless-Tipin complex affects directly the biochemical activities of the DNA polymerases and the replicative MCM DNA helicase at replication forks (Cho et al., 2013). This implies that the TIMELESS-Tipin complex directly affects the stability of the DNA replication fork, and thereby the activation of the ATR-Chk1 kinases pathway.

How the Period proteins affect the ATM-Chk2 pathway is much less clear. Both Per1 and Per3 are reported to interact with both kinases (Im et al., 2010, Gery et al., 2006). Their role at a broken chromosome in the context of ATM activation is, however, unknown. Per2 could indirectly affect the TIMELESS, as Per2 competes with TIMELESS for binding to Cryptochrome1 (Engelen et al., 2013, Kang & Leem, 2014). Whether this extends to TIMELESS when bound to Tipin at the replication fork is unclear.

In addition, the proteins of the circadian system are also involved in modulating the function of the ATM and ATR pathway (Rana & Mahmood, 2010). Because of the close control of the circadian system over the cell cycle and DNA repair, changes in their expression are expected to affect normal cellular responses to DNA damage, thereby resulting in an increased cancer risk.

1.7 The Main Splice Variants of human Per1

The human *Per1* gene is on Chromosome 17: 8,140,472nt - 8,152,435nt on the reverse strand containing 23 exons. The Ensembl database curates currently 9 potential protein-coding splice variants (Table 1-1).

Table 1.1: Protein coding splice variants of the human *Per-1* gene (http://www.ensembl.org; accessed 06 June 2016)**.**

Name	Transcript ID	bp	Protein	Uniprot ID
Per1-001	ENST00000317276	4707	1290aa	O15534
Per1-005	ENST00000581082	4521	1267aa	J3KRL7
Per1-002	ENST00000354903	3115	859aa	O15534
Per1-006	ENST00000582719	3677	824aa	J3KTM2
Per1-003	ENST00000581395	3209	572aa	J3QSH9
Per1-008	ENST00000577253	589	117aa	J3KSL6
Per1-010	ENST00000584202	576	112aa	J3QLQ5
Per1-009	ENST00000581703	528	175aa	J3QL55
Per1-016	ENST00000583559	523	168aa	J3QL46

The literature currently provides no information on the potential function or expression patterns of these variants. A full alignment of the five main Per1 splice variants is included in Appendix 2. Closer inspection of these protein variants, however, reveals several interesting observations. As shown in Figure 1-6, the only two differences between the two long variants, Per1-001 (1290aa) and Per1-005 (1267aa), are two deletions: one 20 amino acid deletion, which affects the PAS-A domain, and a smaller 3 amino acid deletion that sits behind the region required for the phosphorylation by Casein Kinase 1-epsilon. The deletion in the PAS-A domain may

affect the ability of Per1-005 to form homo- or heterodimeric complexes. Interestingly, the three shorter variants lack all different sections of the C-terminus, but retain the two PAS domains. Per1-003 (572aa) is the shortest variant, which lacks the section required for CK1-epsilon phosphorylation. The two main differences between Per1-002 (859aa) and Per1-006 (824aa) is a small, 16 amino acid deletion in the N-terminus, and the loss of (19aa) at the very end of the C-terminus.

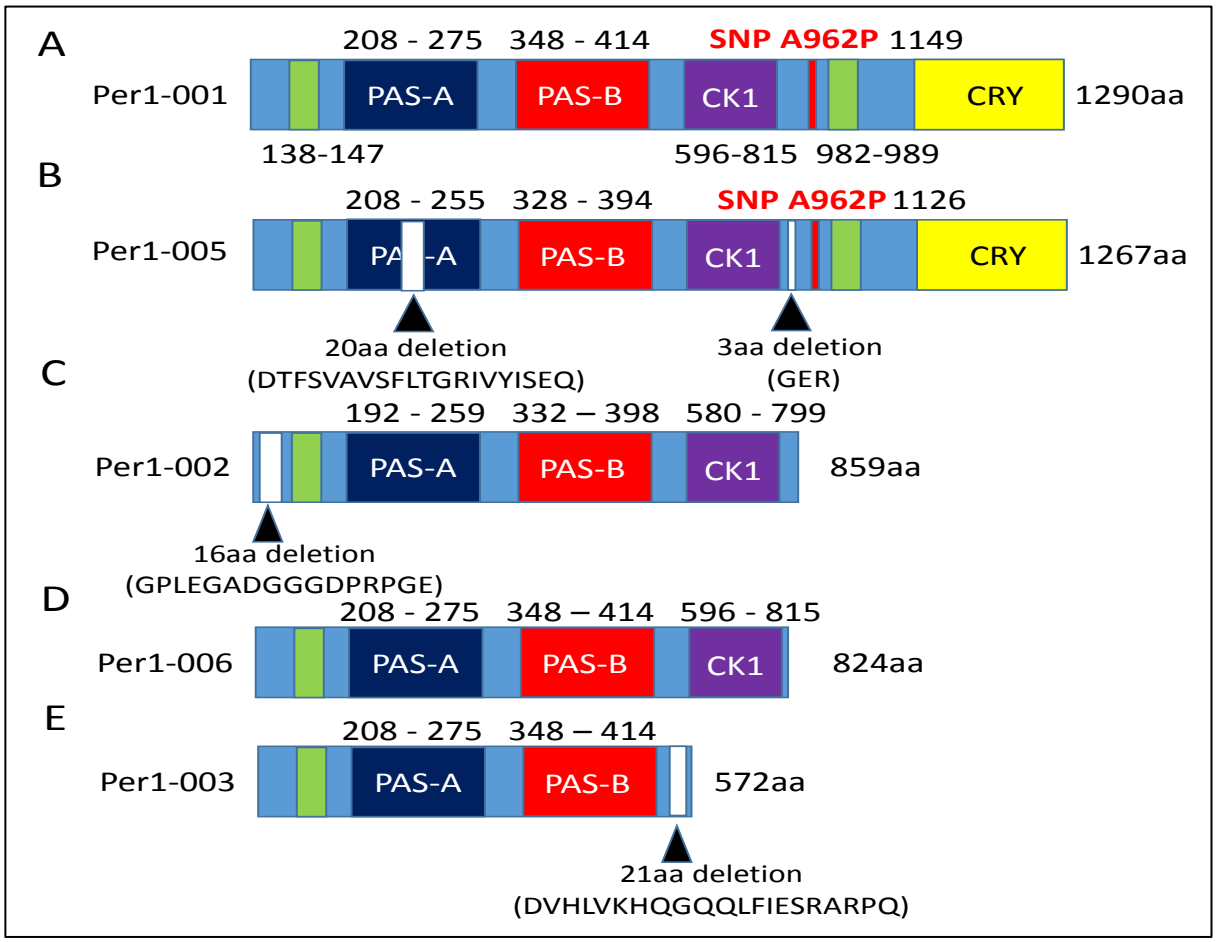


Figure 1–6: Domain organizations of the five main protein variants of human Per1. The PAS-A, PAS-B domains are indicated. The domains required for Caseine kinase-1 (CK1) phosphorylation are shown, as is the domain required for the association with Cryptochrome (CRY). The position of the SNP A962P is indicated. The two nuclear export sequences are shown in green. The full alignment is shown in Appendix 2.

1.8 Single Nucleotide polymorphisms (SNP)

The typical human genome differs at 4-5 million sites, of which 99.9% are either single nucleotide polymorphisms (SNPs) or small deletions (1000 Genomes Project Consortium, 2015).

Like all genes, the PER1 gene is also subject to single nucleotide polymorphisms. The human *Per1* gene is associated with 1095 missense variations, which are in the Ensemble database (accessed 06 June 2016). So far, only a small number of these missense mutations have been linked to a human disorder or a change in behavior. Mice that express human PER1-S714G feed earlier than do normal mice (Liu et al., 2014).

In humans, variations in the *Per2* and *Per3* genes have been associated with an increased risk of a metabolic disorder, diabetes (Valenzuela et al., 2016). Two mood or behavioral disorders were also linked to SNPs, *Per2* was related to an increased risk of high alcohol intake and *Per3* was correlated with an increased risk of bipolar disorder (Valenzuela et al., 2016). As for associations with cancer, seven amino acid variations and one deletion/insertion variation were linked to an increased risk of malignant growth in humans, Table 1-2.

Human Per1 SNP rs2289591 (W824C), which replaces tryptophane-824 with a cysteine residue is connected to prostate cancer, and is also associated with risk of glioma (Valenzuela et al., 2016). The rs3027188 polymorphism is in the intron of PER1, specifically in the CK1ε binding domain's non-coding region (Taniyama et al., 2015, Toh et al., 2001). CK1ε binding activity has been shown to be vital for PER/CRY protein phosphorylation. Maintenance of duration of circadian rhythm is a consequence of PER protein phosphorylation. The timing of the circadian rhythm is determined by the phosphorylation status of the PER protein. Single nucleotide phosphorylation occurring within introns have been shown to adversely affect transcription, RNA splicing as well as other downstream processing of RNA as well as its stability. Therefore, single nucleotide polymorphisms in the intron of PER1 are likely to cause a decrease in level of mRNA transcript and/or altered splicing status. This adversely modulated PER1 has been shown to be common in people with shift work disorder, the chronic occurrence of which has been implicated as one of the causes of cancer. Single nucleotide

polymorphisms of PER1 can also adversely affect other circadian clock genes like CRY2, which is transcriptionally suppressed by PER1 (Richards et al., 2013). Female workers with shift work disorder are more prone to develop breast cancer while male workers with similar disorders have been shown to be at risk for developing lung, bowel, prostate and bladder cancer. However, none of these risk associations turned out to statistically significant (Grundy et al., 2013).

Table 1.2: Polymorphisms of Per genes associated with increased risks of cancer in humans. Adapted from Valenzuela et al. (2016). * Relationship with risk of cancer was marginally significant, however, the relationship with risk of cancer was not significant following adjustment for the false discovery rate (Grundy et al., 2013).

Gene	SNP	Variation	Cancer risk	Reference
Per1	rs2585405 this study	C>G A962P	glioma	(Madden et al., 2014)
	rs2289591	G>T W824C	prostate cancer	(Zhu et al., 2009)
	rs3027188*	G>C	breast cancer	(Grundy et al., 2013)
Per2	rs7602358	G>T	prostate cancer	(Zhu et al., 2009)
	rs934945	G>A	breast cancer	(Dai et al., 2011)
Per3	rs1012477	G>C	prostate cancer	(Zhu et al., 2009)
	rs228669	T>C	hepatocellar carcinoma	(Zhang et al., 2014)
	rs228644	G>A	non-small cell lung cancer	(Couto et al., 2014)
	rs57875989	indel	colorectal adenoma, breast cancer, prostate cancer, hepatocellar carcinoma	(Dai et al., 2011, Wirth et al., 2014, Alexander et al., 2015, Chu et al., 2008, Geng et al., 2015, Grundy et al., 2013)

The missense SNP affecting alanine-962 (rs2585405) did catch our attention, due to its interesting population frequencies. The mutation **G**CC/**C**CC changes alanine (**G**CC) at position 962 to a Proline (**C**CC) residue. As shown in Figure 1-7, the mutation is in the C-terminal Proline-rich domain, close to the second nuclear export motif, and only presents in the two longer Per1 protein variants, Per1-001 and Per1-005. The mutation **G**CC to **C**CC is on the forward strand of chromosome 17, at position 8143454. The ancestral nucleotide is G (i.e. **G**CC = Alanine).

PAEGPPTPASHSPSPSLPAL **A**^{962}PSPPHRPDSPLFNSRCSSPL

Figure 1–7: Amino acid sequence next to the SNP A962P in human Per1. The amino acid sequence contains several SP and TP motifs, which could be phosphorylated by CDK (cyclin-dependent kinases) or by MAP kinases like ERK or p38 kinase (Li et al., 2006).

The population frequency indicates that both alleles, the Alanine and the Proline, have a balanced distribution in East Asia, whereas there may be a selection pressure favoring the Alanine over the Proline in most other populations (Figure 1-8). The selection against the Proline at position 962 appears to be strongest in Europe. This could indicate that either the Proline carries a negative impact or that the alanine results in a positive consequence outside of East Asia. Alternatively, the difference in allele frequencies may be due to genetic drift.

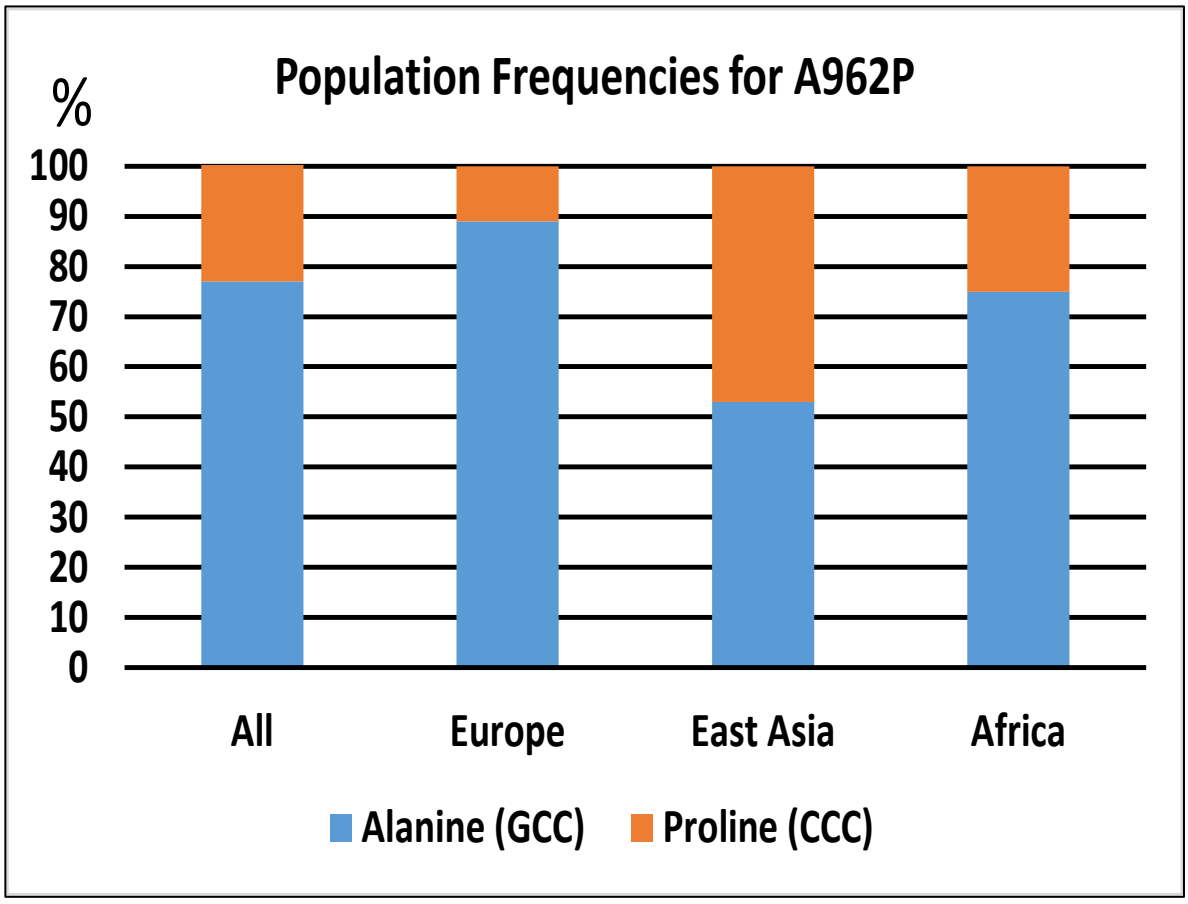


Figure 1–8: Population frequencies for A962P across all tested populations. https://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=17:8142954-8143954;v=rs2585405;vdb=variation;vf=362585851

Project Aims

The overall purpose of the present study was:

1. To confirm the expression of the *Per1* transcript in different human tissues.
2. To test for the association of Per1 with ATM-Chk2 and/or ATR-Chk1 kinases under genotoxic and environmental stress conditions.
3. To construct two stable cell lines (HEK293), which either over-express EGFP-PER1-A962 or EGFP-PER1-P962, to study possible impacts of the SNP on ATR-Chk1 and/or ATM-Chk2 signaling.
4. To down-regulate the endogenous Per1 protein by siRNA, without affecting the FLAG-EGFP-PER1 proteins.

Key Experimental Results

1. Expression analysis of an array of human tissues by PCR shows the presence of the *per1* transcript in all human tissues.
2. HEK293 embryonic kidney cells are homozygote for the proline residue at position 962 in Per1 (SNP rs2585405, A962P).
3. Biochemical studies of the endogenous Per1 protein show its cytoplasmic localization in HEK293 cells and the existence of four splice variants.
4. The two larger splice variants consist of at least two forms with distinct isoelectric values. Each of the two more positively charged Per1 forms increase in abundance in the response to DNA damage. The largest, most negatively charged Per1 form becomes post-translationally modified, probably with ubiquitin, when DNA replication forks break in the presence of the topoisomerase 1 inhibitor camptothecin.
5. Colon carcinoma cells (HCT116) have higher Per1 protein levels which correlates with an increase in ATM-Chk2 signalling.
6. Down-regulation of the endogenous Per1 protein by siRNA, which targeted the 3` - untranslated region of the messenger RNA, resulted only in a partial decline in endogenous Per1 protein levels.
7. Two stable cell lines were constructed which either express Flag-EGFP-PER1 (A962) or Flag-EGFP-PER1 (P962) proteins under the control of a doxycycline-inducible promotor.
8. Unlike the endogenous Per1, both EGFP-PER1 proteins accumulate in the nucleus independently of the A962P SNP and independently of DNA damage.
9. UV-induced DNA damage results in the specific degradation of both EGFP-PER1 proteins suggesting that Per1 needs to be removed from the nucleus when DNA damage has been detected.
10. UV light and the topoisomerase inhibitor camptothecin result both in the phosphorylation of Chk1 kinase at serine-345. While expression of EGFP-PER1-A962 allows this modification to occur, expression of EGFP-PER1-P962 strongly reduces Chk1 phosphorylation at this site. This suggests that the nuclear accumulation of EGFP-PER1-P962 interferes with ATR-Chk1 signaling.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Media

- **LB and LBA (Luria Bertani Agar)**

10g/L of Bacto ® -Tryptone; 211705, 5g/L of Bacto ® - Yeast Extract; 212750, 10g/L NACL and 20g/L Bacto ® Agar; 214030 for (LBA).

2.1.2 Buffers

- **50x TAE (Tris-Acetate- EDTA) buffer**

242g Tris base, 57.1ml glacial acetic acid, 37.2g Na_2EDTA, $2H_2O$ and H_2O up to 1L.

10x PBS (phosphate-buffered saline) buffer

360g NaCl, 43.6g Na2HPO4, 7H2O, 12.8g NaH_2PO4 and dH2O up to 4L, pH=7.2.

- **10x SDS buffer (sodium dodecyl sulfate)**

576g Glycine, 40g SDS, 121g Tris-HCl and dH_2O up to 4L.

- **10x Transfer buffer**

124g Tris-base, 56g Glycine and dH_2O up to 4L.

- **10% APS (ammonium persulfate)**

Weigh 1g Ammonium persulfate in a 50ml tube and add water up to 10ml.

- **4% Paraformaldehyde (PFA)**

-Weigh 2g Paraformaldehyde powder in a 50ml tube.

- Add approximately 20ml of 1xPBS and 1ml of 1M NaOH.

- Heat in a 65°C water bath and shake frequently for about 30min.

- Adjust the pH= 7.0 using 1M HCl, add more 1xPBS up to 50ml.
- Cool down to room temperature, and store as single aliquots at -20°C.

- **DEPC treated water**

Prepared by adding 0.1% of Diethylpyrocarbonate (DEPC) to dH_2O in RNase-free glass bottle (already autoclaved). The solution was incubated in the fume cabinet for overnight at room temperature, and then autoclaved to inactivate the DEPC residues.

2.1.3 Restriction enzymes

The following Restriction enzymes were used in this project:

Table 2.1: Restriction enzymes used in this study.

Restriction enzymes	Restriction site	Cat.no	Company
SalI	G▼TCGA C C AGCT▲G	R6051	Promega
NotI	GC▼GGCC GC CG CCGG▲CG	R6431	Promega

2.1.4 Cell lines

The following cell lines were used during the project:

Table 2.2: The Human cell lines used in this study.

Cell Line names	Source
HEK293 cells	A human embryonic kidney cell line.
HCT116	The HCT116 is a line of malignant cells derived from a human male diagnosed with colon cancer.
Flip-In T-REx-HEK293 Cells	Kind gift from Dr.Fumiko Isashi, Oxford University.
Flip-In T-REx-HEK293 Cells-PER1-962A-GFP	This study
Flip-In T-REx-HEK293 Cells-PER1-962P-GFP	This study
Flip-In T-REx-HEK293 Cells-GFP	This study

2.1.5 Plasmids

The following Primers were used during the project:

Table 2.3: Plasmid used in this study.

Plasmid	Source
pBluescript	Kind gift from Dr.Takahiro Nagase. Japan
pcDNA5/FRT-TO-Flag-EGFP	Kind gift from Dr.Fumiko Isashi, Oxford University
pOG44	Kind gift from Dr.Fumiko Isashi, Oxford University
pRFP-C-RS (TR30015)	OriGene
pRFP-C-RS-shRNA-PER1-001 (HT129860A)	OriGene
pRFP-C-RS-shRNA-PER1-001 (HT129860B)	OriGene
pRFP-C-RS-shRNA-PER1-001 (HT129860C)	OriGene

2.1.6 Primers

The following Primers were used during the project:

Table 2.4: Primers used in this study.

Primers	Sequence
Per1-001-pcDNA5-F-Not1	5’ ATC CGT TAC ATT ATG CGG CCG CAT GAG TGG CCC CCT AGA AGG G 3’
Per1-001-pcDNA5-R-Not1	5’ CTA CGA TGG AAT GCG GCC GCC TAG CTG GTG CAG TTT CCT GC 3’
Per1P962A-F	5’ TGC CCG CCC TCG CCC CGA GTC CTC 3’
Per1P962A-R	5’ AGG ATG GAG AAG GGG AGT G 3’
Per1A962-F	5’ TCA CGC CAC CAC CAG AAC CC 3’
Per1A962-R	5’ CTC TGG CTC AGC AGC CTC CGC 3’
FRT-F	5’ CAA CGG GAC TTT CCA AAATGT CG 3’
CMV-F	5’ CGC AAA TGG GCG GTA GGC GTG 3’
EGFP-F	5’ GCA GCG TGC AGC TCG CC 3’
BGH-R	5’ TAG AAG GCA CAG TCG AGG 3’
PER1-201-F	5’ CAG ACA TGA GTG GCC CC 3’
PER1-202-R	5’ GAG TCC AAC GGG CAT GA 3’
PER1-205-R	5’ GGA GAA GTC CGT CTT CTGC CG 3’
ACTIN-F	5’ AGA AAA TCT GGC ACC ACA CC 3’
ACTIN-R	5’ GGG GTG TTG AAG GTC TCA AA 3’

2.1.7 Antibiotics

The following Antibiotics were used during the project:

Table 2.5: Antibiotics used in this study.

Antibiotics name	Stock Solution	Required concentration	Company	Cat.no
Zeocin™	100mg/ml	100µg/ml	MELFORD	E24474
Blasticidin S	20mg/2ml	15µg/ml	MELFORD	E24375
Hygromycin B	100mg/ml	150µg/ml	FORMEDIUM™	HYG1000
Ampicillin	100mg/ml	100µg/ml	MELFORD	A0104
Chloramphenicol	100mg/ml	34µg/ml	DUCHEFA BIOCHEMIE	C01130025
Puromycin	10mg/ml	1µg/ml	MELFORD	P0121
Doxycycline	100mg/ml	2µg/ml	Sigma-Aldrich	D9891-5G

2.1.8 Antibodies

The following Antibodies were used during the project:

Table 2.6: The Primary antibodies used in this study.

Antibody	Source	Cat. No.	Host	Clonality	Dilution
PER1	Thermo scientific	PA1-524	Rabbit	Polyclonal	WB=1:1000
PER1	ABCAM	AB-174860	Rabbit	Polyclonal	WB=1:2000
GFP	Thermo scientific	MA5-15256	Mouse	Monoclonal	WB=1:1000
ATM	Thermo scientific	PA1-16503	Rabbit	Polyclonal	WB=1:1000
ATR	Cell signalling	2790	Rabbit	Polyclonal	WB=1:1000
Chk1	Cell signalling	2360S	Mouse	Monoclonal	WB=1:1000
Phospho-Chk1 (Ser345)	Thermo scientific	MA5-15145	Rabbit	Monoclonal	WB=1:1000
Chk2	Cell signalling	3440S	Mouse	Monoclonal	WB=1:1000
P.chk2 (Thr68)	Cell signalling	2661	Rabbit	Polyclonal	WB=1:1000
P21Waf1/Cip1	Cell signalling	2947	Rabbit	Polyclonal	Wb=1:1000
H2AX	ABCAM	AB-26350	Mouse	Polyclonal	WB=1:2000

β-Actin	Sigma	A2066	Rabbit	Polyclonal	Wb= 1:1000
GAPDH	Abcam	AB128915	Rabbit	Polyclonal	Wb=1:10000

Table 2.7: The Secondary antibodies used in this study.

Antibody	Source	Cat. No.	Dilution
Anti-mouse secondary AB- HRP anti-light chain	Jackson ImmunResearch	115-035-174	Wb=1:10000
Anti-rabbit secondary AB- HRP anti-light chain	Calbiochem	401315	Wb=1:10000
Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody	Invitrogen	A11008	IF=1:500
Alexa Fluor® 488 Rabbit /Anti-Mouse IgG (H+L)	Invitrogen	A11059	IF=1:500

2.2 Methods

2.2.1 Cell culture protocols

2.2.1.1 Thawing frozen cells

This procedure needs to be conducted in a short time. Cells were taken out from Liquid Nitrogen tank and thawed in a water bath immediately at 37˚C until only a small amount of ice remained (approximately 2 minutes). The vial was then decontaminated with 70% ethanol and the contents of the cryotube transferred quickly to the maintaining medium. Cells were incubated in 37˚C incubator at 5% CO_2 for 4hr to attach and the media was replaced with a fresh complete medium to avoid any residues of DMSO.

2.2.1.2 Cells maintaining

This project used two types of cells, which will be described in detail:

HEK293: A human embryonic kidney cell line. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (SIGMA, D5546), supplemented with 10% Fetal Bovine Serum (FBS) (SIGMA-F2442), 1% Penicillin- Streptomycin antibiotics (SIGMA-P4333) and 200mM L-Glutamine solution (SIGMA- G7513). The media were supplemented with 100µg/ml of Zeocin and 15µg/ml of Blasticidin when it used for HEK293 cells maintaining only. Constructed cell lines were maintained at the same conditions of HEK293 cells, but Zeocin was replaced with 100µg/ml of Hygromycin and then incubated at 37˚C and 5% CO_2 until they reached the required cells confluence percentage.

HCT116: The HCT116 (ECACC # 91091005) is a line of malignant cells derived from a human male diagnosed with colon cancer. The cells were grown in McCoy's 5A (Modified), GlutaMAX™ (Gibco®, #36600-088) supplemented with 10% FBS. They were cultured in an atmosphere of 5% CO_2 at 37˚C.

2.2.1.3 Dissociation of Adherent Cells from Culture

Firstly, the medium was removed and discarded using a sterile pipette .The attached cells were washed with 1x Dulbecco's phosphate-buffered saline (DPBS), (Gibco® #14190-250) to remove all traces of FBS. An appropriate amount of 1x trypsin solution (SIGMA, T3924) was added to cover the cell layer surface and the plate was moved into the incubator. The cells were generally dissociated for 2 to 5 minutes. Medium-containing serum was added to the plates to inhibit the trypsin activity.

2.2.1.4 Cells freezing

Cells were de-attached from the plate, washed with 1xPBS, trypsinized and centrifuged at 1500rpm for 5 minutes. The pellets were re-suspended with 1ml of freezing media (10% dimethyl sulfoxide "DMSO"(Sigma-Aldrich; D8418) and 90% complete media). The cells were transferred into a cryotube and then to the Mr. Frosty equipment and incubated for 24hrs in -80˚C, then transferred later to the Liquid Nitrogen for longer-term storage. In order to awaken frozen cells, vials were gradually warmed up prior to being placed in pre- warmed media.

2.2.1.5 Cells counting

Many techniques in this project involve the counting of cells. To obtain specific cell number, media was aspirated, cells were washed with 1x PBS, trypsinized, spun down at 1500rpm for 5min, and re-suspended with 1ml of 1xPBS. A 10µl of the suspension was removed and mixed with 10µl of 0.4% Trypan Blue (SIGMA, T8154) in a clean Eppendorf tube. A 10µl volume of this mixture was then loaded onto BIO-RAD counting slides (cat.145-0011), and cells were counted by BIO-RAD/TC10™ Automated cell counter.

2.2.1.6 cDNA synthesis

1µg of the total RNA from the normal human tissue panel II (Clontech; 636643) was converted to their corresponding cDNA using the Tetro cDNA synthesis kit (BIOLINE-BIO-65042). Thereafter, a general PCR was done using the produced cDNA as a template. Primers for human β-ACT were used in PCR reaction as a control for the quality of the cDNA samples (see table 2-4 for primer sequence). The PCR protocol was

as follow:

PCR set-up:

Component	Volume (µl)
My Taq HS Red Mix, 2x	25
DNA template	≈100ng
10µM 5'-primer-F	2.5
10µM 5'-primer-R	2.5
Water (dH_2O)	Up to 50

PCR cycling conditions:

Initial Denaturation	95°C	30sec
Separation	98°C	30sec
Annealing	55°C	30sec
Extension	72°C	4min
Final Extension	72°C	10min

PCR products were run on 1%Agarose gel in 1xTBE buffer and 0.5µg/ml ethidium bromide (Sigma-Aldrich; 46067) was added to the gel .5µl of 50bp DNA marker (BioLabs; N3236L) was loaded for size determination of PCR products .Gel was run at 85V for 1hr. Specific fragments were cut out under long wave UV light using a sterile scalpel. The DNA purification process was carried out using GenElute™ Gel Extraction Kit (SIGMA-NA1010) as manufacturer's instructions. Finally, the DNA concentration was determined by a NanoDrop (ND_2000). The DNA amounts was put in a clean Eppendorf tube and in other tubes, 2pmol/µl in a minimum volume 15µl of the forward and/or

reverse primers were transferred. The tubes were then sent at room temperature to Eurofins MWG for DNA sequencing. The result sequencing of each product was subjected to blast analysis and aligned against the expected sequence of PCR product to compare a query sequence with the NCBI databases of sequences.

2.2.2 Cloning onto the pcDNA5/FRT/TO-Flag-EGFP vector

2.2.2.1 cDNA plasmid

The cDNA fragment (A kind gift from Dr.Takahiro Nagase from Japan) was inserted at the *Sal*I-*Not*I site of the *pBluescript II SK (+)* vector (GenBank Accession NO.X52328, AmpR). The 5' end of the cDNA was inserted at the *Sal*I site of the vector, and the 3' end of the cDNA was inserted at the Not1 site of the vector. A Plasmid DNA was extracted from the transformed cells using GenElute™ plasmid Midiprep Kit (SIGMA, PLD35) for higher concentrations as Manufacturer's instructions and digested by SalI and Not1 to test it (table 2-8). I used the basic digestion protocol from New England BioLabs (NEB). The digestion reaction was incubated at 37˚C for 1-1:30 hours. (https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions)

Table 2.8: Digestion components.

Component	25µl Reaction	50µl Reaction
DNA	0.5µg	1µg
10x NEB buffer	2.5µl	5µl
100xBSA(if needed)	0.25 µl	0.5 µl
Nuclease-free water	To 25 µl	To 50 µl
Restriction Enzyme 1	5 units	10 units (=1µl is used)
Restriction Enzyme 2	5 units	10 units (=1µl is used)

2.2.2.2 Cassettes amplifying by PCR Reaction

The PCR was used to amplify the ORF of interest, which is the longer hPER1-001 (4663bp) fragment used *Per1-001-PCDNA5-F-Not1* and *Per1-001-PCDNA5-R-Not1* primers (see table 2-4 for primer).

The following materials were mixed in a PCR tube. All the additions were done on ice:

Component	Volumes (µl)
5x GC buffer (NEB)	10
2mM dNTPs	5
DNA template	≈100ng
10µM 5'-primer-F	2.5
10µM 3'-primer-R	2.5
Phusion polymerase (NEB)	0.5
Sterile dH_2O	Up to 50µl

PCR tubes were placed in a Thermo-cycler and the following program (usually performed as 35 cycle) was used:

Initial Denaturation	95°C	30sec
Separation	95°C	30sec
Annealing	60°C	30sec
Extension	72°C	4min
Final Extension	72°C	10min

2.2.2.2.1 PCR Products Examination by Agarose Gel

The 50µl of PCR reaction were loaded onto 1% (w/v) gel in 1xTAE buffer stained with ethidium bromide. DNA samples (≈5µl) were mixed with 3µl of DNA loading dye (Promega, G5711) and run through the agarose gel in parallel with either 1Kb DNA ladder (Promega, G2101). At the end of the electrophoresis time, the products were analyzed using the Gel Doc2000 machine with the assistance of Quantity One® software.

2.2.2.2.2 PCR Products Isolation by Gel Purification

PCR products were run in 1% agarose gels stained with ethidium bromide in 1xTAE buffer. Specific fragments were cut out under long wave UV light using a sterile scalpel. The DNA purification process was carried out using GenElute™ Gel Extraction Kit (SIGMA-NA1010) as manufacturer's instructions.

2.2.2.3 Digestion of the PCR Products

In order to achieve the cloning, the cassette of hPER1 was digested with the NotI restriction enzyme and inserted into the NotI cloning site. I used the basic digestion protocol from New England BioLabs (NEB), (see table 2-8).

2.2.2.4 Preparation and digestion of pcDNA5/FRT/TO plasmid

The mammalian expression vector, *pcDNA5/FRT/TO* (A kind gift from Dr.Fumiko Isashi,see figure 2-1) used in this thesis contains the Tetracycline repressor, in addition to the N-terminal Flag-EGFP tag, which was already inserted in the KpnI cloning site of the plasmid by Dr. Fumiko Isashi research group. This plasmid was digested with the *NotI* restriction enzyme and was dephosphorylated to stop religation by TSAP (Thermosensitive Alkaline Phosphatase) (Promega, M9910) following the manufacturer's instruction. The mixture was incubated at 37°C for 15 minutes, Thereafter, heat inactivation step for 5 minutes at 65˚C.

2.2.2.5 Ligation of digested PCR and plasmid

The concentration for digested insert and plasmid were measured using a NanoDrop (ND-2000) to determine how much digested insert and plasmid were required for ligation. The ligation of the digested insert and plasmid which are resulted

from the previous step, were ligated to each other using the following protocol:

Usually a 1:3 ratio of plasmid: Insert DNA was used to achieve the ligation process using T4 DNA ligase (Promega, M180A).

Ligation mixture:

Component	Volume
Plasmid	100ng
DNA insertion	17ng
1x T4 ligation buffer	1µl
T4 ligase	1µl
H_2O	Up to 20µl

Ligation mixture was incubated at 16˚C water bath for overnight, and it was preceded with *E.coli* transformation.

2.2.2.6 Preparation of LB and LB agar media for *E.coli* culture

The reagents for each medium (section 2.1.1) were added to a sterile bottle containing 900 ml of distilled water and stirred. Distilled water was added up to one liter, followed by autoclaving for 20 minutes at 120°C on the same day they were made up. The media were cooled and Ampicillin antibiotic (MELFORD; A0104) was added to make a final concentration of 100µg/ml. The LB agar/ amp/ were poured onto each labelled Petri dish. The dishes were then left to dry at room temperature on a clean bench. The LB media and plates were stored at 4°C.

2.2.2.7 Transformation of recombinant plasmids into competent *E.coli* cells

I performed the protocol according to NEB High Efficiency transformation protocol (https://www.neb.com/protocols/1/01/01/high-efficiency-transformation-protocol-c3019):

1. Competent cells were removed from -80°C and thawed on ice for 5 minutes.
2. (1pg-100ng ≈ 1-5µl) of Plasmid DNA was added to the cells, mixed Gently by pipetting, and kept on ice for 30min.
3. Cells were heat shocked at 42oC for 30 seconds, and then the tube placed on ice for 5min.
4. Cells were mixed with 950 µl of room temperature LB medium.
5. Cultures were shaken vigorously (250rpm) for 60min at 37°C.
6. Selection plates were warmed up to 37°C.
7. According to the expected ligation efficiency, cells were re-suspended in 50-100µl of LB medium, and 50µl of cells were spread out onto LBA plates with (100mg/ml of ampicillin) using a sterile spreader.
8. Plates were incubated for overnight (up to 16hr) at 37°C until the colonies grow.

2.2.2.8 Colony screening

White colonies were picked up from the overnight plates by using a micropipette tip into 10ml of LB liquid medium with 100mg/ml ampicillin, and incubated at 37°C overnight with vigorous shaking at 250 rpm.

2.2.2.9 Extraction from transformed *E.coli* Top10 competent cells

Plasmid DNA was extracted from 10ml of *E.coli* overnight bacterial culture using GenElute™ Plasmid Midiprep Kit (SIGMA, PLD35) for higher concentrations as Manufacturer's instructions.3µl l of purified plasmid DNA was run onto 1xTAE agarose gel to check DNA.

2.2.2.10 Cloning confirmation

The plasmid DNA was digested with *NOT1* enzyme to confirm corrects cloning, (see table 2-8). 3µl of digested plasmid DNA was run onto 1xTAE agarose gel to check DNA. Samples with correctly cloned gene were sent for sequencing to confirm the correct orientation and to detect any gene mutations. Plasmids carrying the desired insertion were ready for transfection into human cells.

2.2.2.11 DNA sequencing

DNA was sequenced by the Eurofins MWG Company (Germany) to confirm that the ORF of interest was cloned in the correct orientation. For DNA purified from PCR reaction, the amount sent (together with corresponding forward and reverse primers) was: 5ng/µl of (300-1000bp) 10ng/µl (>1000bp) For plasmid DNA, this amount was 50-100ng/µl (up to >3000bp). The gene sequencing results were blasted and aligned against corresponding genes using the Basic Local Alignment Search Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi and EMBL European Bioinformatics Institute Website http://www.ebi.ac.uk/.

2.2.3 A962 replacement (SNP) using Q5 mutagenesis kit

In order to generate the mutation, the process was carried out using Q5® Site-Directed Mutagenesis Kit (NEB ♯E0554S) as Manufacturer's instructions. . The Plasmid DNA was transformed into *E.coli* Top10 competent cells as described in Section (2.2.2.7). Plasmid DNA was extracted using GenElute™ Plasmid Midiprep Kit (SIGMA, PLD35) for higher concentrations as Manufacturer's instructions. 3µl of purified plasmid DNA was run onto 1xTAE agarose gel to check DNA. The plasmid DNA was digested with *NOT1* enzyme to confirm correct cloning, (see table 2-8), then 3µl of digested plasmid DNA was run onto 1xTAE agarose gel to check DNA. Samples with correctly cloned gene were sent for sequencing to confirm the correct replacement. Plasmids carrying the desired insertion are ready for transfect them into human cells.

2.2.4 Construction of Flag-EGFP tagged hPER1-001-P and hPER1-001-A stable cell lines

2.2.4.1 Transfection into Flp-In™ T-REx™-293 Cell Line

A. Overview

Commercially available Flp-In™ T-REx™-293 cells (ThermoFisher) were used in order to generate a number of HEK cell lines which expressed various genes of interest. These cells express lacZ-Zeocin fusion protein and Tetracyclin (Tet) repressor, and contain single integrated Flp Recombination Target (FRT) site. These cells are Zeocin and Blasticidin resistant. The Flp-In™ T-REx™-293-cell line was used to create stable cell

lines expressing EGFP-PER1-P, EGFP-PER1-A and GFP itself proteins. That was achieved by co-transfecting with pcDNA5/FRT/TO (see section 2.2.2.4 above for description), which contains insertion of the EGFP-PER1-A, EGFP-PER1-P in addition to pOG44 vector which expresses FLP-recombinase. Once the cassette has integrated into the genome ,the inactive hygromycin gene becomes active (integrating downstream of an SV40 promotor and the ATG initiation codon, see figure 2-1) making subsequent cells hygromycin resistant as well as Zeocin sensitive .

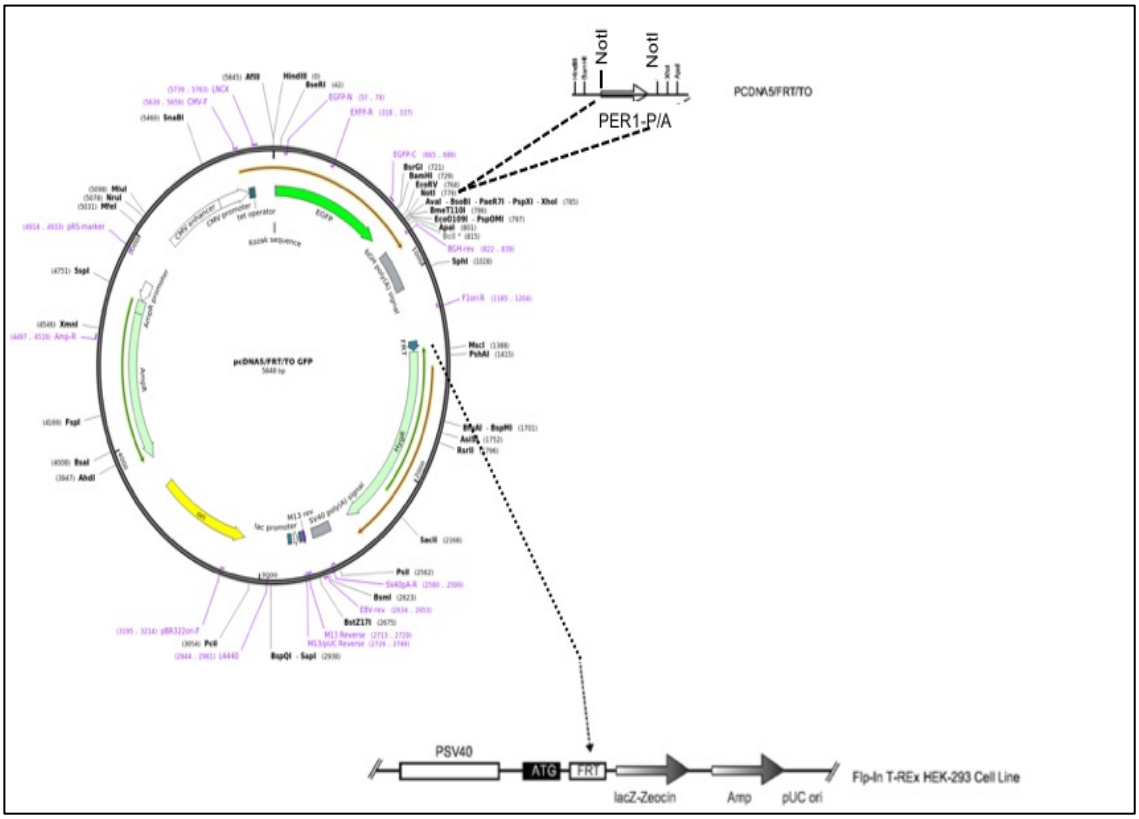


Figure 2–1:The above plasmid map, pcDNA5/FRT/TO, (figure modified from addgene, originally deposited from (Hageman & Kampinga, 2009)**)** shows the positions of the Per1-A/Per1-P and EGFP tags cloned into the NotI site. In addition there is an inactive hygromycin cassette adjacent to an FRT site (at position 1388bp).

B. Preparation of transfection and DNA complexes

The Transfection was carried out using Lipofectamine® 2000 Reagent (Invitrogen™, 11668-019). 1µg of pcDNA5/FRT contains EGFP-PER1-P, EGFP-PER1--A and GFP itself (negative control) plus 10µg of pOG44 in a ratio of 1:9, respectively were taken to a sterile 1.5ml Eppendorf tube containing 500µl of Opti-MEM® I Reduced Serum Medium (Invitrogen™, 11058021), In addition, another 1.5ml Eppendorf tube was prepared with 500µl of Opti-MEM Medium plus 70µl of Lipofectamine® 2000 Reagent, then both Eppendorf tube were mixed together and incubated 5 mints at room temperature to allow the formation of the complex.

C. Transfection of Flp-In T-REx™-293 cells

The Flp-In T-REx™-293 cells were seeded at 2×10^6 cells in 10cm dishes and incubated with complete DMEM medium supplemented with 100µg/ml Zeocin (MELFORD, E24474) and 15µg/ml Blasticidin (MELFORD; E24375) for 24 hours. Next day, the Lipofectamine and DNA complexes were prepared (section 2.2.4.1/B). The medium was removed from cells and replaced with fresh complete DMEM lacking of any antibiotic. The Lipofectamine-DNA complexes were added directly to the medium and the dish was swirled to distribute the complex. After 24hr of transfection, the media were removed and replaced with a complete media contain Blasticidin (15µg/ml). 48 hours post-transfection the cells were split 1:4 into medium supplemented with (150µg/ml) Hygromycin B (FORMEDIUM™; HYG 1000) as a selection marker (Bleuyard et al., 2012). Cells were maintained under these conditions for approximately two weeks. The complete DMEM + Blasticidin + Hygromycin was refreshed every 2-3 days until single clones appeared.

D. Isolation the colonies from transfected cells

After two weeks adding hygromycin to cell culture medium, few hygromycin resistance foci appeared. At this state, the medium was removed from the dish and cells were washed once with 10ml of 1xPBS. The cylinder (Sigma-Aldrich; C1059) was placed over a colony of cells by using a sterile curved forceps. The cylinder was filled with the appropriate amount of trypsin and incubated at 37°C for 3-5 minutes. The cells were then checked for detachment under a microscope and media were added to resuspend

the cells, and then the cells were transferred to a 6-well plate containing 5ml of media supplemented with (150µg/ml) hygromycin. The cells from 6-well plates was transferred to 10cm dish and grown to confluence.

E. Induction of hPER1-001-GFP-P, hPER1-001-GFP-A genes in Flp-In™ T-REx™-293 Cell Lines

The transfected cells were seeded in 10cm plates at the desired density with complete DMEM containing Blasticidin +Hygromycin and incubated until attached to the plate. The media were replaced with fresh media supplemented with freshly prepared (1µg/ml) of Doxycycline (SIGMA; D9891-5G) (prepared in H_2O) to induce expression and then incubated at 37°C for 24 hours. The cells were harvested for SDS PAGE and Western blot (refer to section 2.2.9.4).

F. Isolation of genomic DNA from individual colony

To confirm the transfected genes' integration in HEK293 cells genome, genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (SIGMA, G1N10), as the manufacturer's instructions.

G. Integration confirmation

The investigated genes were then amplified from their corresponding genomic DNA by general PCR reaction (refer to section 2.2.1.6 for PCR protocol) using the primers set: FRT-F (binds in CMV promoter), CMV-F and EGFP-F with hPER1- 205-R respectively.

2.2.5 Time course Induction of the EGFP- hPER1-962P and EGFP-hPER1-962A proteins

EGFP-hPER1-A and EGFP-hPER1-P Proteins were tested for their optimal induction period by using (1µg/ml) of Doxycycline. This was achieved by plating the engineered stable cell lines in 10 cm petri dishes at 80% cells confluence for overnight. Then, the medium was supplemented with freshly prepared (1µg/ml) of Doxycycline (prepared in H_2O), and one plate was left without Doxycycline treatment as a negative control. Whole cell protein extracts were prepared at 2,4,6,24,and48 hours post-treatment. Protein extracts were analyzed using SDS- PAGE and Western Blot. PVDF membranes were probed with anti-PER1 antibody to check the stability of the induction.

2.2.6 hPER1 Down-regulation

- HuSH shRNA Plasmid Panels (29-mer) system (OriGene)

A. Overview

The aim of this experiment was to reduce hPER1 protein production. The shRNAs that target the gene of interest were designed by the OriGene company and constructed using synthetic oligonucleotides cloned into the BamHI / Hind III cloning sites of the pRS vector. Each of the shRNA expression plasmids has a 29 nucleotide and inserted immediately downstream of a U6 RNA promoter, a 7 nucleotide loop, and the 29 nucleotide sequence in reverse complement, followed by a TTTTTT termination sequence. All inserts have the sequence structure shown below:

U6 promoter-GATCG-29nt sense-TCAAGAG–29nt reverse complement-TTTTTT (termination) – GAAGCT

B. pshRNA vector features

This system performs the gene silencing via a range of pRS expression vectors containing gene-specific shRNA insertions, which can be used for shRNA transient and stable expression using Puromycin selection upon the transfection into chosen mammalian cell line. Specifically, I used the *pRFP-C-RS* Vector containing hPER1-specific silencing shRNA insertions as well as a pCMV promoter that controls the constitutive expression of the tRFP gene (Red Fluorescence Protein) and a Chloramphenicol bacterial selectable marker. The system provides a *pRFP-C-RS* plasmid containing a non-effective shRNA cassette as a specific negative control for gene down regulation (see Table 2-9).

Table 2.9: pRFP-C-RS Plasmids that carrying shRNA oligonucleotides targets hPER1 mRNA and their sequences.

Plasmid name	shRNA sequence
HT129860A	Not disclosed
HT129860B	Not disclosed
HT129860C	Not disclosed
TR30015	Scrambled negative control non-effective shRNA cassette in pRFP- C-RS vector

C. Plasmids amplification

E.coli Top10 competent cells were used to amplify *hPER1-shRNA* plasmids using Chloramphenicol selection (34µg/ml as a final concentration prepared in ethanol) (DUCHEFA BIOCHEMIE; C0113) and following the previously mentioned transformation protocol (section 2.2.2.7).

D. Transfection into Human cell line

hPER1-pshRNA vectors were transfected into HEK293 cells using Lipofectamine® 2000 Transfection Reagent (Invitrogen™, 11668-019). The transfection reaction was mediated by Opti-MEM® I Reduced Serum Medium (Invitrogen™, 11058021), as manufacturer's instructions. hPER1- pshRNA transfection into chosen cell line was achieved firstly as transient single plasmids transfection and protein extraction was done after 72hr under Puromycin selection (3µg/ml) as the manufacturer's instructions.

2.2.7 DNA damage treatment

HEK293 and HCT116 cells were treated with different DNA damaging agents (see Table 2-10).

This table shows the DNA damage treatments conditions, which were applied onto HEK293 and HCT116 cells.

Table 2.10: DNA damage treatments and their conditions.

Agent	Abbreviation	Dose	Treatment time
Ultraviolet radiation	UV	$40J/m^2$	30m, 1h, 2h, and 24h
Camptothecin	CPT	1μM	30m, 1h, 2h, and 24h
Heat Shock	HS	43°C	30m, 1h, 2h, and 24h
Hydrogen peroxide	H_2O_2	500μM	30m, 1h, 2h, and 24h

2.2.8 Western blotting protocols

2.2.8.1 Protein extraction

RIPA buffer (Radio Immuno Precipitation Assay buffer) was used to extract the whole cell proteins. RIPA was prepared by the following recipe:

150 mM NaCl

1.0% Triton X-100

0.5% sodium deoxycholate

0.1% SDS (sodium dodecyl sulphate)

50 mM Tris, pH 8.0

A plate of cultured cells was washed with cool 1xPBS and the cells were then detached using trypsin. The resulting cell suspension was washed twice with cool

1xPBS. The total number of cells was counted and an appropriate volume of lysis buffer was added as a ratio of 100µl lysis buffer/2x10^6 cells, and commercial Protease Inhibitor Cocktail III for Mammalian Cells (MELFORD- P2202) was added to the lysis buffer (1µl/100µl of the lysis buffer), The solution was then mixed by vortex. Protein samples were left on ice for 30 min, then spun at 16,000xg for 10min at 4˚C. The extracted proteins were stored at -20°C until used.

2.2.8.2 Cells fractionation

Subcellular fractionations of proteins was performed by separating five fractions using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, 78840), as the manufacturer's instructions.

2.2.8.3 Protein concentration assay using BCA

Total proteins concentration were measured using the Pierce® BCA Protein Assay Kit (Thermo Scientific, 23225) following the manufacturer's instructions. A set of protein standards was prepared from the bovine serum albumin provided with the kit. A working reagent was then prepared by combining 50 parts of Reagent A with one part of Reagent B. Standards and test samples were then added to the working reagent and incubated for 30minutes at 37˚C in the dark. Analysis and standard curve measurements were carried out with 1µl of each sample using a NanoDrop (ND_2000) Spectrophotometer (Thermo, # WZ-83061-12).

2.2.8.4 Detection of target protein

SDS-Polyacrylamide Gel Electrophoresis was used to analyze the protein extracts. It was performed either by one or by two dimensions (2D) electrophoresis:

A. SDS-PAGE

Proteins were separated according to their sizes on different Acrylamide-gel percentages. Regarding our protein samples, I used either 6% or 10% gels. The protocol was achieved as following:

1. Resolving gels were set at specific percentage, casted in between the Bio- Rad Wet/Tank Blotting system's glasses and were over-laid with isopropanol to remove

air bubbles. Gels were left to settle down at room temperature.

❖ **Resolving Gel**

Component	6% gel	10% gel
1M Tris-HCl pH=8.8	11ml	11ml
40% Acrylamide/bis-Acrylamide 37.5:1 solution	4.5ml	7.5ml
dH_2O	14.5ml	11.5ml
20% Sodium dodecyl Sulfate (SDS)	150µl	150µl
10% Ammonium Persulfate (APS)	150µl	150µl
Tetramethylethylenediamine (TEMED)	30µl	30µl

2. Isopropanol was washed off and the stacking layer was prepared as follow:

❖ **Stacking Gel**

Component	Volumes
1M Tris-HCl pH=6.8	2.5ml
40% Acrylamide/bis-Acrylamide 37.5:1 solution	2ml
dH_2O	15.5ml
20% Sodium dodecyl Sulfate (SDS)	100µl
10% Ammonium Persulfate (APS)	200µl
Tetramethylethylenediamine (TEMED)	40µl

3. Gel was poured immediately on the top of the resolving layer, and combs were inserted. Gels were left to set for at least 10min at room temperature.
4. Combs were removed and plates were placed in the electrophoresis tank, which contains 1xSDS running buffer. Samples were loaded in parallel to a standard proteins ladder (ThermoScientific PageRuler™ Pre-stained Protein Ladder, cat.no.26616), and were run for 1:30 hour on 120V.
5. Gels were taken out of the tank and prepared for transferring to the PVDF membrane (GE Healthcare Life Sciences, cat.no: 10600023). The membrane was usually cut to match the gel size and activated with methanol before use.
6. Western Blot sandwiches were set up by placing the sandwich's components in a tray filled with 1xTransfer buffer (100ml of 10x Transfer buffer, 150ml Methanol and 750ml dH_2O), according to the following order: black part of the sandwich, one sponge, filter paper (GE Healthcare Life Sciences, cat.no: RPN6101M), the resolving gel, PVDF membrane, filter paper, one sponge and the red color part of the sandwich.
7. Sandwiches were placed in the transfer tank filled with 1x transfer buffer, and proteins were transferred for 2 hours at 70V.
8. PVDF membranes were blocked with 5% blocking buffer either with non-fat dry milk or serum (such as BSA), (5% Milk buffer or serum: 7.5g of Milk powder or BSA powder, 15ml of 1X PBS, and 0.05% Tween-20) for 1hour at room temperature on rocking platform.
9. Membranes were incubated with the primary antibody diluted in the blocking buffer for overnight at 4˚C on rocking platform (see list of primary antibodies in Table 2-6).
10. Membranes were washed 3 times, 10min each time, with 1x washing buffer (200ml of 10xPBS, 0.05% tween-20 and dH_2O up to 2 liters) at room temperature on a rocking platform.
11. Membranes were incubated with the secondary antibody (see list of secondary antibodies in Table 2-7) diluted in the blocking buffer, for 1hr at room temperature on a rocking platform.
12. Membranes were washed again three times, 10 minutes each time, with washing buffer at room temperature on rocking platform.
13. ECL detection using Western Lightning® Plus-ECL, Enhanced

ChemiluminescenceSubstrate (PerkinElmer, NEL105001EA) was used in 1:1 dilution and incubated with membrane for 3 minutes at room temperature to detect the protein, and the membrane was then placed between two transparency films in X-Ray Cassettes and exposed to CL- XPosure film (Thermo, #34088) within an appropriate amount of time. The film was developed in a dark room according to manufacturer's (MI-5) guide.

B. 2D protein electrophoresis

The protocol to run the 2D protein electrophoresis was adapted (Janes et al., 2012).

1. Approximately 15-20µg of soluble protein extract (section 2.2.9.1) were loaded onto ReadyStrip™ IPG Strips 7cm pH3-10NL (Bio-Rad) embedded in Destreak™ rehydration solution with 2% of the corresponding IPG buffer.
2. Strips were covered by Drystrip Cover Fluid (GE Healthcare immobiline Drystrip cover fluid, 17133501).
3. Strips were rehydrated and focused in the Bio-Rad PROTEAN IEF cell at 50V for 12hr followed by the rapid focusing programed10, 000Vh (Janes et al., 2012).
4. Strips were placed in an IPG tray, and washed with 2ml of equilibration buffer I (6M urea 0.375M Tris-HCl (pH8.8), 2% SDS, 20% glycerol, 2% (w/v) DTT) for 10min at room temperature on a rocking platform.
5. Strips were washed at the same way with 2ml of equilibration buffer II (6M urea, 0.375M Tris-HCl (pH8.8), 2% SDS, 20% Glycerol, and 2.5% (w/v) Iodoacetamide).
6. Strips were applied to a 10% SDS-PAGE gel composed of the resolving layer only.
7. Proteins were ran normally according to the SDS-PAGE and Western blot steps that mentioned previously.

2.2.9 Immunostaining protocol

Cells were seeded at (2.5×10^4 - 5×10^4) cells per well in 12-well plates on glass coverslips coated with poly-D-lysine (VitroCam; 1445-P01) and incubated under normal growth condition until 50% confluence. The medium was then removed and the cells were washed once with 1ml of warm 1xPBS,and then fixed with 4% paraformaldehyde (PFA) in 1xPBS for 10 minutes at room temperature. The cells were permeabilized by adding 0.2% Triton X- 100 diluted in 1xPBS and incubated for 10

minutes at room temperature. The cells were then washed carefully three times with warm 1xPBS for 10 minutes and blocked with 5%FBS (prepared with 1xPBS) for one hour on the shaker at room temperature to block unspecific binding of the antibodies. The cells were then incubated overnight at 4˚C on the rocking platform with 200µl of the primary antibody (see Table 2-6) diluted in the blocking buffer (5% FBS), followed by three washes for 10 minutes each using 1xPBS. The secondary antibody (see Table 2-7) was diluted in the blocking buffer with the light off in the hood and incubated in the dark for 2 hours at room temperature on the rocking platform, followed by three washes for 10 minutes each in the dark. All antibodies were prepared with 5%FBS in 1xPBS at different concentrations according to the manufacturer's guide. The coverslips were removed carefully from each well and allowed to semi-air dry for about 5 minutes. One drop of the mounting solution containing DAPI (VECTASHIELD; H-1200) was added on the glass slides where the samples will be fixed, The cover slips were placed onto glass slides and sealed with nail polish to prevent drying and movement under microscope. Slides were stored at 4˚C until they were examined by Zeiss LSM710 Confocal Microscope with a 63× objective, and the pictures were analyzed by Zen2010 software and processed by Photoshop (Adobe).

Chapter 3: *hPER1* Expression at mRNA level

3.1 Introduction

Circadian patterns are 24-hour rhythms in physiology and behavior, sustained by a biological timekeeping capability that has evolved in most life forms on earth (DeCoursey et al., 2004). In mammals, a central clock controlling a large number of rhythms is located in neurons of the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Reppert & Weaver, 2002, Klein et al., 1991). The central oscillator governs the peripheral oscillators. Yet, under some circumstances peripheral oscillators can disconnect from the SCN and run independently (Damiola et al., 2000, Stokkan et al., 2001). Moreover, many physiological processes are subject to circadian regulation. In mammals, sleep-wake cycles, body temperature, heartbeat, blood pressure, endocrine secretion, renal activity, and liver metabolism are all under the control of a circadian pacemaker (Lavery & Schibler, 1993, Portaluppi et al., 1996, Rabinowitz, 1996, Hastings, 1997).

Central components of the mammalian circadian clock are the *Period* (*Per*) genes, which are expressed in a diurnal manner in various tissues on the RNA and protein levels (Sun et al., 1997, Field et al., 2000). On the other hand, all three mammalian *Per* genes (*Per-1, Per-2,* and *Per-3*) are rhythmically expressed in tissues outside of the brain. These peripheral molecular oscillations raise the possibility that non-neural circadian oscillators exist in mammals (Zylka et al., 1998).

The human *Per1* gene consists of 23 exons, which are alternatively spliced into 7 protein-coding transcripts and 13 non-protein coding transcripts (Ensembl database, accessed 03 November 2016). Five of the protein coding transcripts will be analysed in this chapter. It should also be noted that the *Per1* transcripts encode several regulatory microRNAs (Nicholas et al., 2007).

In this chapter, I want to find out whether hPER1 is expressed at the mRNA level in different human tissues.

3.2 Results

I examined the expression of *hPER1* gene in 12 human tissues. 1µg of the total tissue RNA was converted to their corresponding cDNA, using the Tetro cDNA synthesis kit. Next, a general PCR was performed using the produced cDNA as a template. Primers for human β-Actin (β-ACT) were used in all PCR reactions, as a control for the quality of the cDNA. To amplify Per1 cDNAs, the primers *Per1-201-F+Per1-202-R* were used to confirm their existence at the mRNA level (Figure 3-1). As shown in Figure 3-2, the Per1 primers bind to the cDNAs from all splice variants and the size was 186bp. The results of the PCR experiment confirmed the expression of hPER1 at the mRNA level in all tested tissues, including prostate, thymus, spleen, placenta, stomach, adrenal gland, thyroid, skeletal muscle, brain, lung, salivary gland, and kidney (Figure 3-3). To confirm the identity of the hPER1, PCR products obtained from lung, spleen and adrenal gland were isolated using GenElute™ Gel Extraction Kit (SIGMA-NA1010) and sent for DNA sequencing. The cloned variant was identical to the corresponding sequence from the Ensemble genome database and so on, confirming the existence of the hPER1 at RNA level (Appendix 3).

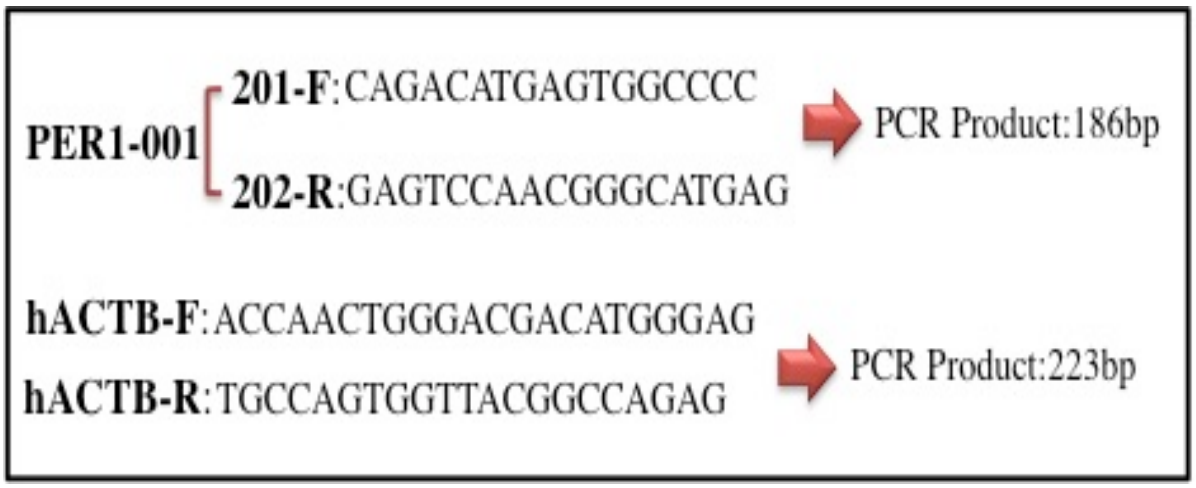


Figure 3–1: Primers to check the existence of hPER1 in the aforementioned human tissues.

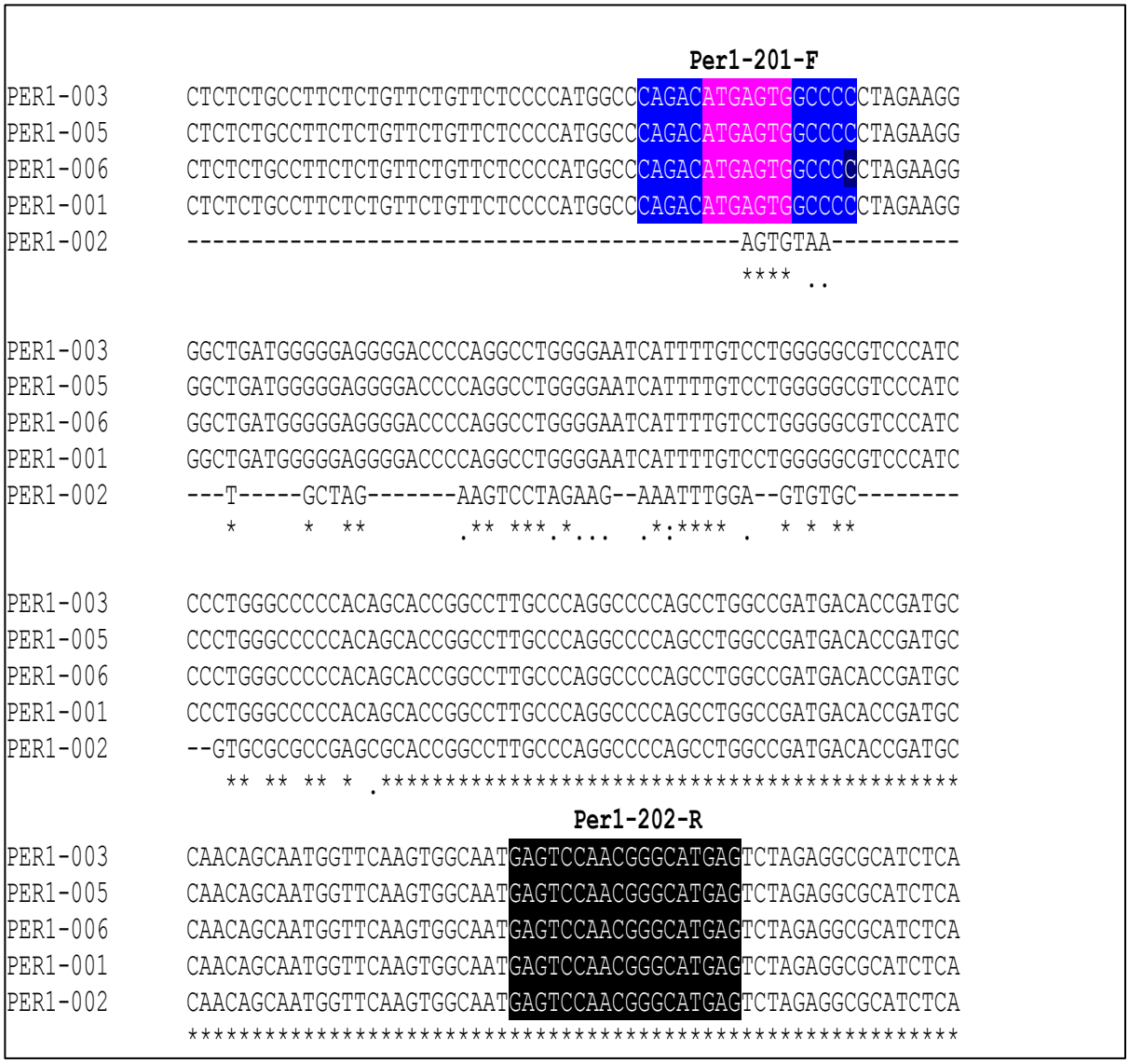


Figure 3–2: Alignment of the section of the Per1 cDNAs with the primers *Per1-201-F* and *Per1-202-R* bind. The ATG start codon of the open reading frame is highlighted.

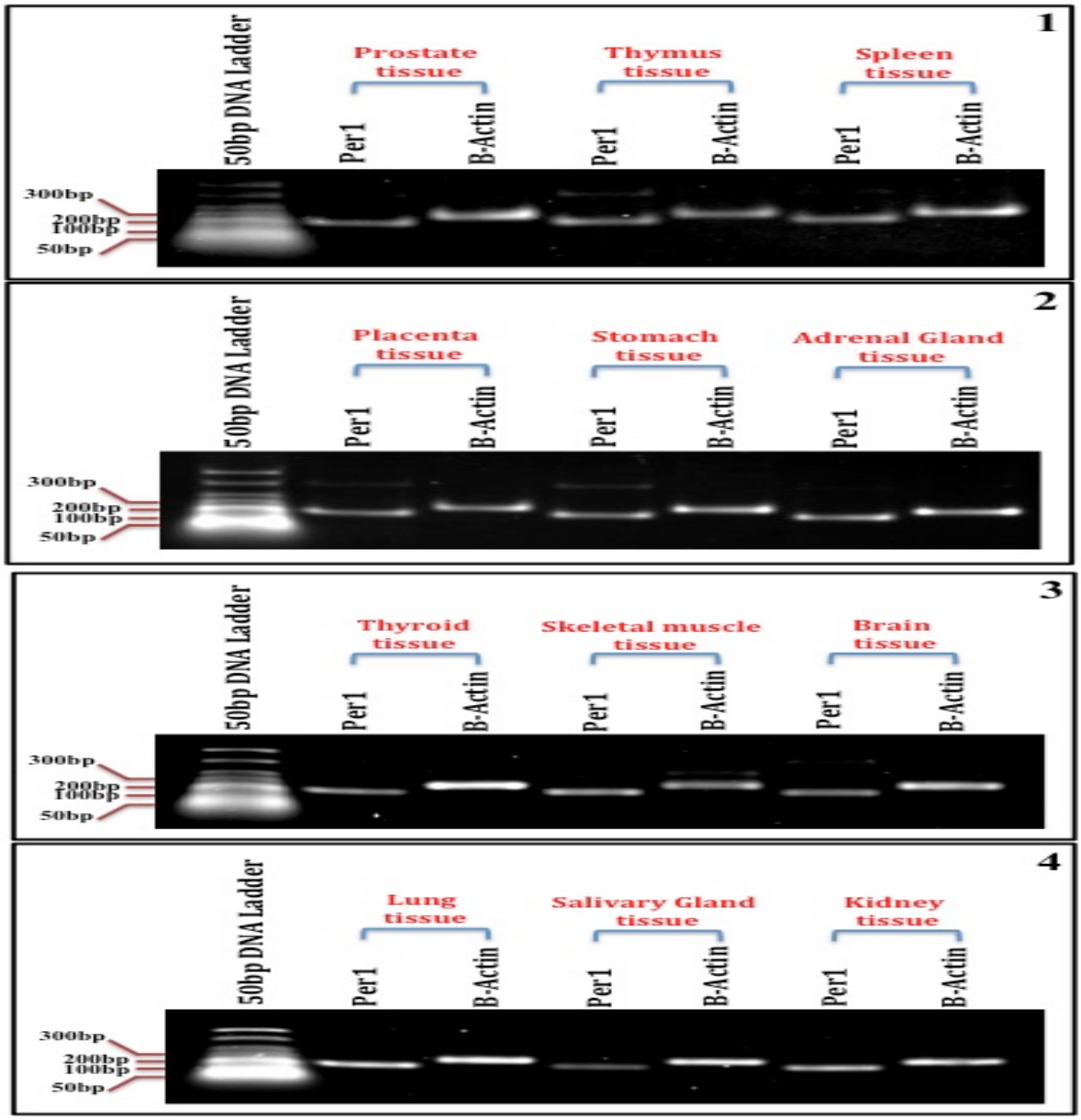


Figure 3–3: Expression of the *hPER1* gene in different human tissues. cDNAs were isolated from the total RNA from 12 tissues. The cDNA was produced from the total RNA purchased for the different human tissues (Human Total RNA Master Panel II, Clontech Laboratories, cat. 636643), using the Tetro cDNA synthesis kit (BIOLINE). A general PCR was performed using cDNA as a template. β-Actin primers were used as a positive control. The primers *Per1-201-F* and *Per1-202-R* were used for Per1. PCR products were run on 1% agarose gel and stained with SYBR-green. The Bio-Rad gel documentation system was used to take the images. PCR results were positive for *hPER1* transcript in all tested human tissues, and the size was at 186bp.

3.3 Discussion

Using PCR primers that detect the *Per1*, the results indicate expression of *Per1* in all tested human tissues. This finding is in line with the expression of the circadian clock in all human tissues (Reppert & Weaver, 2002). Changes in *Per1* expression levels at the mRNA level have been reported for a wide range of cancers, including tumours of the breast, the prostate, the liver, and the colon. Whether *Per1* expression is up or down-regulated depends on the type of malignancies. For example, *Per1* is down-regulated in colon cancer (Gery et al., 2006), but up-regulated in liver cancer (Sato et al., 2009). Given the different expression levels in different cancers, it is unclear which factors contribute to the increase or decrease in *Per1* transcription. The latter may be tumour specific. It is also unclear whether the expression levels reflect the circadian, cell cycle, or DNA damage response function of Per1.

Down-regulation of BMAL, for example, which results in reduced Per1 expression levels, promotes tumour growth by increasing the cell cycle regulators Cdc2 kinase and the cyclins B1, D1 and E at protein level (Zeng et al., 2010). On the other hand, over-expression of human Per1 leads to an increase in the protein levels of the c-myc oncogene (Gery et al., 2006). A recent report by (Fu et al., 2016), reported that down-regulation of *Per1* in SCC15 squamous cell carcinoma cells increased the mRNA levels of the cell cycle regulators CyclinD1, Cyclin E, CyclinB1, CDK1 kinase, and WEE1 kinase. Meanwhile, the mRNA levels of the tumour suppressor p53 and the CDK inhibitors p16 and p21 were reduced. This correlated with faster cell growth and a reduction in cell death.

Even though PER1 expression is high in the suprachiasmatic nucleus, PER1 expression has been detected in a wide range of other normal tissues (Shearman et al., 1997, Tei et al., 1997). PER1 expression has been detected in heart, placenta, liver, skeletal muscle, lymph node, thymus, ovary, testis, small intestine, pancreas, stomach, urinary bladder, kidney, salivary gland, lung, brain and prostate. It is also expressed in hair follicles. Among non-brain tissues, highest expression of PER1 has been shown to occur in skeletal muscle. High expression of PER1 has also been shown to be present in other tissues like adrenal, appendix, bone marrow, endometrium, oesophagus, fat, gall bladder, spleen and thyroid. Lowest expression of PER1 was noted in duodenum and

skin. This is suggestive of key roles for the circadian genes in other tissues. It is also possible that PER1 is involved in other non-circadian activity such as suppression of tumour formation and also apoptosis. In the brain, PER1 protein has also been implicated in the maintenance of long term memory (Sakai et al., 2004). Studies have shown that knock-out of PER1 gene abolishes long term memory, which can be expressed by restoration of protein levels. Similarly, overexpression of PER1 has been shown to potentiate long term memory. PER1 expression has also been implicated to play a key role in ageing since some experimental studies have shown increase in lifespan, suggestive of its role in other tissues (Krishnan et al., 2009). However, this has not been corroborated by other similar studies. In conclusion, all data in this chapter show that Per1 is expressed in all of the tissues tested and that were supported by work carried out by others.

Chapter 4: Expression levels of the *hPER1* Protein and checkpoints in HCT116 and HEK293 cell lines

4.1 Introduction

Given the association of Per1 with the ATM-Chk2 pathway, this chapter summarises the analysis of endogenous Per1 in the context of ATM-Chk2 and ATR-Chk1 signalling in the response to UV light, the Topoisomerase 1 inhibitor Camptothecin, oxidative, and heat stress.

Under stress conditions, the mammalian DNA damage response pathway triggers activation of checkpoints. The sensor proteins that detect DNA damage consist of two members of the phosphatidylinositol serine/threonine protein kinase family: Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-related (ATR). Activation of ATR and ATM kinase relay the DNA damage signal through the phosphorylation of Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2), respectively (Takai et al., 2000). Chk1 is known to be activated by phosphorylation at Ser-345 (Mailand et al., 2000, Peng et al., 1997), while the activation of Chk2 in checkpoint signalling is initiated by phosphorylation at Thr-68 in an ATM-dependent manner (Ahn et al., 2000). These response pathways are induced by a variety of DNA-damaging agents that appear to orchestrate the damage response, sometimes in concert and sometimes separately. One primary difference between ATM and ATR is the type of damage to which each responds (Abraham, 2001). While ATR is recruited to single-stranded DNA by its subunit ATRIP (Ball, Myers & Cortez, 2005). ATM binds to double-stranded DNA breaks through its interaction with Nbs1 in the MRN complex (Lavin, 2007).

The experiments were conducted with HEK293 cells, since this cell line was also used to generate the stable cell lines expressing the engineered GFP-PER1 proteins , and HCT116 colon cancer cells. HEK293 cells are derived from human embryonic kidney cells by the random integration of adenovirus 5 DNA. The transformation resulted in the incorporation of approximately 4.5 kilobases of the viral genome into chromosome 19 of the HEK cells (Graham et al., 1977), and encodes for the E1A/E1B proteins, which interfere with the cell cycle control pathways and counteract apoptosis (Berk, 2005,

Sha et al., 2010). While the consequence of the integration on cell morphology and function is not known, the cell line is considered a standard laboratory cell line without oncogenic changes.

The cell line HCT116 originated from a single human colon carcinoma from a male patient. The cell line is almost diploid (Brattain et al., 1981). The HCT116 cells were chosen to compare (HEK293) cells with malignant cells (HCT116).

In this chapter, I wanted to determine whether Per1 expression could be influenced by the DNA damage response. Ultraviolet (UV) light activates ATR-Chk1 signalling, when ATRIP associates with ssDNA-binding protein RPA at damaged replication forks or sites of nucleotide excision repair (Zou & Elledge, 2003). UV light also leads to ATM activation at stalled replication forks through ATR (Stiff et al., 2006). The topoisomerase inhibitor camptothecin (CPT) was used, as it generates double-stranded DNA breaks at a collapsed DNA replication fork. This strongly activates ATM (Zhao, Traganos & Darzynkiewicz, 2008), but also can lead to ATR activation once the break has been converted into a single-stranded tail (Patro et al., 2011). Oxidative DNA damage activates ATM-Chk2 and ATR-Chk1 signalling (Yan, Sorrell & Berman, 2014), and heat stress activates ATM in G1 and G2, whereas ATR is activated in S phase. The heat-induced activation (HS43°C) of both kinases is, however, different: ATM no longer requires the MRN complex, while ATR is activated by nucleoline rather than RPA (Turner & Caspari, 2014).

4.2 Results

Several chemical agents known to cause DNA damage were assessed for their ability to induce ATM and ATR activates in HEK293 and HCT116, as measured by induction of phosph-Chk2 at Thr-68 and phosph-Chk1 at Ser-345. I have tested 4 groups of DNA-damaging agents (UV, CPT, H_2O_2, and HS/43°C).

UV affects cells by damaging their DNA, as it generates thymidine dimers and 4-6 photoproducts that interfere with DNA replication and transcription(Mao et al., 2017). Here, UV-C light of 254nm was used (CL-1000 UV Crosslinker from UVP Inc). CPT, also known as camptothecin, refers to a cytotoxic quinolone alkaloid that affects cells by inhibiting the topoisomerase1-DNA cleavage complex, which forms in front of moving DNA replication forks. The collision of forks with the immobilized Topo1-DNA complexes causes S-phase specific DNA breaks (Pommier, 2006).

Hydrogen peroxide (H_2O_2) forms hydroxyl radicals whenever chromatin-bound iron (Fe2+) reacts with H_2O_2, resulting in damage that is responsible for the death of cells (Imlay, Chin & Linn, 1988). Oxidative stress causes single-stranded and double-stranded DNA breaks in human cells (Imlay, Chin & Linn, 1988). Heat stress affects cells mainly in the S phase, by the introduction of single-stranded DNA breaks, although double-stranded breaks can be detected in G1 and G2 cells (Velichko et al., 2012).

Different commercially available anti-Per1 antibodies were screened for their use in this set experiment. It was decided to use the Pierce/ThermoScientific PA1-524 antibody, as it binds to a defined epitope (S L A D D T D A N S N G) in the N-terminal section of all potential Per1 protein variants (Figure 4-1).

Figure 4–1: Epitope recognized by the anti-Per1 antibody (Pierce/ThermoScientific PA1-524). The epitope is highlighted.

4.2.1 hPER1 expression as a response to different DNA-damaging agents

4.2.1.1 Ultraviolet light (UV)

Cells were exposed to 40J/m^2 UV light and cell extracts were prepared after 30mins, 1h, 2hrs, and 24hours post-treatment. Cells were left to recover at 37°C. The UV-induced degradation of the CDK inhibitor p21 WAFI/CIP1 was used as a control for the UV treatment (Soria et al., 2006). As reported previously (Zou & Elledge, 2003), the dose was very effective on both cell lines, as it triggered checkpoint-dependent phosphorylation of Chk1 at Ser-345. This was very clear in both cell lines and much stronger in HCT116 cells (Figure 4-2). Interestingly, the degradation of p21 WAFI/CIP1 was faster in HEK293 cells compared to HCT116 cells. HEK293 cells responded within the first 30 min, whereas HCT116 cells responded after 2-hours. The UV treatment did not show phosphorylation of Chk2 at Thr-68 in HEK293 cells, but showed a phosphorylation of Chk2 at Thr-68 after 24 hours in HCT116 cells. This suggests the accumulation of DNA breaks in HCT116 colon cancer cells, at 24 hours post-treatment. The late appearance of DNA breaks could be caused by a checkpoint defect that allows HCT116 cells to re-enter the cell cycle after UV treatment, as this could convert single-stranded gaps left behind by incomplete nucleotide excision repair into DNA breaks when cells go through the S phase. GAPDH was used as a loading control.

The anti-Per1 antibody (Thermo Scientific/PA1-524) recognizes a peptide epitope that is present in Per1-001 (1290aa), Per1-005 (1267aa), Per1-003 (572aa), Per1-006 (824aa) and Per1-002 (859aa)(Figure 4-1). Consistent with this, the antibody recognizes five strong bands in total extracts prepared from HCT116 cells, but only three dominate bands in extracts from HEK293 cells (Figure 4-2). Since the size of Per1-001 (1290aa) and Per1-005 (1267aa) differs only by 23 amino acids, the band (band 1 in Figure 4-2) with an apparent molecular weight of 136KDa is very likely to contain both variants, if they are expressed in the analysed cell line. This is supported by the presence of a double band, depending on the acrylamide percentage, and by the shift of this band when the larger engineered EGFP fusion proteins are expressed (Figure 6-10). The protein above band 1, the very top band (Figure 4-2) above Per1-001 and 005, may well be a covalent modified from Per1-001 or Per1-005.

The second band of around 85kDa, band 2 in Figure 4-2, could also be a double band containing both Per1-002 (859aa) and Per1-006 (824aa), as they also have a very similar size of around 85KDa. Interestingly, this band is much weaker in the HEK293 extracts, which could mean that HEK293 cells do not express both Per1 forms.

The third band, number 3 in Figure 4-2, could be Per1-003 (572aa), as the size of around 50KDa would be in line with the molecular weight of this form. The identity of the band below band 3 in HCT116 cells is not clear.

The expression levels of all Per1 bands remained unchanged by UV treatment in HCT116 cell lines, but band 3 declined after UV treatment in the HEK293 cell line, indicating the down-regulation of Per1-003 after UV treatment. It was interesting to find that HTC116 cells had a stronger overall expression of the Per1 forms and that an additional band appeared above the 136kDa band (Figure 4-2). This band could be a modified form of Per1 in the malignant cell line. A similar band may also be present in HEK293 cells, but is more difficult to detect as the Per1 protein levels are lower in this cell line.

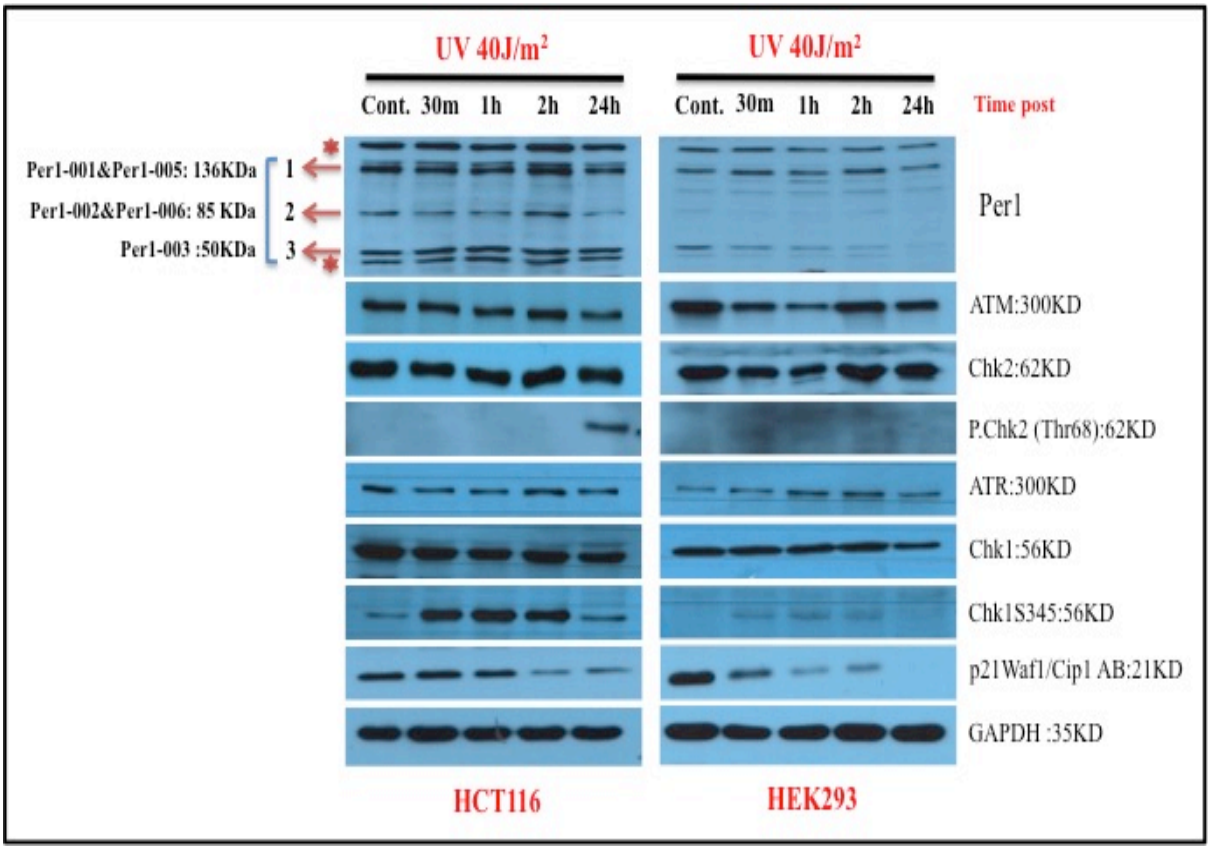


Figure 4–2: UV treatment of HEK293 and HCT116 cells. Cells were exposed to 40J/m² of UV light and cells were allowed to recover for the indicated times at 37°C. Protein extracts were then prepared and analysed by Western blot. The DNA damage signal was very strong, which is clearly shown by the degradation of the p21 WAFI/CIPI protein in both cell lines. The signal of Chk1S345 phosphorylation indicates ATR activation, which was strong in both cell lines, especially in HCT116 cells. By using the anti-Per1 antibody from Thermo Scientific (PA1-524), that recognizes a peptide epitope that is present in Per1-001 (1290aa), Per1-005 (1267aa), Per1-003 (572aa), Per1-006 (824aa), and Per1-002 (859aa) several bands were detected in total extracts. Band N.1 (which is Per1-001 and Per1-005 [136KDa]); band N.2 (which is Per1-002 and Per1-006 [85KDa]); and band N.3 (which is Per1-003 [50KDa]) showed the same expression level at all time-points in HCT116 cells, but displayed a double-band above band of N.1 in HCT116 cells that had a higher Per1 expression level. Band N.3 declined after UV treatment in HEK293 cells, indicating the downregulation of Per1-003 after UV treatment. HEK293 did not show the phosphorylation of Chk2 at Thr-68 by ATM, but HCT116 did at 24 hours post-UV treatment. GAPDH was used as a loading control. (*) Unspecific band

4.2.1.2 Replication fork damage by Camptothecin (CPT) treatment

Cells were treated with 1μM of CPT for 30-min, 1-hour, 2-hours and 24-hours at 37°C, then protein extracts were prepared and analyzed by Western blot. In this experiment, the ATM-Chk2 checkpoint response was clear in both cell lines especially at the 2-hours time point, as indicated by the phosphorylation of Chk2 at Thr-68 (Figure 4-3). This modification was much stronger in HCT116 cells. Phosphorylation of Chk1 at Ser-345 by ATR was only detected in HEK293 cells again at the 2-hour time point. Per1 expression levels did not change in either cell line, and in this experiment, HEK293 cells displayed also a double band at 136kDa. The level of p21 WAFI/CIP1 remained high in both cell lines, in line with its UV-specific degradation. The accumulation of the phosphorylated Chk2 at Thr-68 signal at the 2-hour time point indicates that a larger number of cell must have entered S phase by this time, since CPT only breaks DNA during DNA replication (Ryan et al., 1991).

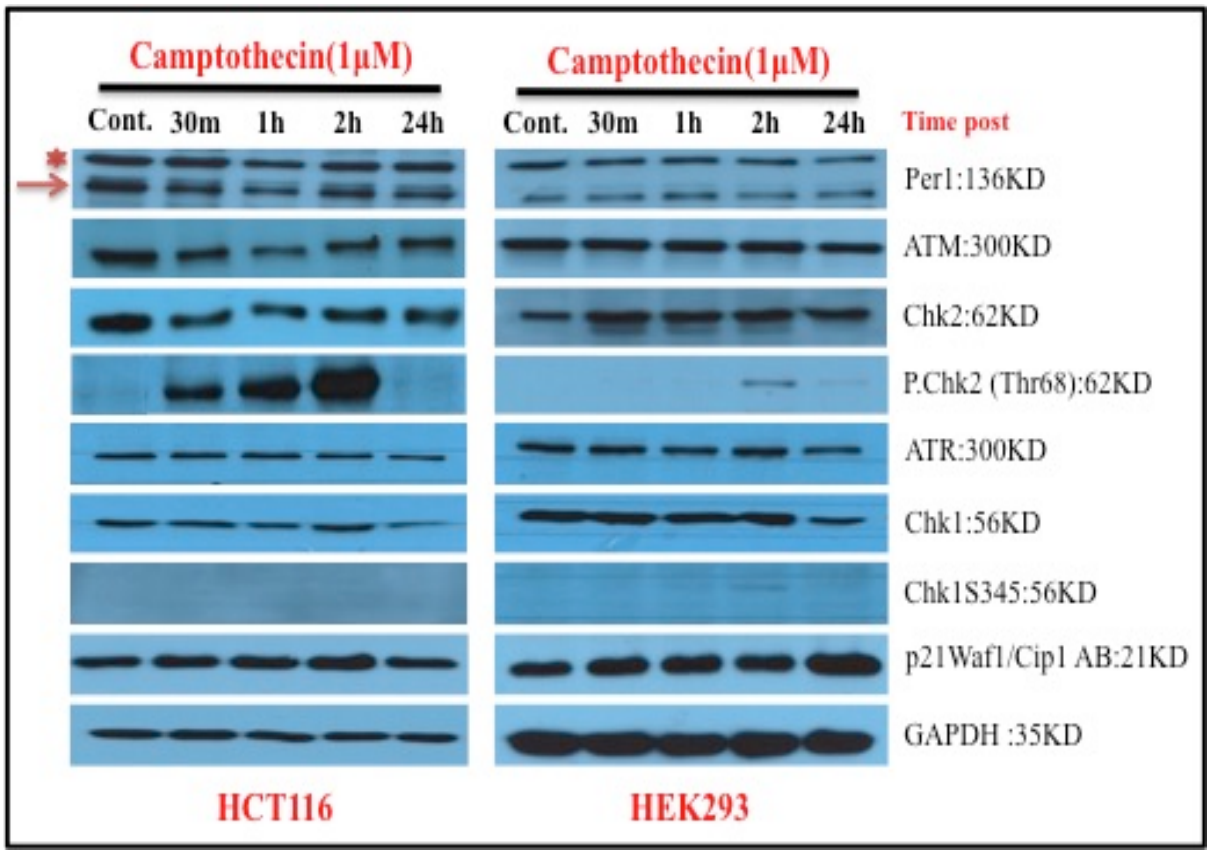


Figure 4–3: Camptothecin treatment of HEK293 and HCT116 cells. Cells were treated with 1μM of CPT for the indicated times at 37°C, then protein extracts were prepared and analyzed by Western blot. By using the anti-Per1 antibody from Thermo Scientific (PA1-524), the expression level of hPER1 (arrow) was the same at all time-points in HEK293 and HCT116 cells, but this time a double-band at 136kDa was evident in both cell lines. The phosphorylation of Chk2 at Thr-68 was very clear in both cell lines, especially at the 2-hours time point, but the signal was much stronger and appeared earlier in HCT116 cells. A weak signal for Chk1 S345 phosphorylation was only present at the 2-hours time point in HEK 293 cells. The level of p21 WAFI/CIPI protein did not decline in either cell line. GAPDH was used as a loading control. A repeat experiment for the anti-Chk2 antibody with HCT116 and HEK293 cells is shown in Appendix 4. (*) Unspecific band.

4.2.1.3 Oxidative stress (H_2O_2)

Cells were treated with 500µM hydrogen peroxide (H_2O_2) for 30 min, 1-hour, 2-hours and 24-hours at 37°C, then protein extracts were prepared and analyzed by Western Blot. Both cell lines were affected by H_2O_2 treatment, as they triggered the phosphorylation of Chk2 at Thr-68 (Figure 4-4), which was most obvious after 2 hours and 24hrs of treatment. The phosphorylation of Chk1 at S345 was evident earlier, after 30-min and 1-hour, but only in HCT116 cells. This indicates that HEK293 cells do not activate the ATR-Chk1 pathway in the presence of oxidative damage. The HEK293 cells showed also a transient down-regulation of p21 at the 1-hour and 2-hours time points, which was not detected with HCT116 cells. Taken together, both observations show that HEK293 cells respond in a different way to oxidative stress, when compared to the cancer HCT116 cell line. GAPDH was used as a loading control.

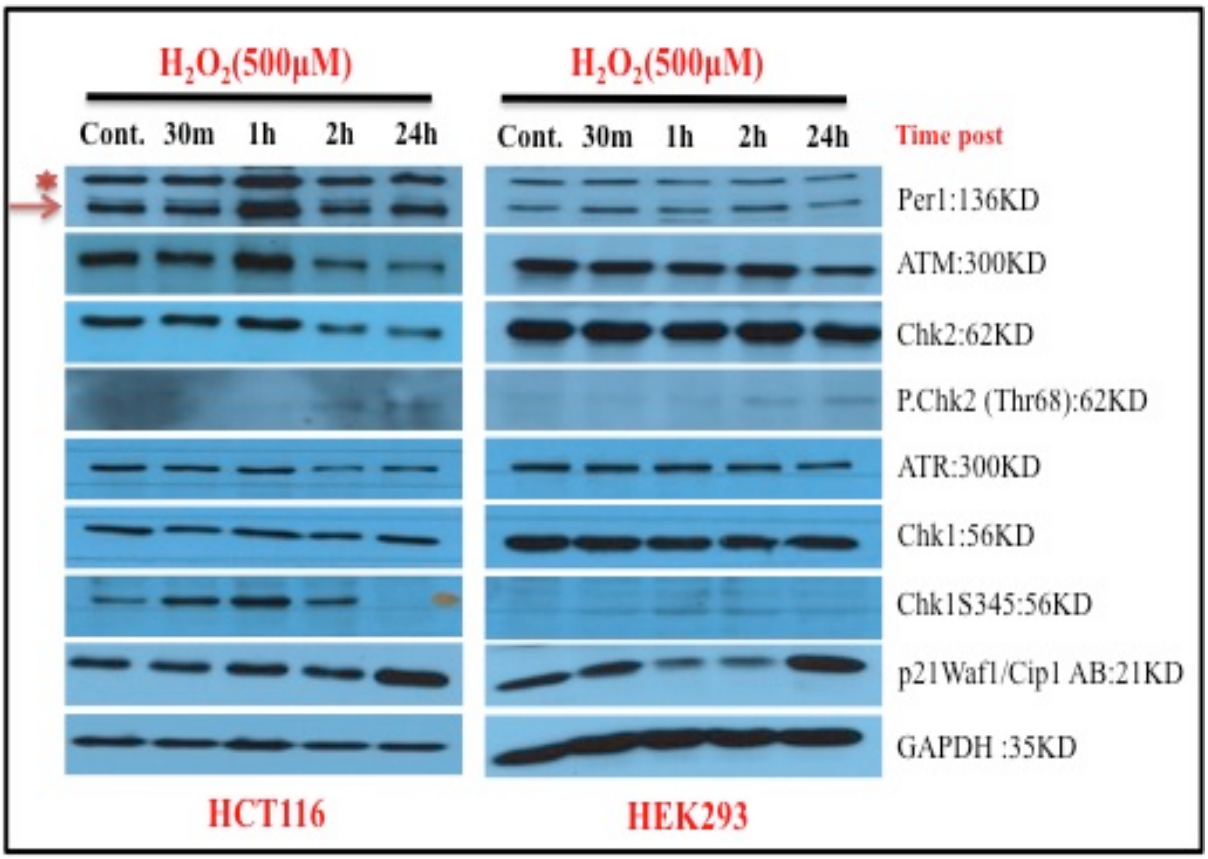


Figure 4–4: Treatment of HEK293 and HCT116 cells with oxidative stress. Cells were treated with 500µM H_2O_2 for the indicated times at 37°C, then protein extracts were prepared and analyzed by Western blot .The treatment triggered Chk1 phosphorylation at Ser-345, mainly in HCT116 cells, and Chk2 modification at Thr-68 in both cell lines. Interestingly, Chk1 phosphorylation occurred earlier than Chk2 phosphorylation. HEK293 cells showed downregulation of p21Wafi/CIPI protein at the 1-hour and 2-hours time points, which was not observed in HCT116 cells. Interestingly, hPER1 (arrow) showed a double band at all indicated times in HCT116 cells, which have a higher expression level of Per1 using the anti-Per1 antibody from Thermo Scientific (PA1-524). GAPDH was used as a loading control.

(*) Unspecific band.

4.2.1.4 Heat stress

HEK293 and HCT116 cells were heat-shocked for 1-hour at 43°C in complete medium, then left to recover at 37°C for 30 min, 1-hour, 2-hour, and 24-hours. Protein extracts were than prepared and analyzed by Western blot. While the expression pattern of hPER1 did not change in either cell line, heat stress affected the two cell lines in different ways. In HEK293 cells, Chk1 at Ser-345 increased within 30 min and peaked at the 1-hour time point (Figure 4-5). This implies activation of ATR kinase by heat stress. This conclusion is consistent with a recent report showing ATR-Chk1 activation after 30 min at 42.5°C (Tuul et al., 2013). Interestingly, ATR activation is dependent on Rad17 and the 9-1-1 ring, but some down-stream targets of ATR, RPA and FANCD2 are not modified. There may also be some activation of Chk2 at Thr-68 by ATM at the 2-hour time point. A delayed activation of ATM, 2-hour after a 30-minute exposure to 44°C, has been reported previously (Furusawa et al., 2012). The situation in the HCT116 cells was, however, different. As observed in Figure 4-5, Chk1 at Ser-345 increased within 1 hour, which means that the Chk1 at Ser-345 phosphorylation occurred later in colon cancer cells. p21WafI/CIPI protein levels remained at the same in HCT116 cells, whereas in HEK293 cells the CDK inhibitor appeared to be up-regulated between the 30-minute and 2-hours time points. The latter would indicate a transient cell cycle arrest, or the up-regulation of a DNA repair activity of p21.

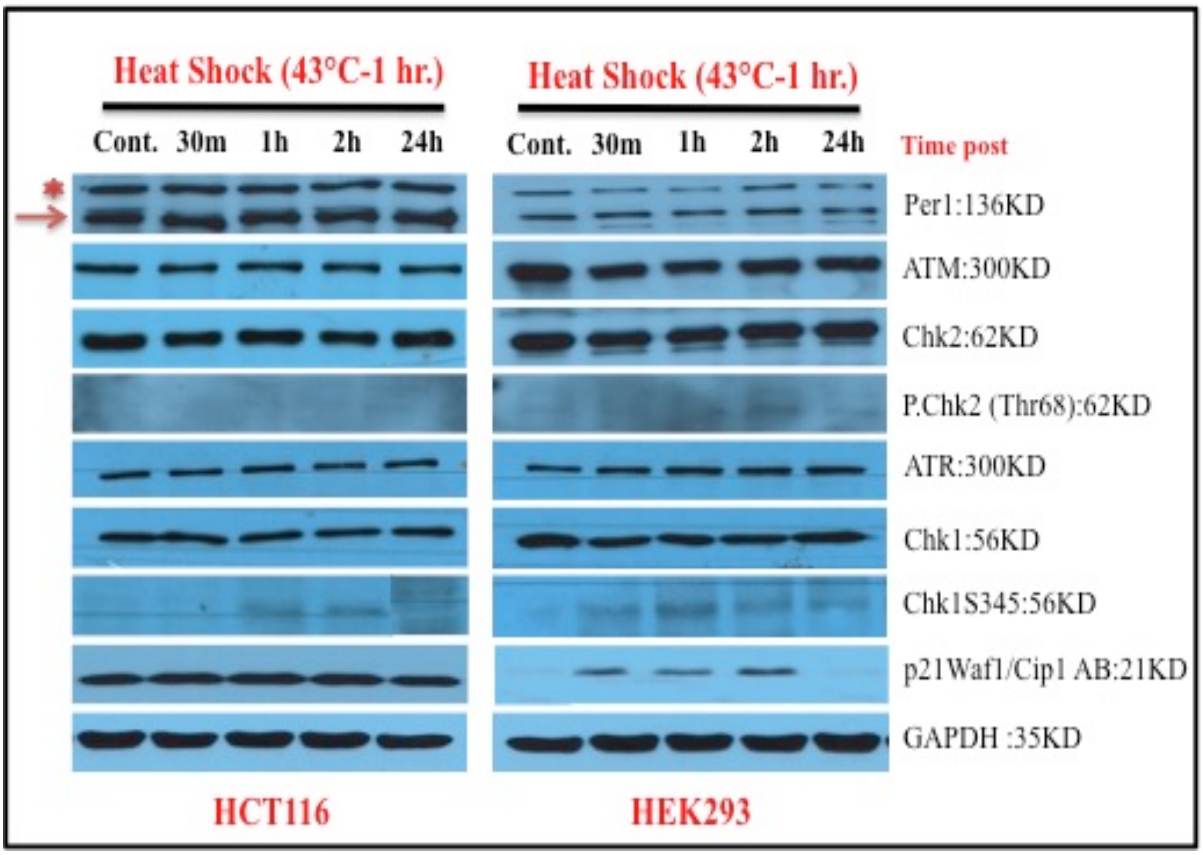


Figure 4–5: Heat-shock treatment of HEK293 and HCT116 cells. Cells were heat-shocked at 43°C for 1-hour in complete medium, and left to recover at 37°C for the indicated time points. Protein extracts were prepared. Using the anti-Per1 antibody from Thermo Scientific (PA1-524), the expression level of hPER1 (arrow) did not change after the heat shocks. HEK293 cells showed phosphorylation of Chk2 after 2hrs of recovery. Chk1 phosphorylation at S345 increased within 30min in HEK293 cells, while increasing within 1h in the HCT116 cells.

(*) Unspecific band.

4.2.1.5 2D Analysis

To analyze the post-translational modification of Per1 in greater detail, protein extracts obtained from HEK293 cells were subjected to isoelectric focusing (pH 3=10). Isoelectric focusing is very sensitive to post-translational changes, which affect the overall charge of proteins like phosphorylation. Cells were untreated or treated with 4 types of agents (UV, CPT, H_2O_2, and HS/43°C). Protein extracts were taken at the end of recovery time, which was after 2-hrs of treatment, and then subjected to the isoelectric focusing. PVDF membranes were probed with the Anti-PER1 antibody (ABCAM-AB174860). Based on the possible existence of 5 main protein variants, bands of 130kDA (twice, Per1-001: 1290aa; Per1-005: 1267aa), 86kDa (Per1-002: 859aa), 82kDa (Per1-006: 824aa), and 57kDa (Per1-003: 572aa) could be expected. The results showed that this antibody detects 4 bands with 136kDa, 100kDa, 70kDa, and a smaller band at around 45kDa on a normal 8% acrylamide gel (Figure 4-6A). Separation of the same sample on the isoelectric focusing system revealed that the two larger bands of 100kDa and 136kDA both consist of two protein species with different isoelectric points (Figure 4-6B). The smaller, 70kDa band is only one species. Treatment of cells with UV light strongly increases the abundance of the more positively charged species of the 100kDA form, which runs closer to the positive end of the strip. A similar change was observed upon all other treatments. Camptothecin treatment and, to a lesser extent, heat and oxidative stress resulted in the appearance of two larger molecular weight forms above the more negatively charged species of the 136kDA form (Figure 4-6D). These larger forms could indicate that Per1 is ubiquitinylated, as each ubiquitin protein would add approximately 8KDa to the molecular weight of Per1. The *Drosophila* Period proteins are the target of E3-ubiquitin ligases (Grima et al., 2002). Given that CPT triggers this modification, Per1 may be modified with ubiquitin when DNA replication forks are damaged.

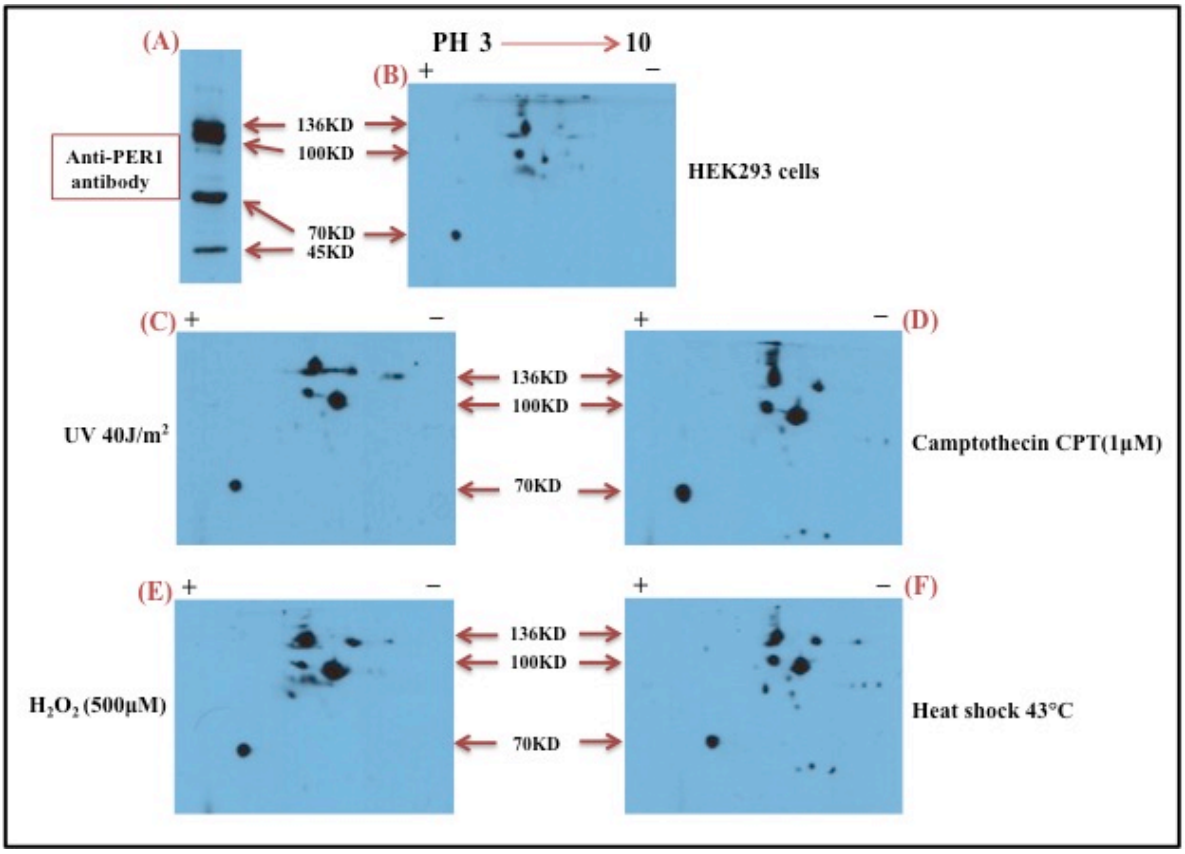


Figure 4–6: Untreated and treated HEK293 cells were subjected to isoelectric focusing (PH 3=10). Cells were untreated or treated with 4 groups of DNA-damaging agents (UV, CPT, H_2O_2, and HS/43°C). All protein extracts were taken at the end of recovery time, which was after 2hrs, and then subjected to the isoelectric focusing. PVDF membranes were probed with Anti-PER1 antibody (ABCAM-AB174860) to analyse the different variants of the Per1 protein. A: Untreated HEK293 cells, which show four bands detected by the anti-Per1 antibody after separation on 8% SDS page. B-F: protein extracts were first separated on an immobilized pH gradient 3-10 (linear) strip, which was then placed on an 8% SDS page. Full-length of PER1 runs at around 136KDa, a second band at around 100KDa, a third band at around 70kDa, and a fourth band at around 45kDa are detected (A). The two large bands separate as two dots with distinct isoelectric values after isoelectric focusing. DNA damage increases the abundance of the more positively charged species of the 100kDA form, whereas CPT treatment results in the appearance of two larger forms of the more negatively charged 136kDa form. The latter forms may indicate ubiquitinylatiobn of Per1.

4.3 Discussion

The key findings of this chapter are (i) the elevated expression level of Per1 in colon carcinoma HCT116 cells, compared to HEK293 cells; (ii) a general tendency of a stronger ATM-Chk2 and ATR-Chk1 response to different types of stress in the HCT116 cell line; and (iii) the existence of at least 4 Per1 forms on a normal 8% SDS page, two of which (the larger forms) can be further resolved in two species by isoelectric focusing, and the possible ubiquitinylation of one of the largest Per1 forms when DNA replication forks collapse in the presence of the Topoisomerase 1 inhibitor camptothecin (CPT).

Work by Gery and colleagues (2006) linked human Per1 with the ATM-Chk2 pathway. In this context, it is very interesting that the HCT116 colon carcinoma cell line expresses higher levels of Per1 and responded more robustly to, for example, CPT (Figure 4-3), with high levels of Chk2 phosphorylation at Thr-68 by ATM. CPT breaks replication forks in S phase and the Thr-68 phosphorylated Chk2 has been reported to localise to DNA double-strand breaks (Ward, Wu & Chen, 2001). This may indicate a role of Per1 at broken replication forks, in the activation of Chk2 Thr-68 phosphorylation (Figure 4-7). Since human Timeless associates with DNA replication forks, and also associates with *Drosophila* and mouse Per1 *in vitro* (Sangoram et al., 1998, Cho et al., 2013), it could be possible that Per1 promotes Chk2 phosphorylation by ATM at broken DNA replication forks through its association with Timeless (Figure 4-7).

A similar positive impact may exist on the phosphorylation of Chk1 at Ser-345 by ATR, in the response to oxidative DNA damage (Figure 4-4). Again, Timeless may be the link between the ATR-Chk1 pathway and Per1, as Timeless associates with ATR and Chk1 in human cells (Unsal-Kacmaz et al., 2005). This association could also take place at DNA replication forks, since recent findings suggest a key role of oxidative stress in the slowdown of human DNA replication forks (Wilhelm et al., 2016).

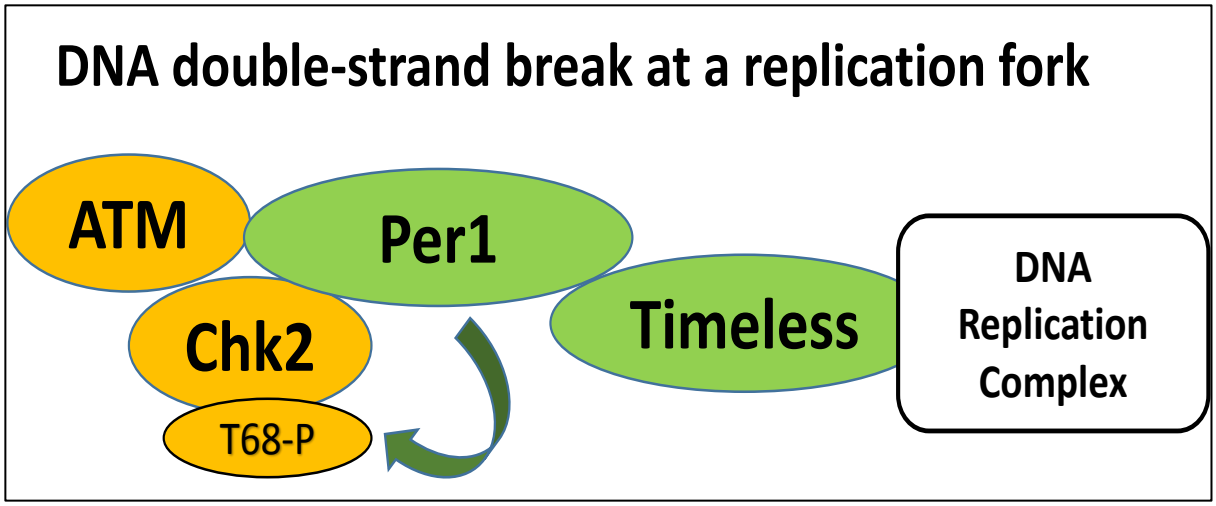


Figure 4–7: Model of the role of Per1 at broken DNA replication forks. Per1 may promote phosphorylation of Chk2 at threonine 68 by ATM at a damaged fork (CPT treatment), through its association with Timeless.

The human Per1 gene may encode at least 5 different splice variants (Figure 1-6). Two large protein forms with 1290aa and 1267aa, respectively. It is interesting that both anti-Per1 antibodies (from ABCAM and FisherScientific) detect a double band in this size range (Figures 4-2,4-3, 4-4,4-5, and 4-6). The lower of the two bands appears to also have an additional, slower-migrating species. Although the latter appears to be specific to HCT116 cells (Figure 4-2), this could be the consequence of the higher expression levels of Per1 in this cell line.

Isoelectric focusing (Figure 4-6) revealed that both large Per1 bands are a mix of at least two protein species with distinct isoelectric points. The more negatively charged form of the largest Per1 species appears to be post-translationally modified after CPT treatment. The two larger forms may represent the attachment of 8kDa ubiquitin units to this form of Per1, when DNA replication forks break. Ubiquitinylation of human Per1 has not yet been shown but is very likely, since Per1 associates with F box proteins that are the substrate recognition subunits of E3-ubiquitin ligases (Shirogane et al., 2005). Interestingly, Timeless has been implicated in the ubiquitinylation of PCNA at stalled

DNA replication forks (Yang & Zou, 2009). This may indicate that Per1 is modified by an F box protein at broken replication forks, in a manner dependent on Timeless.

Per1 does not seem to be phosphorylated in the response to DNA damage, since the main change in the focusing pattern is the increase of a more positively charged species of the smaller of the two big Per1 forms (Figure 4-6). If Per1 were to be phosphorylated, its isoelectric value should become more negative and not more positive. It is also interesting that the size of the more positive species is slightly smaller. An increase in a positive value could indicate that Per1 is de-phosphorylated in the presence of DNA damage. The lack of a cell line without Per1, or the difficulties to down-regulate Per1 expression in HEK293 cells (see Chapter 5), however, made it difficult to fully annotate the different forms detected by the two anti-Per1 antibodies to Per1 and its possible splice variants.

Chapter 5: hPER1 down-regulation and knockdown

5.1 Introduction

This chapter reports the attempt to down-regulate *Per1* expression in HEK293 cells using siRNA (small interfering RNA) gene silencing. This would be beneficial to validate the anti-Per1 antibodies, and to test how a reduction in Per1 would affect ATR-Chk1 and ATM-Chk2 signaling.

Small double-stranded RNA molecules ending in a small loop are processed by the cytoplasmic enzyme Dicer into a short double-stranded RNA duplex, of which only one RNA strand is loaded into the Argonaut complex to form the silencing RISC complex (Li & Patel, 2016). The RISC complex is then guided by the siRNA molecule to the target mRNA. Often the target sequences are in the 3`-untranslated region (3`-UTR) of the mRNA. Binding of the RISC complex to the mRNA can either block its translation or induce its degradation. In this set of experiments, the small double-stranded RNA molecules are produced from a plasmid that encodes a 29nt long stem loop consisting of complementary sequences from the 3-UTR region of the *Per1-001* mRNA. Three siRNA plasmids and one control plasmid were purchased from the company OriGene. The three plasmids encode three distinct sequences from the Per1-001 3`-UTR. The exact sequences were not disclosed by the company.

The aim of this series of experiments was to reduce the endogenous level of hPER1, and to check whether the bands and protein species identified by the anti-PER1 antibodies are all specific to Per1. I used siRNA to inhibit translation of the endogenous Per1 mRNA in HEK293 cells. In the present study, knockdown of PER1 was attempted using three different *siRNA* plasmids. Three oligonucleotide sequences were designed by the company OriGene against the *hPER1* gene, and were used in parallel with a negative control sequence. The company had already cloned all oligonucleotides onto the *pRFP-C-RS* vector. This system works by transfection of the *pRFP-C-RS* plasmid into human cells, which offers both transient and stable shRNA down-regulation. The plasmid carries the shRNA expression cassette, which is formed by 29 nucleotides that are designed to disrupt expression of the gene of interest, followed by a seven-

nucleotide long loop and the reverse complementary sequence of the 29 nucleotides. Expression of the shRNA precursor is controlled by the U6 RNA promoter and terminated by an oligo-TTTTTT sequence, which terminates the transcription by RNA Pol III (Figure 5-1).

The longer sequence of the stem loop (29bp compared to the normal 21bp) appears to enter the Dicer-Drosha pathway more efficiently, resulting in a more potent down-regulation of the target mRNA.

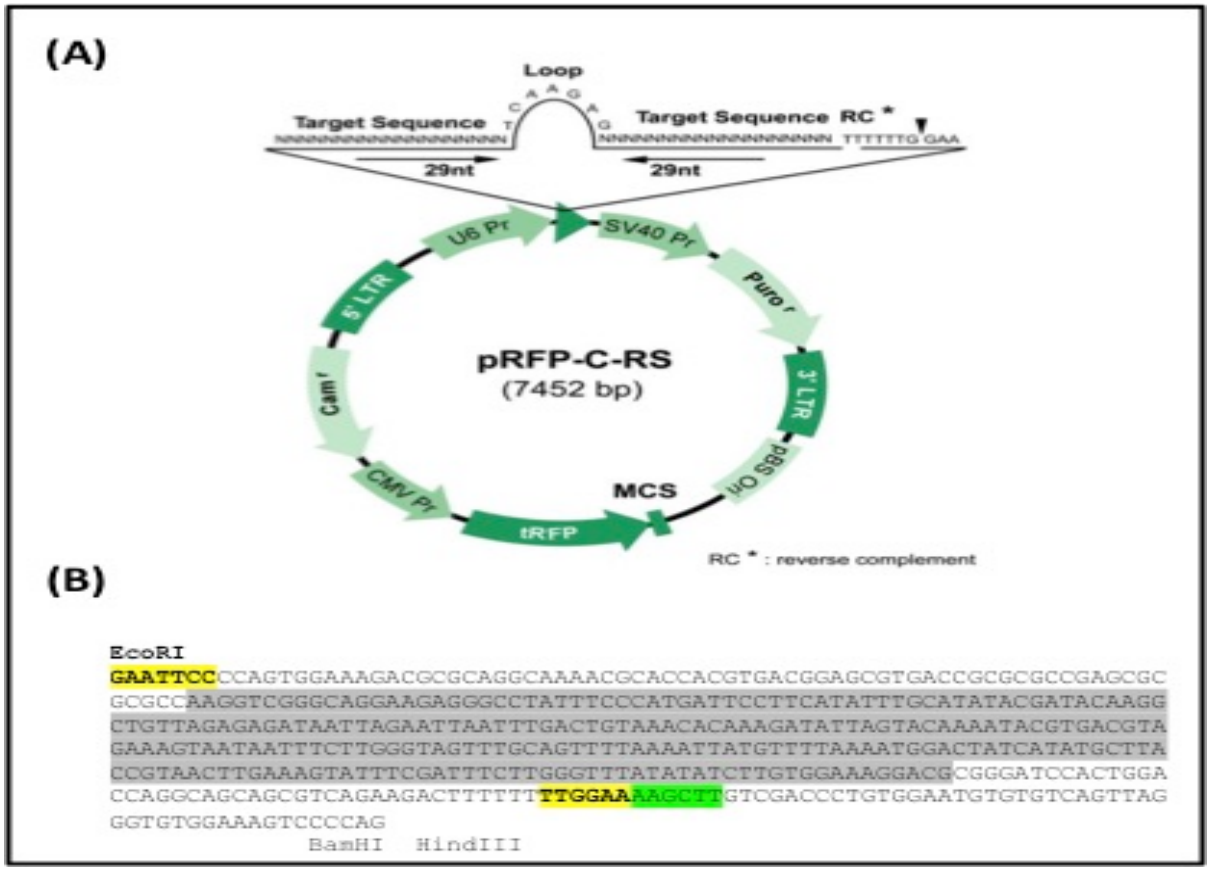


Figure 5–1: Structure of the shRNA down-regulation vector from Origene. A: the stem loop of 29bp complementary sequence, separated by the TCAAGAG loop, is integrated into plasmid down-stream of the U6 promotor. Chloramphenicol allows for the selection in *E.coli*, whereas puromycin allows for the selection of transfected human cells. Transfected cells express the red fluorescent protein RFP from the CMV promotor. B: DNA sequence of the U6 promotor and the relevant down-stream sequence. The complete sequence is in Appendix 5.

https://www.origene.com/products/vectors/shrna-vectors

5.2 Results

The knockdown was carried out in HEK293 cells with four *pRFP-C-RS* plasmids: one scrambled control sequence (control) and three different Per1 shRNA sequences (A, B, C) (Figure 5-2). Single plasmid transfection in parallel, and transfection of the negative control, were achieved using Lipofectamine® 2000 Transfection Reagent (Invitrogen™). Transfected cells were grown in the presence of the antibiotic puromycin, to select for cells containing the *shRNA* plasmid. Protein samples were taken 72 hours post-transfection. Transfected cells were 70% red, due to expression of the red fluorescence protein from the plasmid and were puromycin resistant. Cell maintenances were performed according to the manufacturer's instructions. Protein concentration was measured using the BSA protein assay. Western blot analysis showed a slight effect for plasmid (A) by using anti-PER1 antibody (ABCAM-AB174860), compared to cells transfected with the negative control siRNA. The GAPDH antibody was used to confirm equal loading (Figure 5-3).

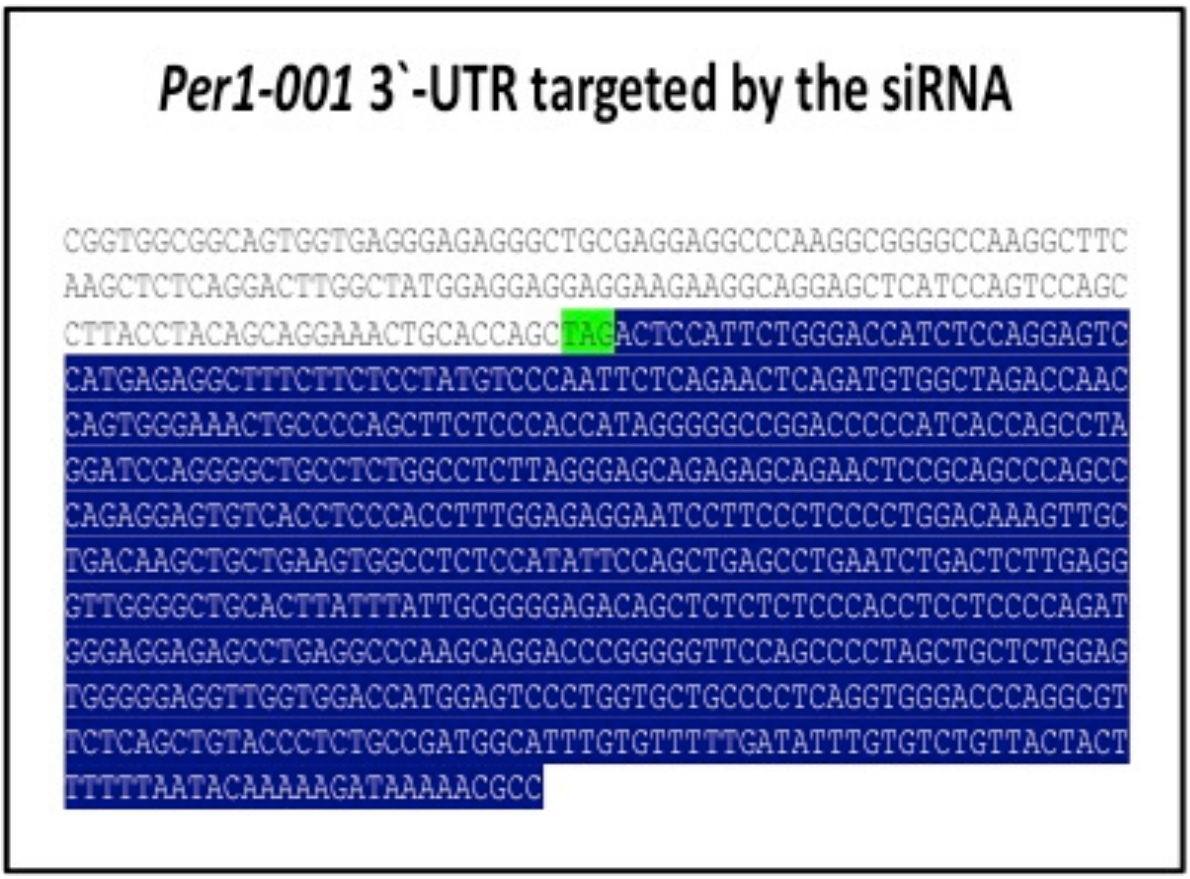


Figure 5–2: Target sequence of the siRNA. The three siRNA molecules encoded by plasmids A, B and C all target the 3`-untranslated sequence of the Per1-001 mRNA (green = stop codon; blue = 3`UTR).

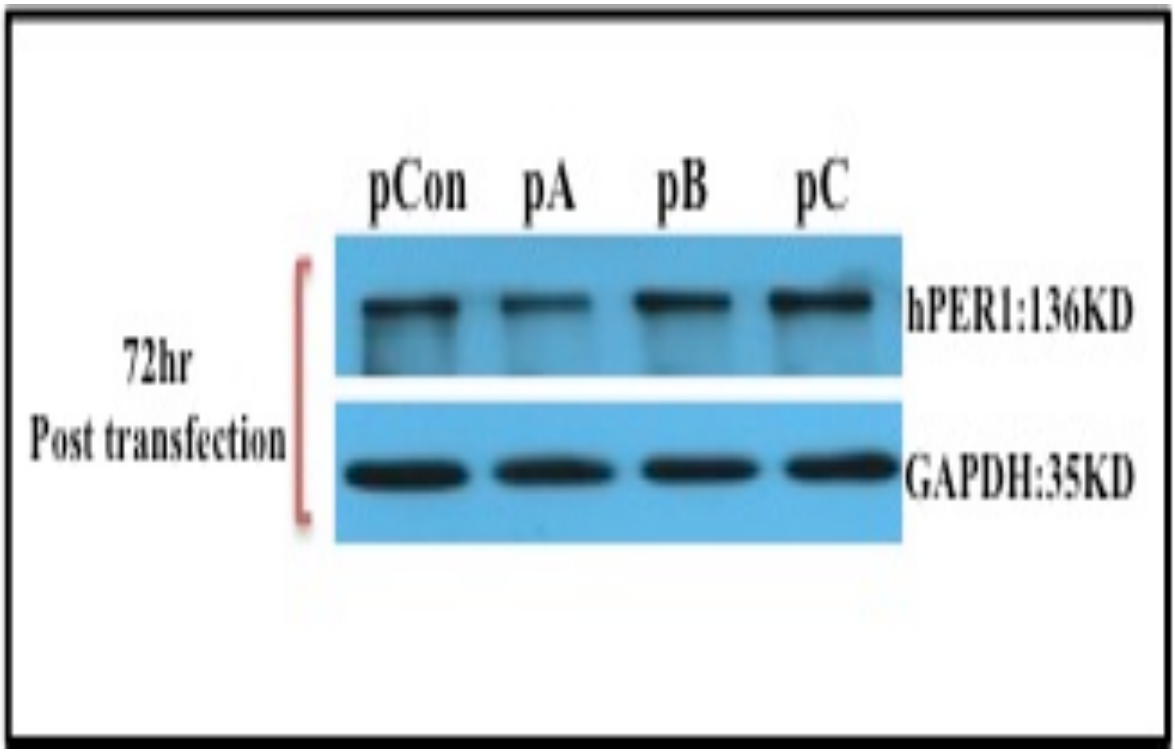


Figure 5–3: Western blot analysis for the three siRNA Plasmids. HEK293 cells were transfected with the three indicated plasmids (A-B-C), separately. Each plasmid contains a different shRNA expression cassette. Protein extracts were prepared 72-hours post-transfection. Using (ABCAM AB), Western blot results showed a moderate down-regulation in the cells transfected with plasmid (A), compared to the control vector (pCon). GAPDH was used to confirm equal loading.

5.3 Discussion

The results from this experiment indicate that down-regulation of Per1 may be difficult to achieve, as only one of the three siRNA plasmids, (plasmid A) resulted in mildly reduced protein levels of Per1. (Gery et al., 2006) reported the down-regulation of Per1 in HCT116 cells, 24-hours post-transfection, with a commercial siRNA plasmid. Although the down-regulation was more efficient, compared to Gery experiment, it was not a complete elimination of Per1 expression. This may indicate that a complete loss of Per1 may be detrimental for the cells. If this were to be true, this does not extend to murine Per1, and homozygote knockout mice are alive (Bae et al., 2001). In this experiment the siRNA did not work either because the sequences were not optimal or because the transfection efficiency was too low.

Chapter 6: Construction of the Flag-EGFP tagged hPER1-001-Proline and hPER1-001-Alanine stable cell lines

6.1 Introduction

A single nucleotide polymorphism, or SNP, is the most common type of genetic variation amongst people. It is a variation at a single position in a DNA sequence among individuals. An SNP may lead to variations in the amino acid sequence, known as missense mutations. However, they can also occur in noncoding regions of the gene like the promotor, introns or the 3` untranslated region. The 1000 Human Genome project reported that a typical human genome differs from the reference genome, at 4.1 million to 5.0 million different sites. Interestingly, the vast majority of these differences (>99.9%) are SNPs and short insertions or deletions (indels). The typical genome also contains between 2,100 and 2,500 structural differences, which can be large deletions (approximately 1000/genome); copy-number variations of genes or loci (around 160/genome); insertions linked with mobile elements (around 915 Alu insertions, 128 L1 insertions, and 51 SVA insertions per genome); or inverted sequences (approximately 10/genome) (1000 Genomes Project Consortium, 2015). As discussed in the overall Introduction, the SNP rs2585405 (A962P; **G**CC/**C**CC) in the human *Per1* gene has interesting population frequencies. As shown in Figure 6-1, the new **C** (**C**CC) allele encoding proline-962 is rare in Europe (9%), but much more frequently found in East Asia (43%). This indicates that the two alleles can freely fluctuate in East Asia, as the distribution is close to 50%, whereas the **C** (Proline) allele is disadvantaged in Europe and other parts of the world. This implies a negative selection against the proline allele in Europe.

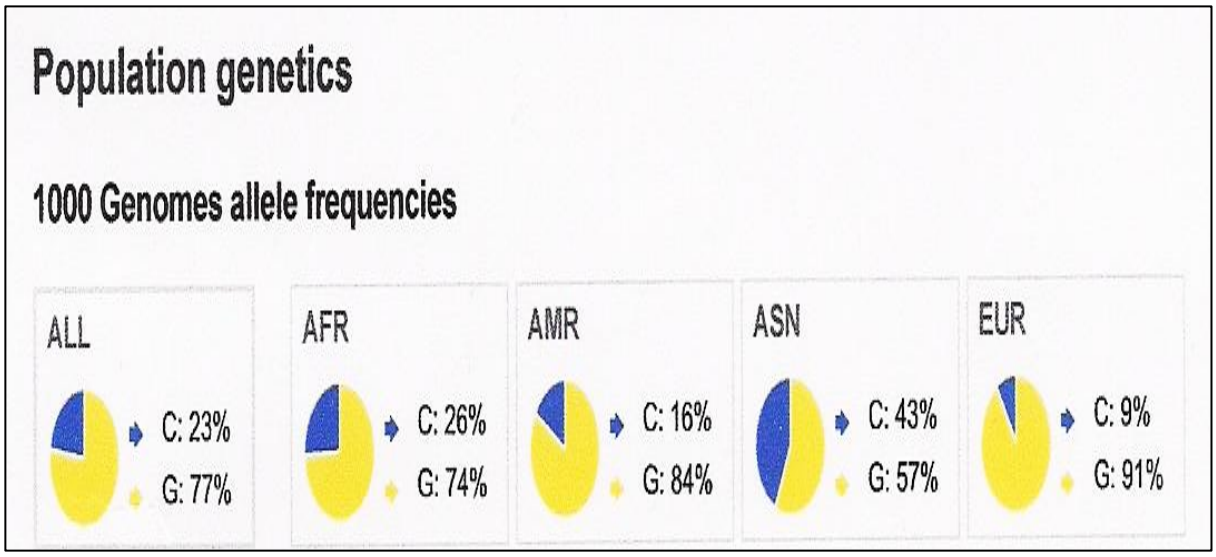


Figure 6–1: Percentage of people who have the new C (CCC) allele encoding proline-962 or the ancestral G (GCC) allele encoding alanine-962, in Africa (AFR), America (AMR), Asia (ASN), and Europe (EUR).

https://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=17:8142954-8143954;v=rs2585405;vdb=variation;vf=362585851

In this part of the project, I decided to construct two stable HEK293 cell lines, which either produce PER1-A962 or PER1-P962, respectively. I proceeded to establish stable cell lines of N-terminally EGFP-tagged hPER1-001-Proline and hPER1-001-Alanin proteins, since this would allow me to perform experiments with the different full-length proteins to compare their impact on ATR-Chk1 and/or ATM-Chk2 signaling. The stable cell lines were constructed using the Flp-In™ T- REx™-293 cells (embryonic kidney HEK293) from Invitrogen (O'Gorman, Fox & Wahl, 1991), for two reasons. First, HEK293 embryonic kidney cells have only one defined integration site for the Flp-In™ T- REx™ cassette, which allows for targeted gene integration. Second, they are easy to maintain and transfect well, using different methods with high efficiency (Thomas & Smart, 2005). The different steps required for the integration of the N-terminally tagged Per1 genes are shown in Figure 6-2. The host HEK293 cell line carries an integrated plasmid on one of its chromosomes, which contains one FRT recombination site integrated in-frame into the LacZ-zeocin gene that renders cells resistant to the antibiotic Zeocin. The gene of interest is cloned into the plasmid *pcDNA5/FRT,* down-

stream of the N-terminal Flag-GFP tag. The recombinant plasmid is then transfected together with the pOG44 plasmid, which encodes the FLP recombinase. This enzyme recognizes the FRT sequence in the *pcDNA5* plasmid and the chromosomal location to facilitate recombination between them. This will result in the integration of the N-terminally tagged gene. During this process, the zeocin resistance is inactivated and a new hygromycin B resistance is added (Buchholz et al., 1996). The stable integration of the EGFP fusion gene at the single FRT site can be selected using hygromycin B resistance and Zeocin™ sensitivity (Figure 6-2).

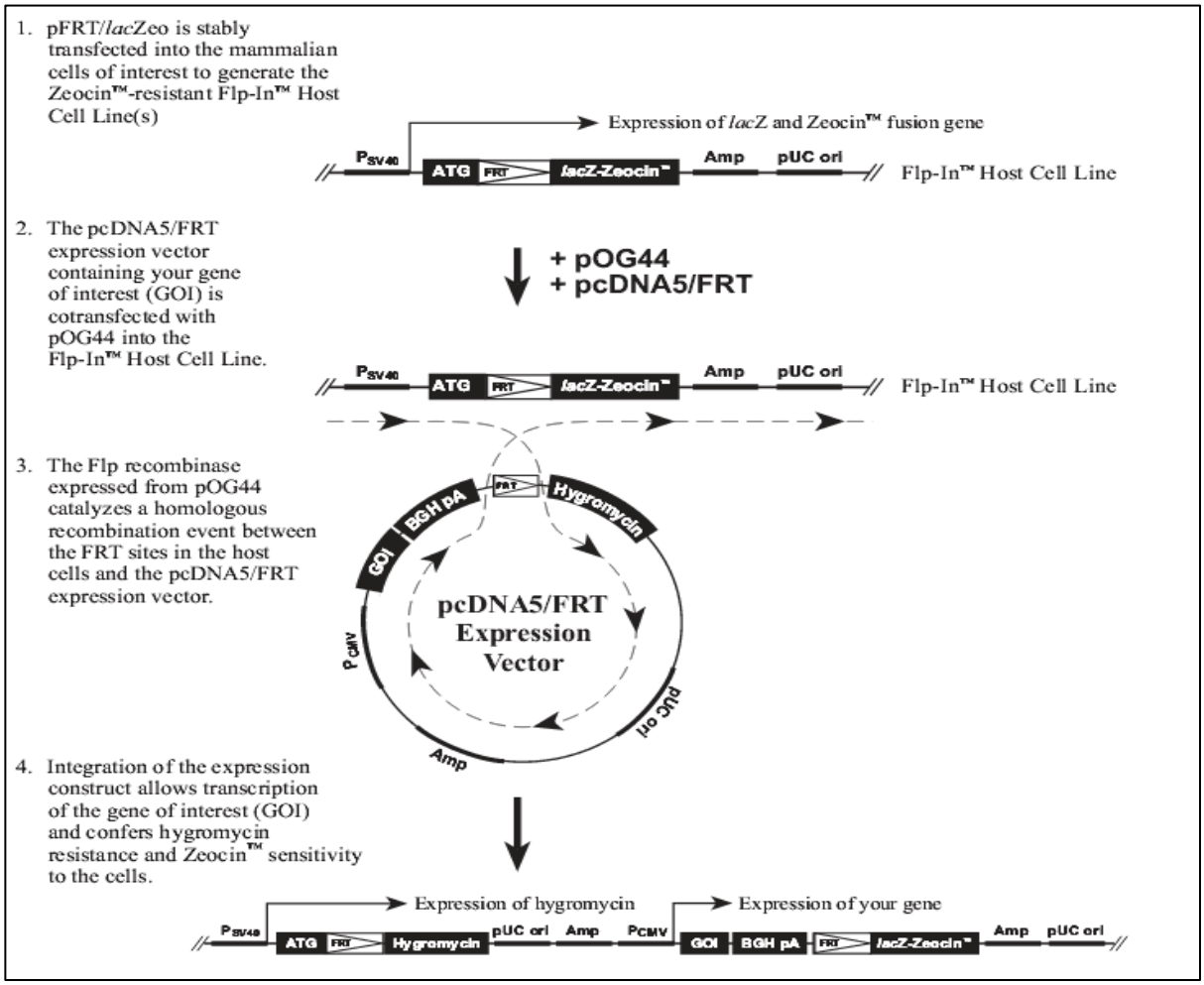


Figure 6–2: Diagram of the Flp-In™ T-REx™ System. The transfection process and the expression of the gene of interest are explained in the main text. (Adapted from Flp-In™ T-REx™ Core Kit for Generating Stable, Inducible Mammalian Expression Cell Lines by Flp Recombinase-Mediated Integration, Invetrogen. Available at http://tools.lifetechnologies.com/content/sfs/manuals/flpintrex_man.pdf

6.2 Results

6.2.1 HEK293 cells are homozygote for the proline at position 962

To find out whether HEK293 cells are homozygote for the alanine or the proline, or contain both alleles, the section of the Per1 gene was amplified using the primers (*Per1-A962-F+Per1-A962-R*), and the PCR fragment was sequenced. The sequence confirmed that HEK293 cells are homozygote for proline at position 962 (Appendix 6).

6.2.2 Cloning of the *hPER1* cDNA into the mammalian pcDNA5/FRT/TO vector

The PER1-001 full-length cDNA encoding for the 1290aa. Per1 protein was amplified from the hPER1 cDNA, inserted at the SalI-NotI sites of the *pBluescript SK (+)* plasmid (a kind gift from Dr.Takahiro Nagase of Japan), using the *Per1-001-pcDNA5-F-Not*I and *Per1-001-pcDNA5-R-Not*I primers (Figure 6-3).

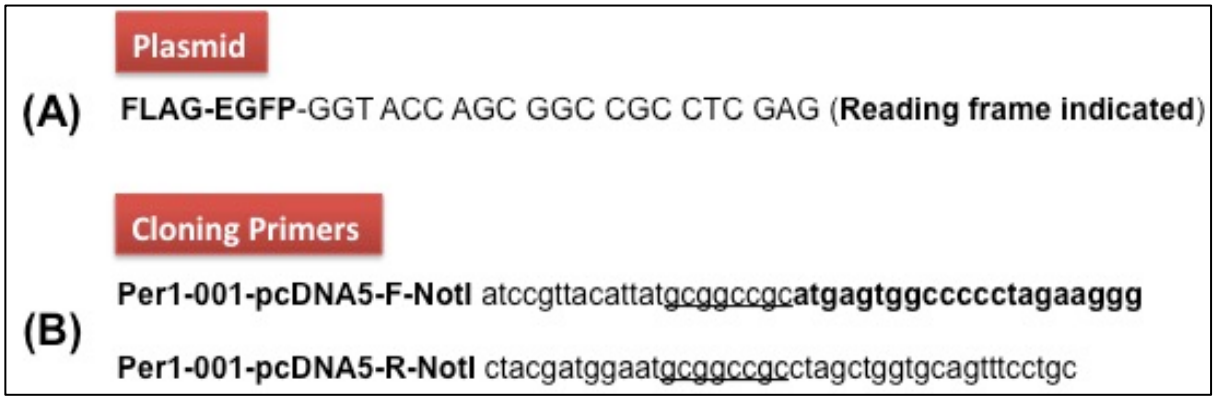


Figure 6–3: pcDNA5 cloning site and primers sequence. A: Multiple cloning site of the plasmid *pcDNA5*. B: Sequences of the cloning primers. The NotI site is underlined.

The amplified fragment was about 4663bp (Figure 6-4). The purified PCR fragment was digested with the restriction enzyme *NotI*; the purified insert was separated on an agarose gel and cloned onto the NotI-digested *pcDNA5/FRT/TO* vector (a kind gift from Dr. Fumiko Isashi, Oxford). The cut vector (5857bp) was dephosphorylated with TSAP (Thermosensitive Alkaline Phosphatase) to prevent relegation. After overnight ligation

at 16°C, the recombinant plasmid was transformed into competent *E.coli* TOP10 cells. The *EGFP-hPER1-001* plasmid was isolated from *E.coli* and re-cut with the *NotI* restriction enzyme. This confirmed the correct integration of the *Per1-001* cDNA (4663bp) into the integration plasmid (5857bp), (Figure 6-4). The plasmid was sent for DNA sequencing; the sequence is shown in Appendix 7. See Figure 6-5 for the cloning process and primers were used to check the integration.

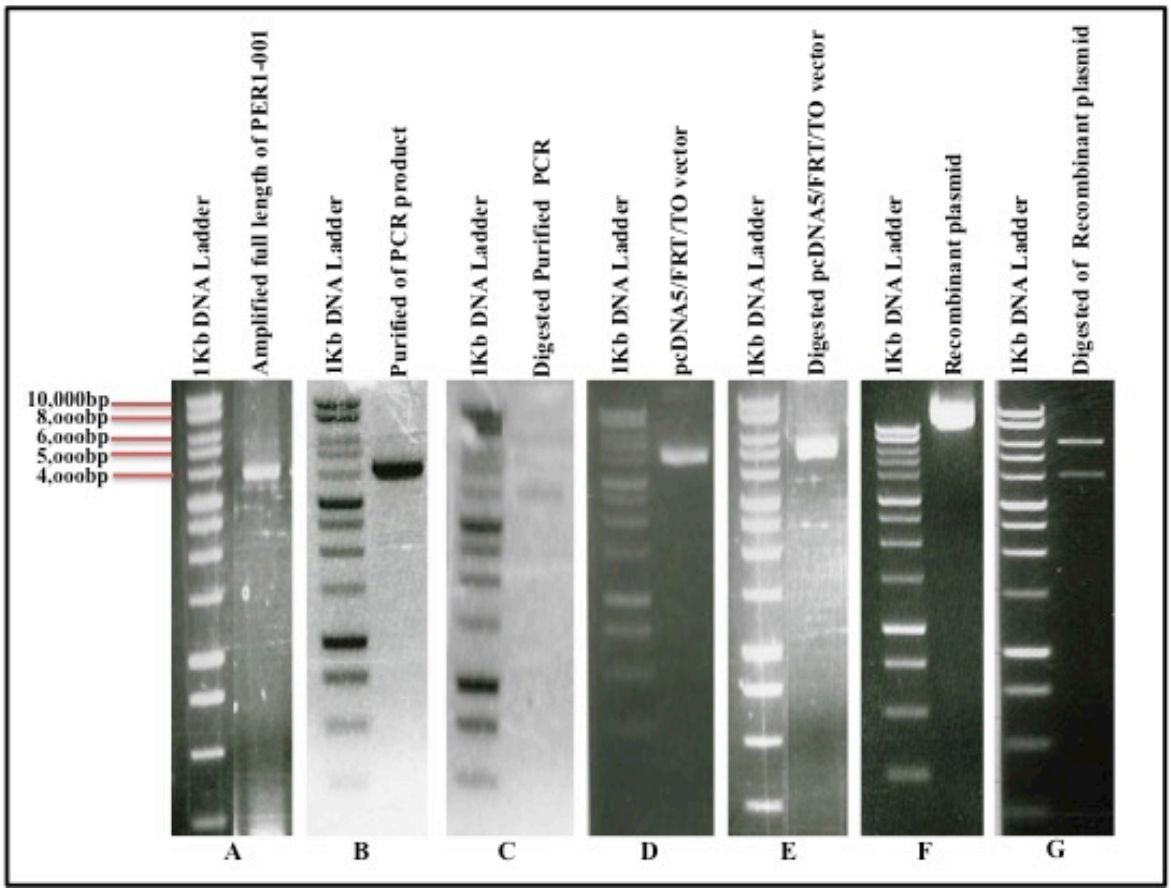


Figure 6–4: Generation of a *PER1-001* cDNA clone in the *pcDNA5/FRT/TO* vector. 1% agarose gels stained with ethidium bromide. (A) Shows the full-length *hPER1-001* gene after amplification by PCR. (B) The PCR product was purified from the agarose gel. (C) The NotI-digested purified PCR product. (D-E) The undigested and NotI-digested *pcDNA5/FRT/TO* vector. (F) The recombinant plasmid after ligation with the digested *pcDNA5/FRT/TO* vector and the digested *PER1-001* gene. (G) The digestion of the recombinant plasmid (*pcDNA5/FRT/TO+hPER1* gene) by the *Not*I restriction enzyme. Two bands are formed; the upper band is *pcDNA5/FRT/TO* vector (5857bp), while the lower band is the full-length *hPER1* gene of 4663bp.

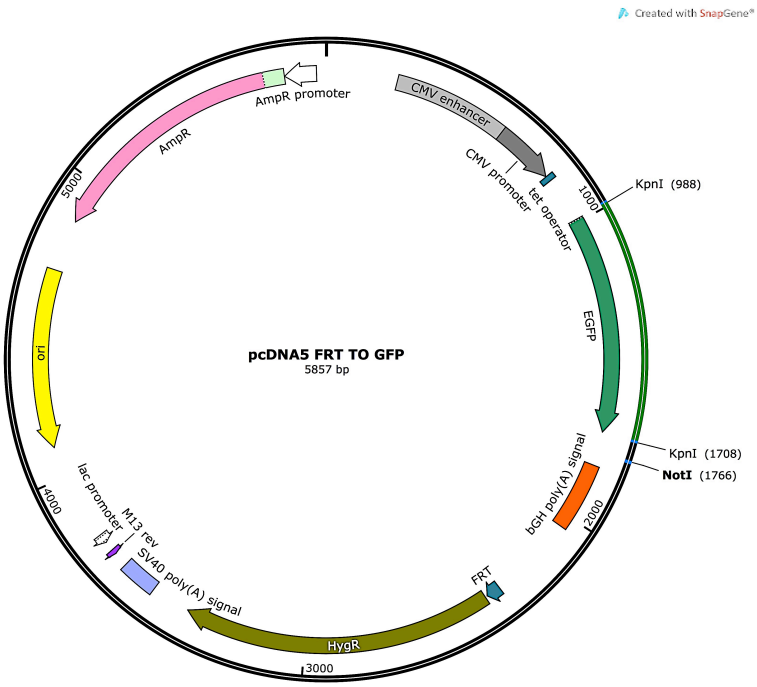
(1)


Created with SnapGene®
SalI (674)
f1 ori
AmpR promoter
AmpR
7000
MCS
1000
ori
6000
pBluescript II SK(+) hPER1-001
7553 bp
2000
MCS
(5330) NotI
5000
hPER1-001
3000
4000

(2)


Created with SnapGene®
(16) NotI
(1 .. 43) Per1-001-pcDNA5-F-NotI
(0) Start
(3915) End
(3897) NotI
Per1-001-pcDNA5-R-NotI (3875 .. 3915)
1000
2000
3000
hPER1-001

(3)


Created with SnapGene®
AmpR promoter
CMV enhancer
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pcDNA5 FRT TO GFP
5857 bp
KpnI (1708)
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SV40 poly(A) signal
FRT
HygR
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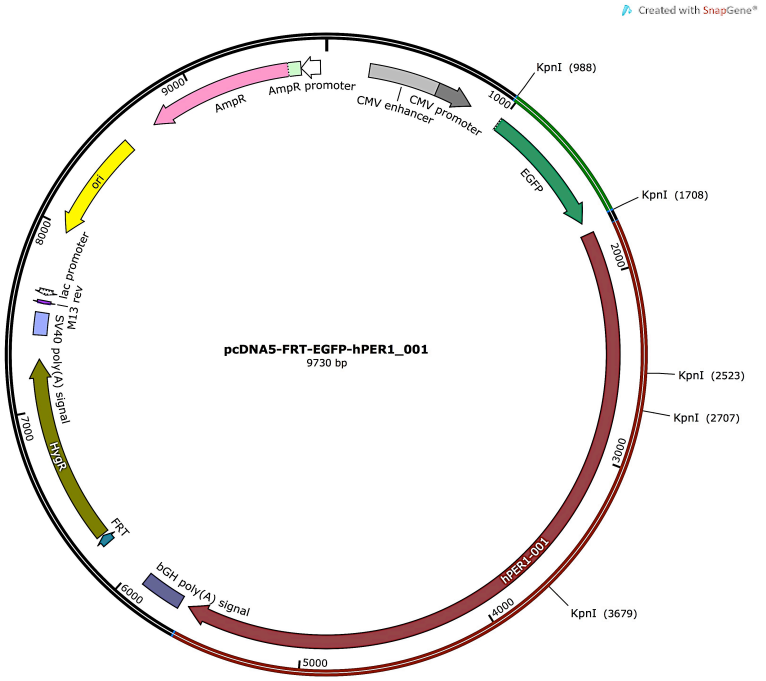

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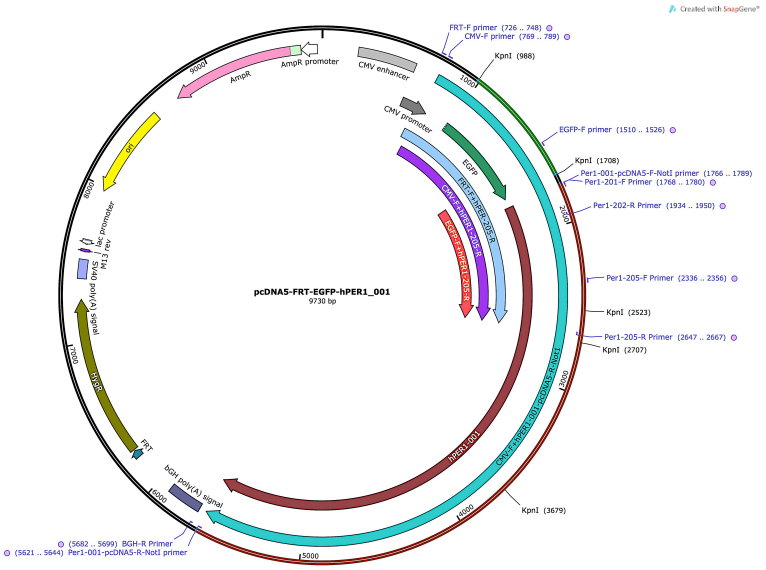

Figure 6–5: The Cloning process and primers used to clone hPer1-001. (1) The *hPER1* cDNA fragment was inserted at the SalI-NotI site of the pBluescript SK (+) (a kind gift from Dr.Takahiro Nagase of Japan). (2) Amplification of the PER1 cDNA using the primers *Per1-001-pcDNA5-F-NotI* and *Per1-001-pcDNA5-R-NotI*. The purified PCR insert was digested with the *Not*I restriction enzyme. (3) pCDNA5/FRT/TO/GFP (Kind gift from Dr. Fumiko Isashi) was digested with *Not*I restriction enzyme and dephosphorylated to stop religation. (4) The digested, purified PCR insert and the digested vectors were ligated. (5) The primers (*FRT-F*, CMV-*F*, and *EGFP-F* and *PER1-205-R*) were used to check the integration, as described in the main text.

6.2.3 Construction of the ancestral G allele (GCC – Alanine 962) by site directed mutagenesis

The plasmid containing the *PER1-001* gene was sent for sequencing, and the sequence of the EGFP-hPER1-001 was confirmed (reference sequence NM_002616). Interestingly, the cloned *hPER1-001* gene showed two different bases from the reference sequence (Figure 6-6). One of the two differences was an A/G mutation at nucleotide 2361: silent mutation changing an **ACG** to an **ACA** codon, which both encode threonine-787. Interestingly, this is the synonymous SNP rs2253820 (AC**A**/AC**G**). The population frequencies for this silent SNP are shown in Figure 6-7. The second difference was the SNP rs2585405 (A962P; **G**CC/**C**CC), which is the subject of this study. To generate the corresponding A962 clone the recombinant pcDNA5-Per1-001-P962, the plasmid was mutated using the Q5 Site-Directed Mutagenesis Kit. The polymerase chain reaction successfully converted the SNP from **CCC**-Proline to the ancestral **GCC**-Alanine codon, using the repairing primer (*Per1-P962A-F TGCCCGCCCTC**G**CCCCGAGTCCTC*+*Per1-P962-R AGGATGGAGAAGGGGAGTG*) (Figure 6-8A). The recombinant plasmid was transformed into competent *E.coli* TOP10. The *EGFP-hPER1-001-A962* plasmid was isolated from the *E.coli* and then cut it with *Not*I restriction enzyme, to confirm the presence of the insert (Figure 6-8B). The replacement was confirmed by the sequencing analysis.

Figure 6–6: The Alignment of the cloned Per1-001 sequence with the reference Ensemble sequence. (A) Position of synonymous SNP rs2253820 (ACA/ACG; T787T). (B, C) Position in the coding sequence of the non-silent SNP-rs2585405 (GCC/CCC; A962P).

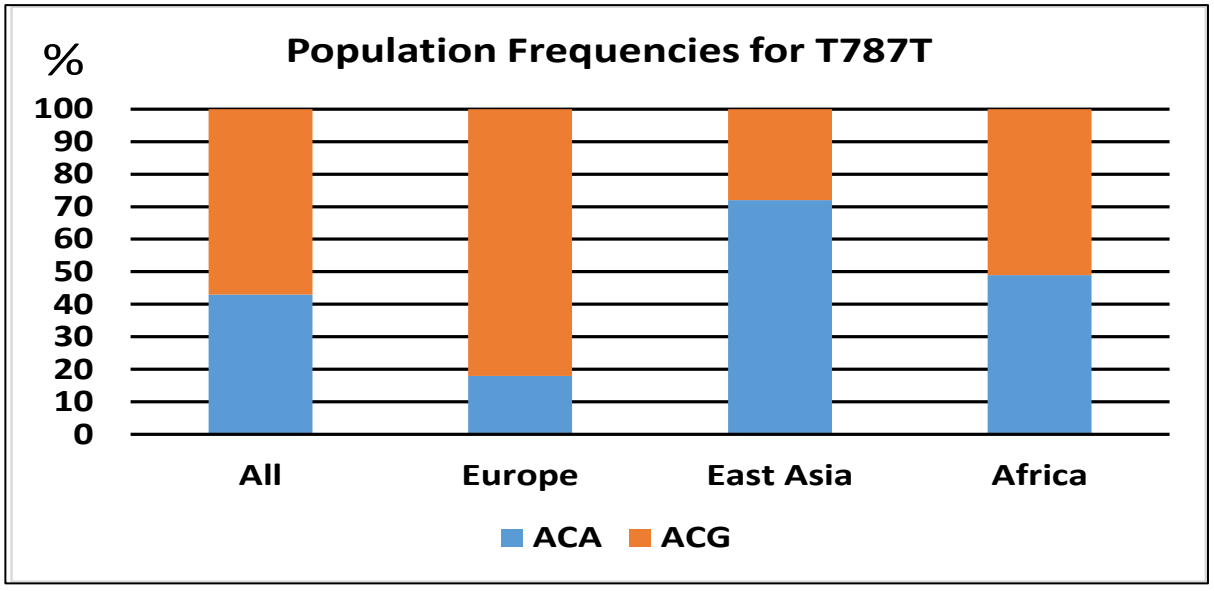

Figure 6–7: Population frequencies for the synonymous SNP rs2253820 (ACA/ACG).

https://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=17:8144351-8145351;v=rs2253820;vdb=variation;vf=362576541

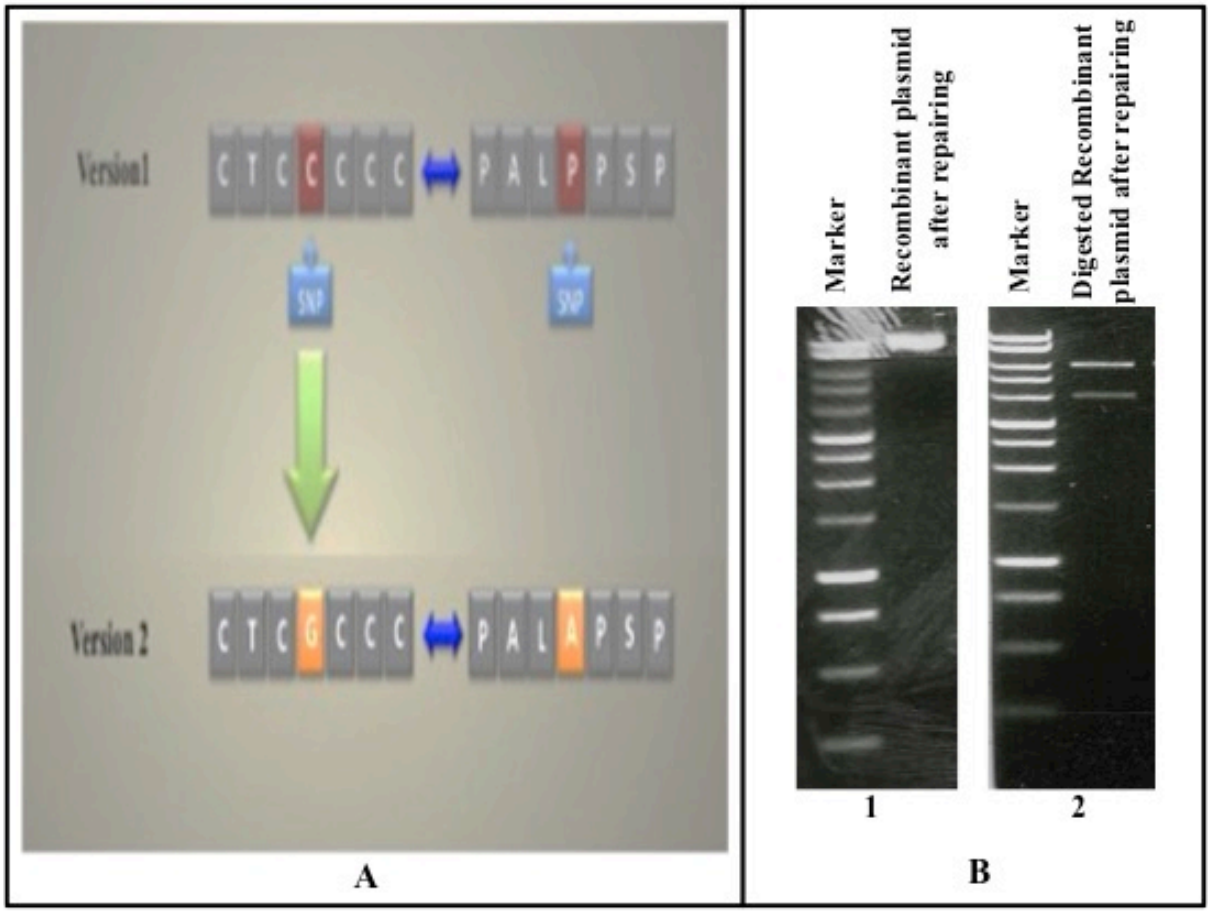

Figure 6–8: The Non-silent SNP-rs2585405 (GCC/CCC; A962P). (A) Version1: the hPer1-cDNA with the SNP C allele-Proline; Version2: The ancestral G allele-Alanine. (B.1) 1% Agarose gel picture showing the PCR product of the recombinant, uncut plasmid after the site-directed mutagenesis of the SNP from C to G. (B.2) The digestion of the recombinant plasmid (*pcDNA5/FRT/TO+hPER1-001*) by *NotI*. Two bands are formed; the upper band is *pcDNA5/FRT/TO* (5857bp), while the lower band is the full-length *hPER1* gene (4663bp).

6.2.4 Integration of the pcDNA5/FRT/TO plasmid containing PER1-001-P and PER1-001-A into the Flp-In™ T- REx™-293 cell line

To establish the stable cell lines, the Flp-In™ T-REx™-293 cells were transfected with the *pcDNA5/FRT/TO* plasmids that carried the constructed genes, and with pOG44 that encodes the Recombinase enzyme at a ratio of 9:1 (pOG44: pcDNA5/FRT/TO). A negative control was set up, via transfection of the Flp-In T-REx-293 cell line with pOG44 (which contais a hygromycin-resistance cassette) and pcDNA5/FRT/TO alone, without the gene of interest. The desired cells should be resistant to hygromycin, but sensitive to Zeocin. As expected, the majority of the cells did not survive due to the presence of hygromycin, which was used for the selection of the stable cell lines which contained the gene of interest. Individual hygromycin resistant clones were selected for each construct.

6.2.5 Analysis of the recombinant Flp-In™ T-REx™-293 cell lines

The integration of the pcDNA5/FRT/TO construct containing hPER1-001-P or hPER1-001-A into the chromosomal *FRT* site was evaluated by PCR screening, using genomic DNA as a template. The genomic DNA was extracted from the transfected Flp-In™ T-REx™-293 cells. Correct PCR products were found in all transfected cells with integration of the *pcDNA5/FRT/TO:: hPER1-001-P* and *pcDNA5/FRT/TO:: hPER1-001-A* plasmids, or the empty *pcDNA5/FRT/TO* plasmid as a control .This was done using the primers *FRT-F*, *CMV-F*, and *EGFP-F,* which bind in the up-stream regions of the integrated plasmids, and the Per1-specific primer *PER1-205-R* (Figure 6-9). The results of PCR screening suggested that the different fragments of the *hPER1-001-P* and *hPER1-001-A* genes were correctly integrated into the genomic DNA of the Flp-In™ T-REx™-293 cells. The integration of hPER1-GFP-001-Proline, hPER1-GFP-001-Alanine, and GFP (empty plasmid-only encoding GFP) into the Flp-In™ T-REx™-293 cell line was followed up by the extraction of the whole cell proteins. The extracts were analyzed by 6% SDS-PAGE and Western Blot. When the integrated PER1-001-GFP-Proline, PER1-001-GFP-Alanine, and GFP (control) proteins were overexpressed by (2µg/ml) of Doxycycline, the PVDF membranes were probed with either a monoclonal anti-GFP antibody or polyclonal anti-PER1 antibody. The extracts, which were probed with the anti-GFP antibody, showed a specific band for hPER1-001-GFP-Proline and hPER1-001-GFP-

Alanine protein of around 166KDa (136KDa for PER1+30KDa for GFP), and a small band of around 30KD for the integrated GFP control plasmid (Figure 6-10A). On the other hand, when the same extracts were probed with anti-PER1 antibody, I noticed a strong band in the molecular weight range of ~166KD, for both the recombinant proteins and the endogenous hPER1 protein (at around 136KD in both). Meanwhile, only endogenous hPER1 was recognized in the GFP cells. The GAPDH was used as a loading control (Figure 6-10B). Interestingly, induction of the EGFP-tagged, engineered Per1 proteins led also to an increase in the two endogenous Per1 bands. The latter could be explained by the ability of Per1 to form homodimers via their PAS domains, which may protect Per1 from degradation. It also indicates that both high molecular weight bands in normal cell lines are Per1. Which post-translational modification leads to the significant size difference is not yet known.

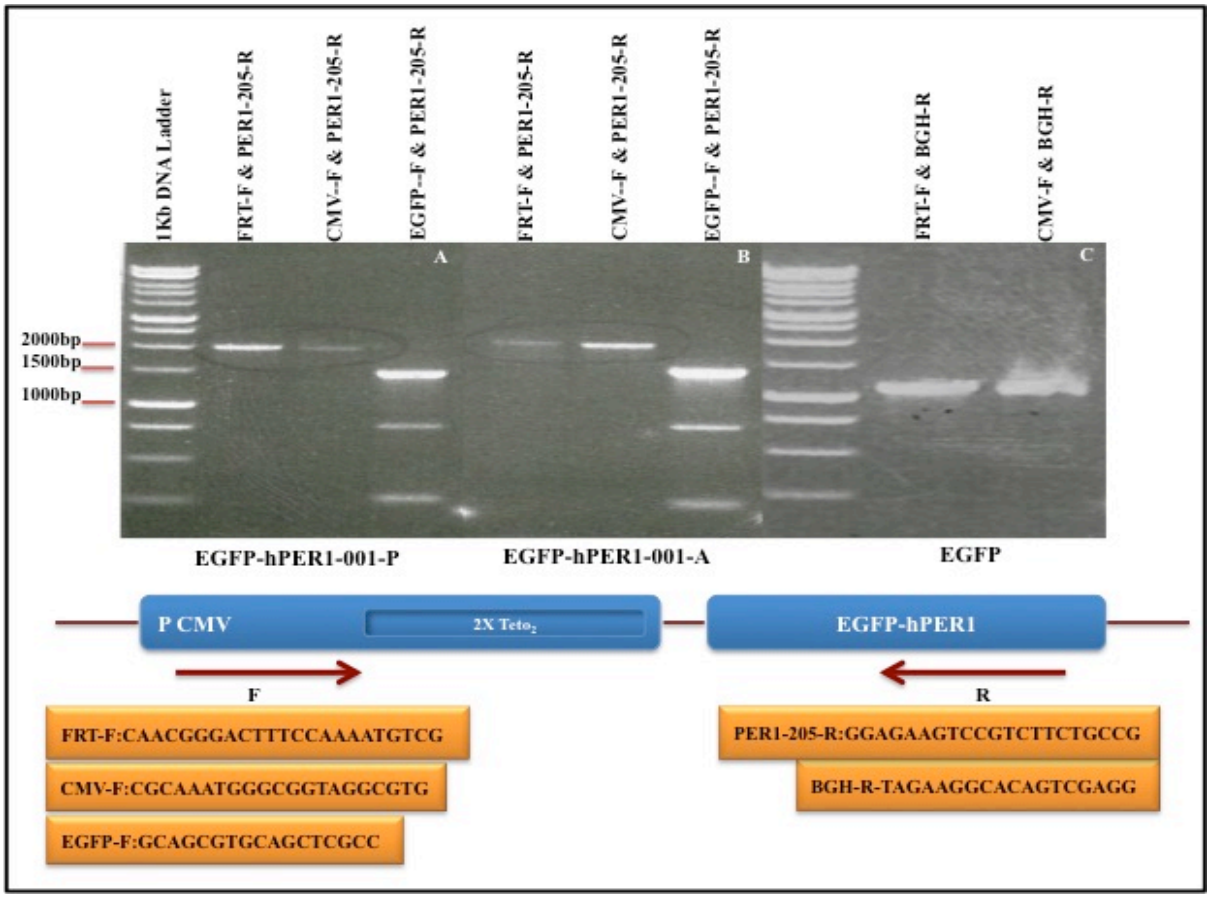

Figure 6–9: Confirmation of the integration of EGFP-PER1-P, EGFP-PER1-A, and EGFP into the cell lines. The confirmation was carried out using genomic PCR with the indicated primers.

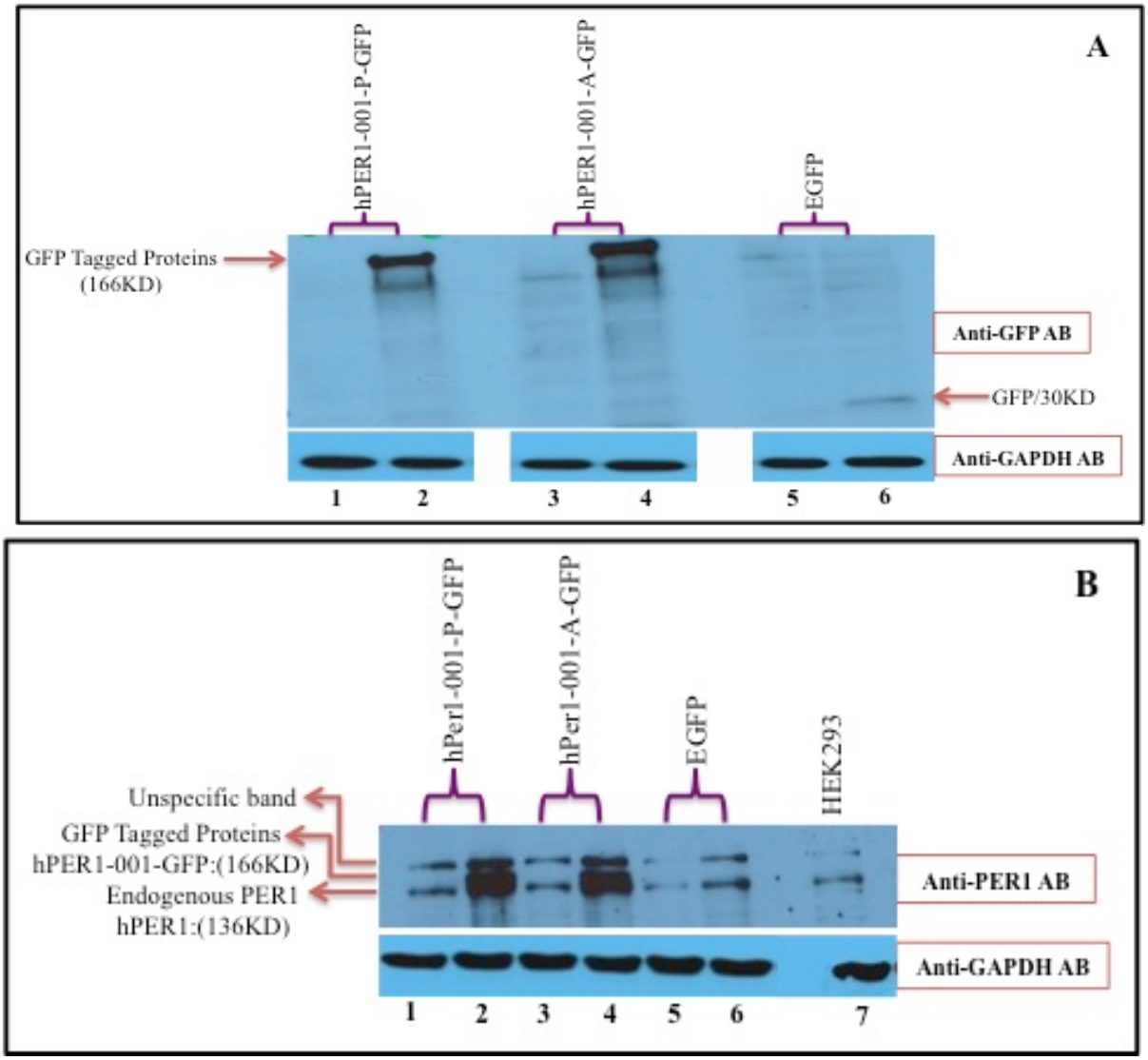

Figure 6–10: Whole cell extracts of the EGFP-PER1-P, EGFP-PER1-A and EGFP cell lines. The proteins were extracted after 24hrs post induction with (2µg/ml) of Doxycycline (lane2, 4 and 6). The lysates were separated on a 6% SDS-PAGE gel. (A) The membrane was probed with the monoclonal anti-GFP antibody. Lanes (2&4) show the engineered EGFP-Per1 proteins, whereas lane 6 shows on the GFP signal. (B) Western blot membranes were probed with the polyclonal anti-PER1 antibody. Lanes (2&4) show both the engineered and endogenous hPER1, whereas only the endogenous Per1 (double band) was present in the absence of doxycycline lanes (1&3) or in the control cell lines. Interestingly, induction of the EGFP-tagged, engineered Per1 proteins led as well to an increase in the endogenous Per1 bands.

6.2.6 Stability of the hPER1-Proline and hPER1-Alanine tagged -EGFP

I started with a time course experiment of Doxycycline induction, to determine when the recombinant proteins are fully induced (Figure 6-11). I induced the cells for 2hrs, 4hrs, 6hrs, 24hrs, and 48hrs with 2µg/ml of Doxycycline. The protein extracts were taken at each indicated time. Expression of the recombinant proteins started after 4hrs, reaching its peak after 24-hours.

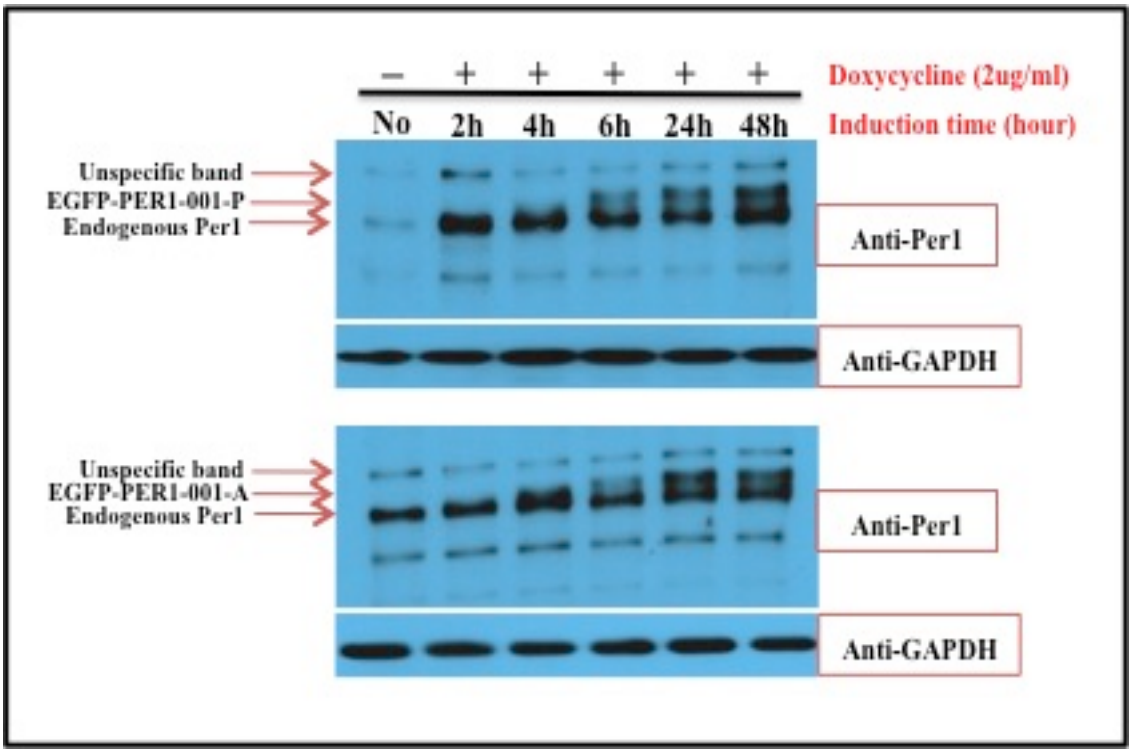

Figure 6–11: Doxycycline time course induction of hPER1-GFP-P and hPER1-GFP-A. Cells were induced with (2µg/ml) of Doxycycline for the indicated times. By using anti-Per1 antibody (Thermo scientific/PA1-524), the upper arrow indicates the engineered EGFP-PER1, while the lower arrow indicates the endogenous PER1. This time course induction experiment shows that the detectable protein expression started after 4-hours, and continued to increase throughout the experiment.

6.2.7 Cell fractionation of hPER1-GFP-Proline and hPER1-GFP-Alanine after UV treatment

To find out in which cellular compartment Per1 resides, and whether this was affected by the Alanine or Proline at position 962, cell extracts were fractionated to five different fractions using a Thermo Scientific, 78840 kit. Figure 6-12 shows that the endogenous and the engineered Per1 proteins resided in the cytoplasmic fraction, which also contained the cytoplasmic marker GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). Some Per1 protein was also associated with the membrane fraction and the soluble nuclear fraction, but so was GAPDH, which indicates that both associations are contaminated by the cytoplasmic protein. To test whether UV-induced DNA damage would affect this pattern, hPER1-GFP-P and hPER1-GFP-A cells were induced with 2µg/ml doxycycline overnight. They were then exposed to UV light at 40J/m^2 and left to recover for 30min, 1hr, 2hrs, 4hrs and 24hours at 37°C. Cell extracts were fractionated into 5 fractions after each indicated time, and analyzed by SDS-PAGE and Western blot using the anti-PER1 antibody (Figure 6-12). UV-induced DNA damage had no impact on the fractionation pattern, but I noticed that both recombinant Per1 proteins decreased in abundance when extracts were prepared after 2-hours of recovery. After 24-hours, the recombinant proteins were no longer detectable, although doxycycline was present throughout the experiment.

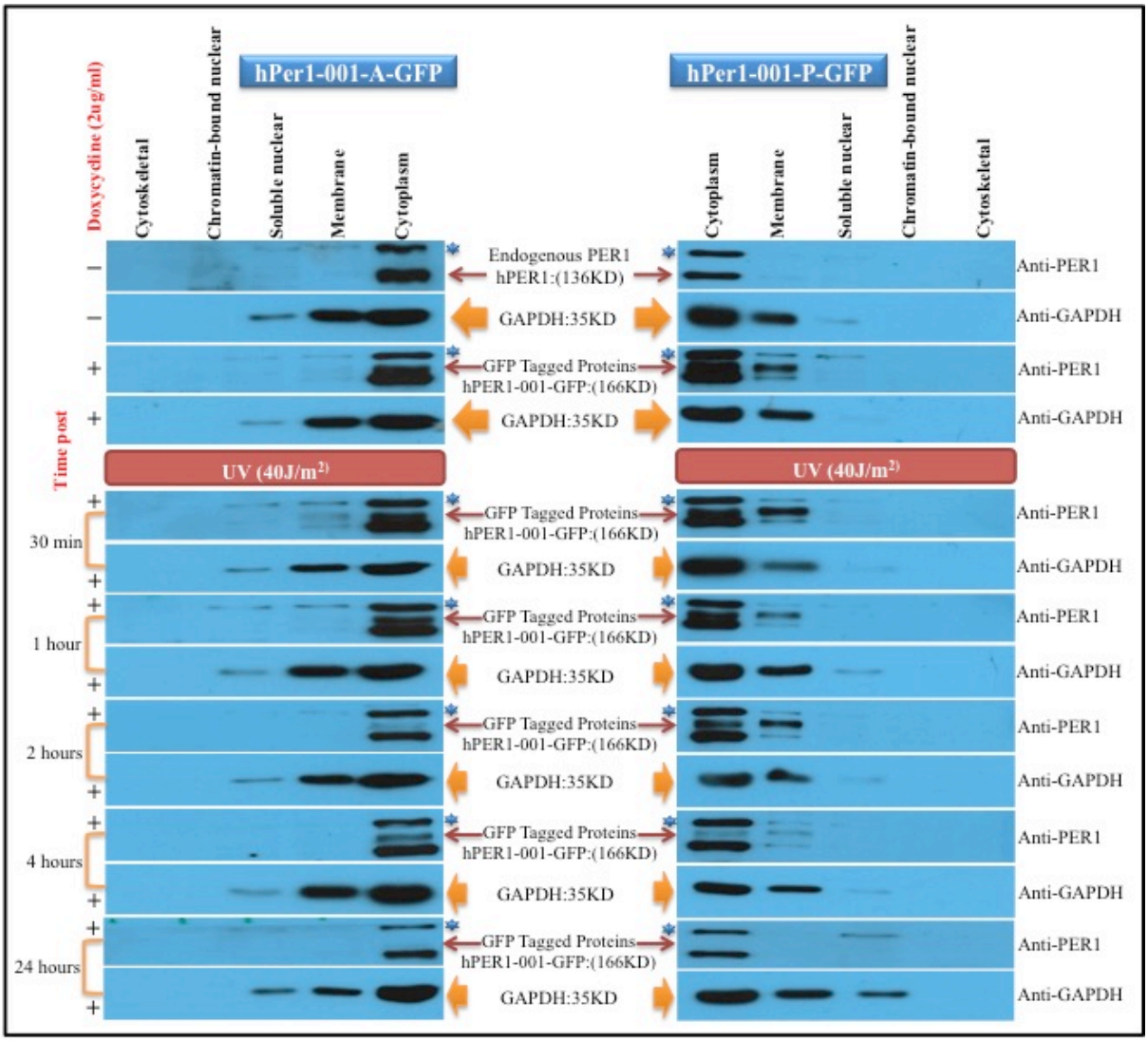

Figure 6–12: Cell fractionation after UV treatment of EGFP tagged hPER1-001-P and hPER1-001-A cell lines. Cells were induced with 2µg/ml of Doxycycline for overnight, exposed to 40 J/m^2 of UV light, and then left to recover for the indicated times at 37°C. Protein extracts were prepared at the end of each recovery time and subjected to the cell fractionation protocol. Western blot membranes were probed with the polyclonal anti-PER1 antibody (Thermo scientific/PA1-524). Western blot results showed that both recombinant proteins and the endogenous hPER1 proteins were located in the cytoplasmic fraction. Please note that the abundance of the recombinant EGFP-PER1 proteins decreased with an increase in recovery time, starting after 2-hours. The GAPDH was used as a control for the cytoplasmic fraction. (*) Unspecific band.

6.3 Discussion

The main findings of this chapter are (i) the successful generation of two stable HEK293 cell lines, expressing EGFP-PER1-A962 or EGFP-PER1-P962: (ii) a probable cytoplasmic localization of Per1, independently of the amino acid, at position 962: and (iii) the degradation of the EGFP-PER1 proteins after UV treatment.

The cytoplasmic localization of human Per1 is consistent with the predominantly cytoplasmic localization of mouse Per1. In approximately 45% of cells, Per1 is cytoplasmic, in around 45%; the protein is in the cytoplasm and the nucleus, whereas in just 10% of the cells Per1 is only in the nucleus (Yang et al., 2014). Per1 is known to shuttle between the nucleus and the cytoplasm, in a manner regulated by its phosphorylation by Casein kinase 1-epsilon and its ubiquitinylation (Yang et al., 2014). It could well be that, during the cell fractionation process, the soluble nuclear Per1 protein fractionates to the cytoplasmic fraction when the integrity of the nucleus is affected.

The UV-induced decline in the abundance of the recombinant Per1 protein is interesting, since the recombinant proteins are continuously induced in the absence of UV light as long as doxycycline is present (Figures 6-12). Since this does not seem to affect the endogenous Per1 proteins, the latter could be due to the EGFP fusion section of the engineered proteins.

Expression of recombinant proteins or the shutdown of continuous protein expression Via T-REx™ System, involves plasmid activation by adding Doxycycline to the culture medium. The current study observed low levels of recombinant proteins after 2h to 24h, which might be the consequence of degradation of Doxycycline upon prolonged exposure to UV light. Indeed, some studies have demonstrated that even though degradation of Doxycycline by UV light is minimal, degradation may build up over time (Naveed et al., 2014, Yuan et al., 2011). Notably, a study reported complete degradation of doxycycline by exposure to UV-C light (Bolobajev, Trapido & Goi, 2016). Thus, in the work reported here, doxycycline might have been degraded upon UV treatment, which would explain the loss of recombinant protein expression.

Chapter 7: The role of the Single Nucleotide Polymorphism A962P in the response to genotoxic stress

7.1 Introduction

The work presented in this chapter aims to find out whether there is a difference in the response to the activation of the ATR-Chk1 and ATM-Chk2 pathways, dependent on whether HEK293 cells express EGFP-PER1-Alanine-962 or EGFP-PER1-Proline-962. ATM is recruited by the MRE11-RAD50-NBS1 (MRN) sensor complex to double-strand DNA breaks, where it phosphorylates Chk2 at threonine-68 (Lee & Paull, 2005). Meanwhile, ATR is activated by its recruitment to single-stranded DNA (ssDNA) by its partner protein ATRIP, where it phosphorylates Chk1 at serine-345 (Dart et al., 2004, Ahn et al., 2000)(Figure 7-1).

In this study, I have used 3 different cell lines: HEK293 cells expressing the ancestral alanine at position 962 of EGFP-PER1; cells expressing the mutated proline at position 962 of EGFP-PER1; and control cells whose only express DNA damage has been inflicted by UV light, a strong activator of ATR-Chk1 signaling (Heffernan et al., 2002), and by topoisomerase 1 poison camptothecin (CPT), which causes replication fork breakage in S phase and thereby activates ATM-Chk2 signaling (Sordet et al., 2009). Using phospho-specific antibodies, the modification of Chk1 at Ser-345 and Chk2 at Thr-68 were analyzed. Based on the work by Gery at al (2006), which reported the association of Per1 with the ATM-Chk2 pathway, I expected to see an impact on Chk2 phosphorylation upon expression of the engineered EGFP-PER1 proteins.

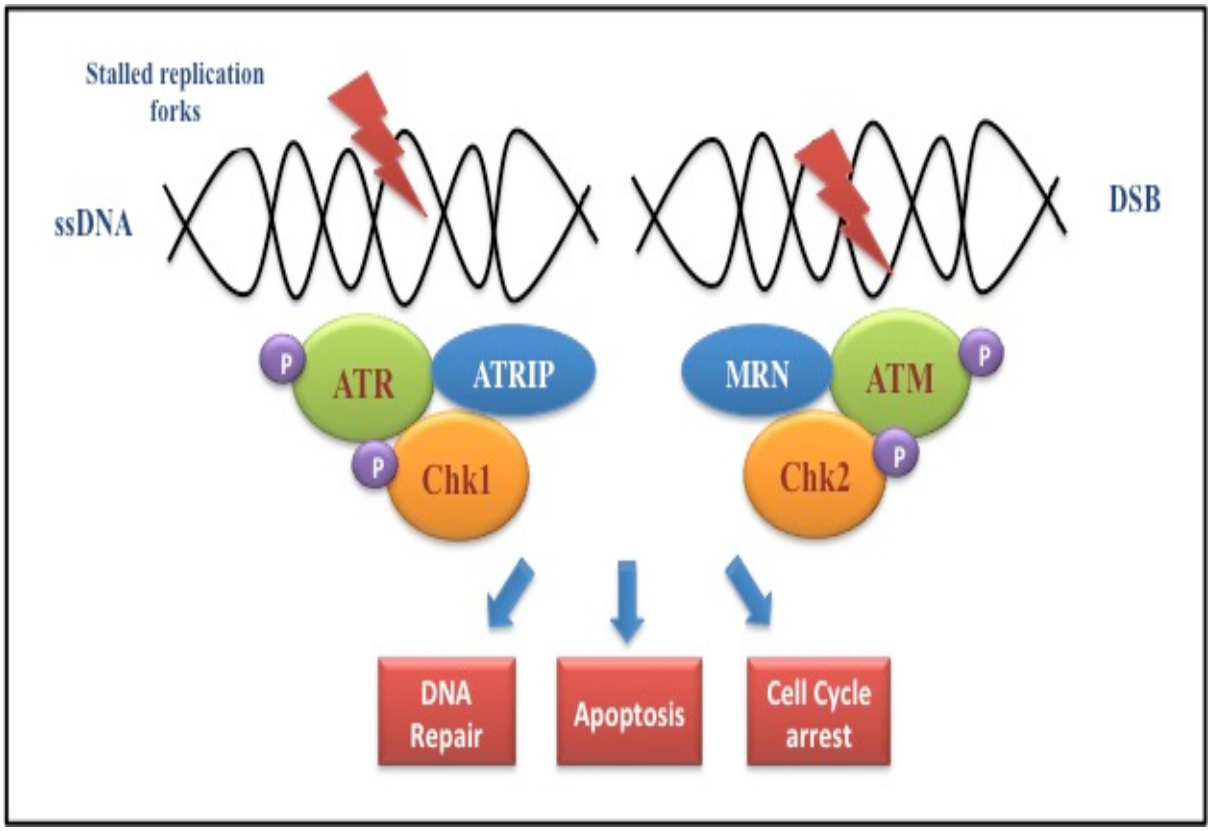

Figure 7–1: Activation of the ATM-Chk2 and ATR-Chk1 pathways. The ATM-Chk2 and ATR-Chk1 pathways are activated selectively by DSBs and ssDNA, respectively. Chk1 and Chk2 are selectively phosphorylated and activated by ATR and ATM, respectively, to trigger a wide range of distinct downstream responses.

7.2 Results and Discussion

7.2.1 Differences in the expression levels of the checkpoint signaling pathways with DNA damage response, upon UV treatment between the ancestral and mutated recombinant proteins

I tested the effect of the ancestral-hPER1 (Alanine-962) and the mutated-hPER1 (Proline-962) overexpression by subjecting cells to UV light, followed by an analysis of the ATM-Chk2 and the ATR-Chk1 pathways. The UV-induced degradation of p21 was used to confirm the efficiency of the treatment. Expression of EGFP-hPER1-001-Proline-962, EGFP-hPER1-001-Alanine-962, and GFP was induced overnight with 2μg/ml Doxycycline. Cells were then exposed to UV light at 40J/m^2, and left to recover at 37°C for 30min, 1hr, 2hrs, 4hrs, and 24hrs. Protein extracts were prepared at the end of each recovery time. It is important to point out that my cell lines express the normal endogenous Per1 plus the engineered Per1 protein. In all three-cell lines, the DNA damage signal was very strong, as apparent by the degradation of the p21 protein, which was used as a control for the UV treatment (Figures 7-2, 7-3, 7-4). Interestingly, both engineered Per1 proteins (ALANINE and PROLINE) seemed to disappear after 4-hours post-treatment and were undetectable after 24hours, although doxycycline was present.

This set of experiments has revealed various interesting observations. Firs, the phosphorylation of Chk2 at T68 by ATM increased throughout the recovery time, while the abundance of both GFP-tagged Per1 proteins declined. Second, the abundance of ATM declined at the 4h and 24h time points correlating with a transient drop in Chk2 abundance at the 4h time point. Both events were independent of the amino acid at position 962. Third, the levels of Chk1 phosphorylation at S345 were reduced throughout the experiment, specifically in the EGFP-PER1-P962 cell line (Figure 7-2). It is not yet clear why Chk2 levels dropped at 4hours post-treatment, but it happened in both experiments at the same time. It is likewise unclear why cells degrade the EGFP-PER1 fusion proteins after UV treatment, as the degradation is not affecting the endogenous Per1 protein. What is, however, noteworthy is the reduced phosphorylation of Chk1 at S345 in cells expressing EGFP-PER1-P962, since this could suggest a negative impact of the proline at position 962 on Chk1 activation.

The experiments were repeated with the control cell line, which only expresses GFP. As shown in Figure 7-4, Chk1 phosphorylation at Ser-345 was as strong as in the EGFP-PER1-A962 cell line, and Chk2 levels did not decline 4-hours post-treatment. Taken together, this implies that the presence of the recombinant Per1 proteins is linked with the transient decline in Chk2 protein levels, and that the proline at position 962 may interfere with the phosphorylation of Chk1 at Ser-345.

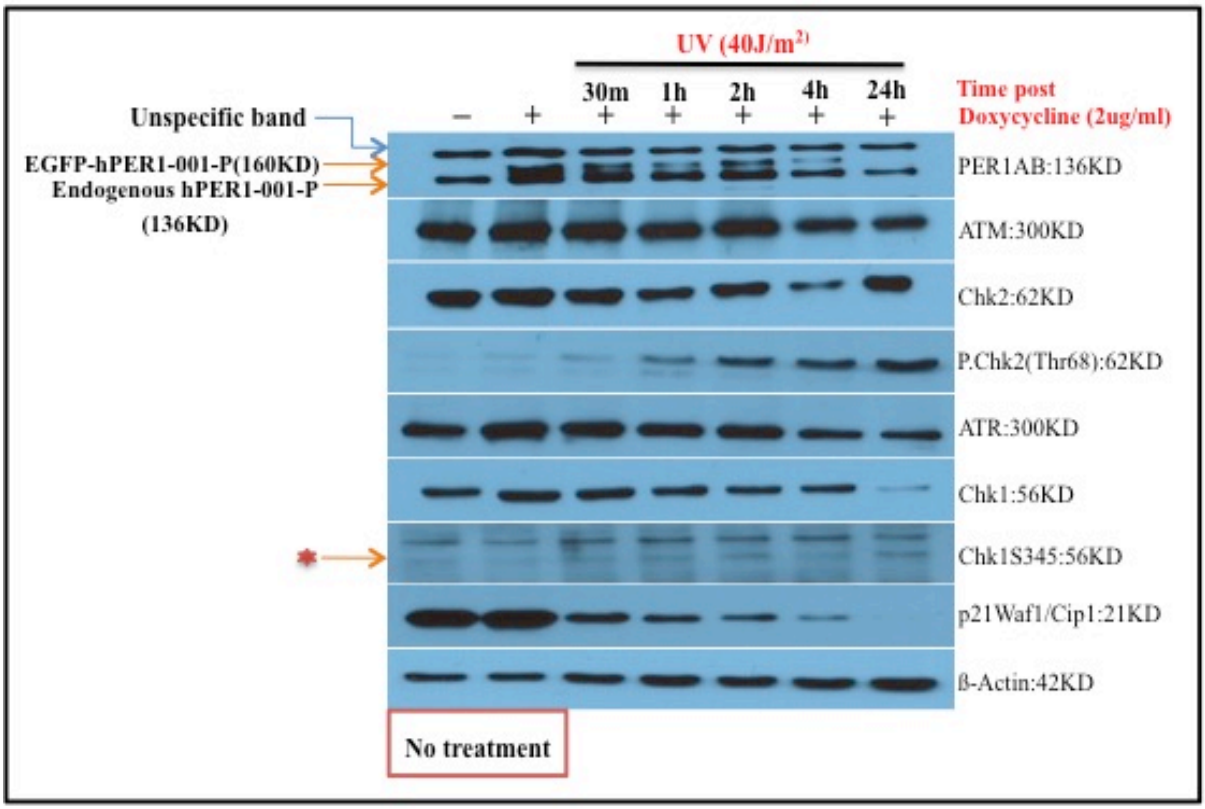

Figure 7–2: UV treatment of EGFP-PER1-001-P962 expressing cells. Cells were induced with 2µg/ml doxycycline overnight, exposed to 40J/m² of UV light, and then left to recover after the exposure at 37°C for 30min, 1hr, 2hrs, 4hrs and 24hrs. Protein extracts were prepared and separated on an 8% SDS-PAGE. The DNA damage signal was very strong, as indicated by the degradation of the p21 WAF1/CIP1 protein that was used as a control for the UV treatment. By using anti-Per1 antibody (Thermo Scientific/PA1-524), EGFP tagged hPER1-001-P962 started to disappear after 4-hours of recovery. The asterisk indicates the phosphorylation of Chk1 at S345. β-Actin was used as a loading control.

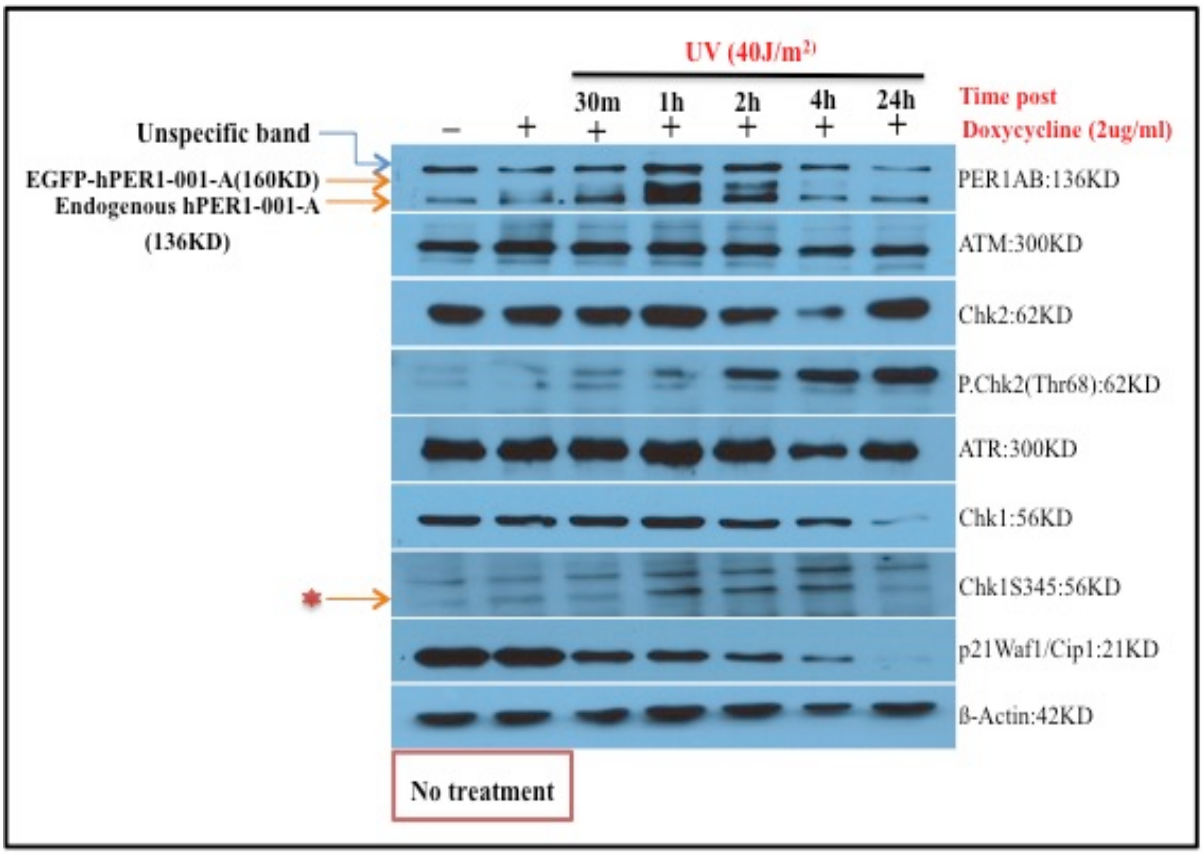

Figure 7–3: UV treatment of EGFP-PER1-001-A962 cells. Expression of the recombinant protein was induced overnight with 2µg/ml doxycycline. Cells were then exposed to 40J/m² of UV light and left to recover at 37°C for 30min, 1hr, 2hrs, 4hrs, and 24hrs. Protein extracts were prepared at the end of each recovery time and separated on an 8% SDS-PAGE. The DNA damage signal was very strong, as indicated by the degradation of the p21 WAF1/CIP1 protein that was used as a control for the UV treatment. Using anti-Per1 antibody (Thermo Scientific/PA1-524) EGFP tagged hPER1-001-A started to disappear 4-hours after the UV treatment. The asterisk indicates phosphorylation of Chk1 at S345 by ATR kinase. Note that the phosphorylation signal is stronger, compared to the EGFP-PER1-P962 cell line. β-Actin was used as a loading control.

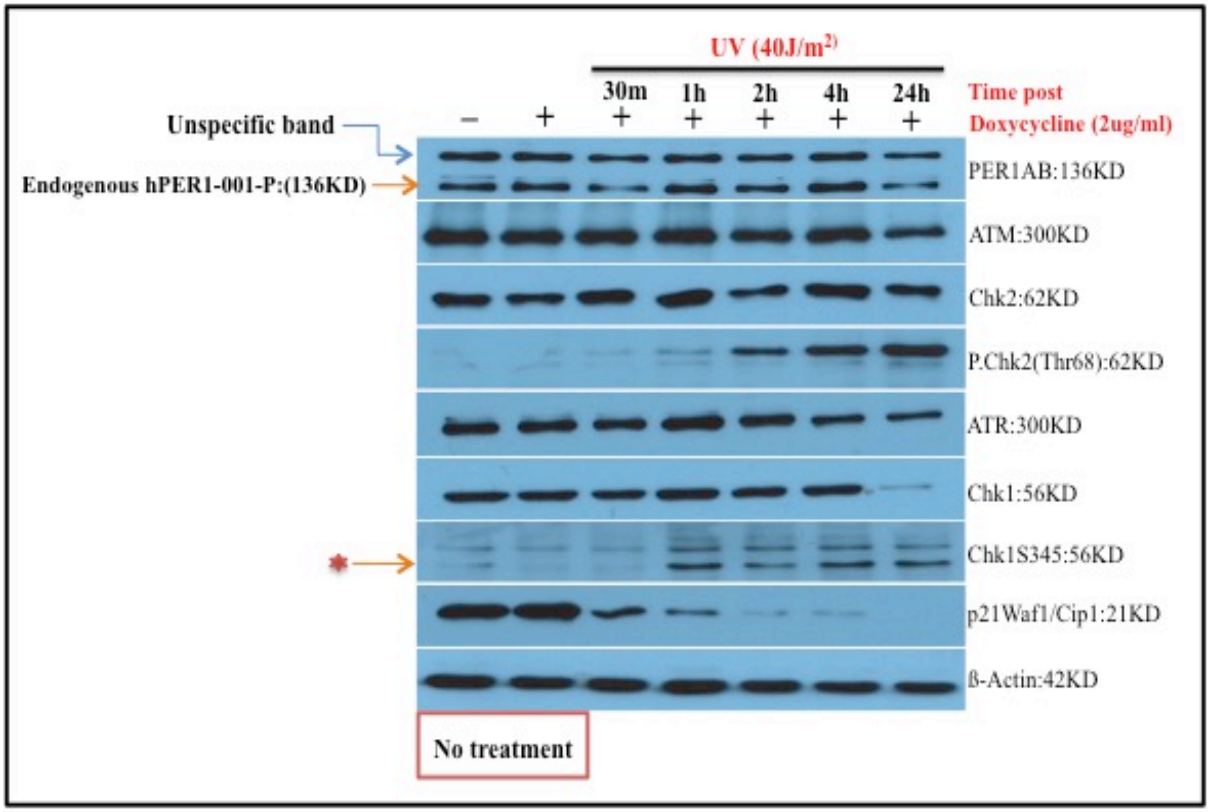

Figure 7–4: UV treatment of the EGFP control cell line. Cells were induced with 2µg/ml doxycycline overnight, exposed to 40J/m² of UV light, and left to recover at 37°C for 30min, 1hr, 2hrs, 4hrs, and 24hrs. Protein extracts were prepared at the end of each recovery time and separated on an 8% SDS-PAGE. The DNA damage signal was very strong, as indicated by the degradation of the p21 WAF1/CIP1 protein that was used as a control for the UV treatment. Please note that Chk2 doesn`t decline at the 4h time point, and the Chk1 phosphorylation level at S345 (asterisk) is high.

7.2.2 Association of EGFP-hPER1-001-Proline with the ssDNA Breaks and EGFP-hPER1-001-Alanin with the DSBs

Camptothecin induces DNA double strand breaks (DSBs), specifically in replicating DNA (Pommier, 2006), which have been revealed to trigger S and G_2 –M arrest (Zhou et al., 2002).

Here, I used CPT to induce DSBs in S phase to compare between EGFP-hPER1-001-Proline and EGFP-hPER1-001-Alanine under DNA double strand breaks. I tested the effect of the ancestral-PER1 (Alanine) and the mutated-PER1 (Proline) cell overexpression by inducing them with (2µg/ml) of Doxycycline for overnight. I then subjected them to 1µM Camptothecin (CPT) and left them to recover at 37°C for (30min, 1hr, 2hrs, 4hrs, and 24hrs), separately. Protein extracts were prepared at the end of each recovery time, followed by analysis by Western blotting for target proteins to assess checkpoint activation. Interestingly, in the case of EGFP-hPER1-001-Proline, the extract that was probed with the anti-Chk-S345 phospho-antibody showed no signal. Chk1 phosphorylation at serine-345 was clearly detectable after 30min and 2 hours, in the cells expressing EGFP-Per1-001-Alanine (Figure 7-3). In contrast, phosphorylation of Chk2 at threonine-68 was detectable in both extracts. The absence of Chk1 phosphorylation, upon CPT treatment in the presence of EGFP-Per1-P962, is in line with its reduced phosphorylation at serine-345 after UV treatment (Figures 7-2, 7-3). Since CPT acts mainly on DNA replication forks, the proline at position 962 may interfere with Chk1 activation by ATR kinase at damaged forks in S phase. Since UV light damages DNA as well outside of S phase, Chk1 phosphorylation may only be reduced and not completely blocked. As in the case of UV light, both recombinant proteins disappear after CPT treatment. Anti-H2AX was used as a control for DNA double strand breaks in response to DNA damage (Dickey et al., 2009, Elvers et al., 2012, Jones & Petermann, 2012), and anti-GAPDH was used as a loading control (Figure 7-5).

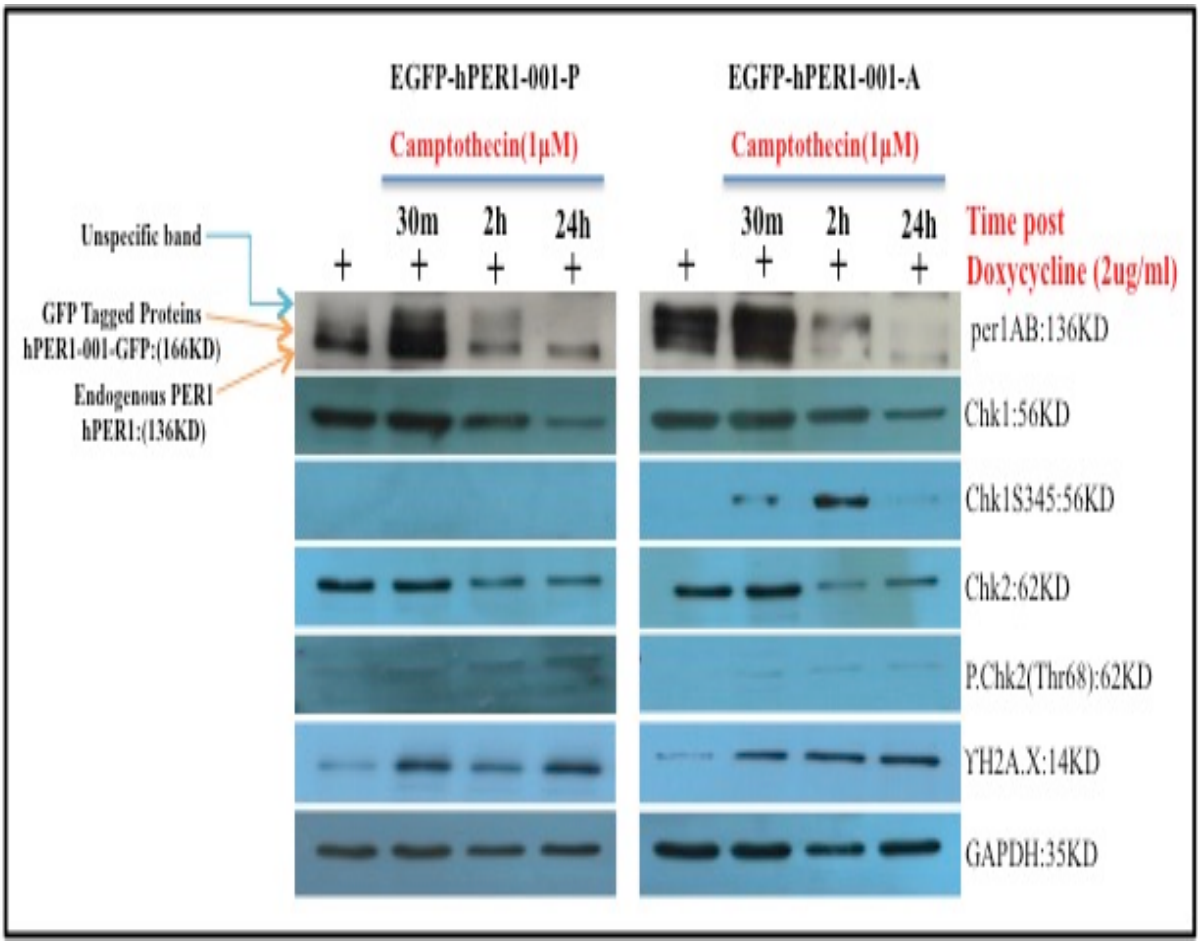

Figure 7–5: Camptothecin (CPT) treatment of EGFP tagged hPER1-001-P and EGFP tagged hPER1-001-A cells. Cells were induced with (2μg/ml) of doxycycline for overnight, treated with 1uM of CPT, then left to recover after the exposure at 37°C for (30min, 2hrs, and 24hrs) separately. Protein extracts were prepared at the end of each recovery time. Regarding EGFP-hPER1-A, the cells showed a much stronger signal for Chk1345Ph, especially after 2hrs of treatment, and a weak signal for P.Chk2 phosphorylation after being exposed for 24hrs. On the other hand, EGFP-hPER1-P cells showed a much stronger signal for P.Chk2 phosphorylation, up to 24hrs. Interestingly, there was no signal for Chk345Ph and the EGFP-hPER1-001-P signal disappeared after 24hrs exposure. GAPDH was used as a loading control for both cell lines, and H2AX was used as a control for double strand breaks (DSBs).

7.2.3 Cellular localization of EGFP-PER1-Alanine-962 and EGFP-PER1-Proline-962 upon UV treatment

To find out how endogenous Per1 and the engineered EGFP-PER1 proteins respond to UV-induced DNA damage, Immunofluorescent staining was performed for Untransfected control HEK293 cells, EGFP-PER1-Alanine-962 cells, EGFP-PER1-Proline-962 cells, and EGFP control cells. Untreated and UV-irradiated cells were fixed with 4% para-formaldehyde (PFA) for immune staining, incubated with the polyclonal anti-PER1 antibody or monoclonal anti-GFP antibody. In the first experiment, un-transfected HEK293 cells were stained with the polyclonal anti-PER1 antibody and the monoclonal anti-GFP antibody, in combination with a secondary anti-rabbit AlexaFlour-488 antibody or an anti-mouse AlexaFlour-532 antibody.

As shown in Figure 7-6, the endogenous Per1 protein was cytoplasmic under both conditions. This result is in line with the cell fractionation experiment, which also showed cytoplasmic staining (Figure 6-12). Since the polyclonal anti-Per1 antibody should bind to the N-terminal S L A D D T D A N S N G epitope, which is present in all predicted Per1 protein variants (Figure 4-1), all Per1 protein variants expressed in HEK293 cells appear to be in the cytoplasm. No GFP signal was observed in un-transfected cells (Figure 7-6), showing that the antibody does not cross-react with a protein in the absence of EGFP. The induction of both, EGFP-PER1-A962 and EGFP-PER1-P962 resulted in a strong nuclear signal in both cell lines (Figure 7-7). Importantly, in untreated cells, there was a striking difference between the two cell lines: while the EGFP-PER1-A962 protein was present in the nucleus and the cytoplasm, the EGFP-PER1-P962 protein was only present in the nucleus. The other key difference was that both over-expressed EGFP-PER1 proteins were located preferentially in the nucleus, whereas the endogenous Per1 protein was cytoplasmic. The latter difference was not caused by the EGFP fusion partner, since EGFP was mainly cytoplasmic in both normal and UV-treated cells, although there was a tendency towards a stronger nuclear EGFP signal after UV treatment (Figure 7-9). The mainly nuclear localisation of both EGFP-PER1 fusion proteins was not affected by UV light (Figure 7-8). Given that the engineered proteins fractionated in the cytoplasmic fraction (Figure 6-12), the mainly nuclear localisation of the EGFP-PER1 proteins suggests that the protein is only loosely associated with nuclear structures and enters the cytoplasmic fraction when the cells

are broken up during the fractionation experiment.

What remains to be explained, however, are the striking differences between the cytoplasmic localisation of the endogenous Per1 (Figure 7-6) and the mainly nuclear localisation of the EGFP-PER1 proteins. The PERIOD proteins accumulate in the cytoplasm, where they are posttranslational modified, which triggers their nuclear entry to down-regulate the activity of the main circadian transcription factor complex CLOCK-BMAL1 (Yagita et al., 2002). Per1 has several nuclear imports and export sequences, which help with the shuttling between cytoplasm and nucleus. Deletion of the second nuclear export motif in mouse Per2 (983-LQLNLLQL-990) results in the nuclear accumulation of the GFP tagged C-terminally truncated protein (Yagita et al., 2002). In Vielhaber et al. (2001), it was demonstrated that multiple mechanisms control the nuclear localisation of PER1, PER2, and PER3 in mammals. Additionally, mPER1's nuclear entry can be controlled by a phosphorylation-reliant masking of its nuclear localisation indicator. Here, the authors presented evidence indicating that PER proteins' nuclear localisation is ascertained by nuclear import and earlier unrecognised nuclear export signals. Cytoplasmic accumulation led the putative nuclear export signals from both mPER1 and mPER2 when combined with a heterologous protein (Vielhaber et al., 2001).

Extension of the human Per1 N-terminus by EGFP may interfere with the nuclear-cytoplasmic shuttling of Per1, resulting in its nuclear accumulation, as found in my experiments. The latter was independent of the amino acid position at 962. One interesting conclusion from these results is that the degradation of both engineered Per1 proteins, starting at 4 hours post UV treatment (Figures 7-2, 7-3), could be triggered by their predominantly nuclear localisation. In other words, maybe cells need to degrade nuclear Per1 in the presence of UV damage.

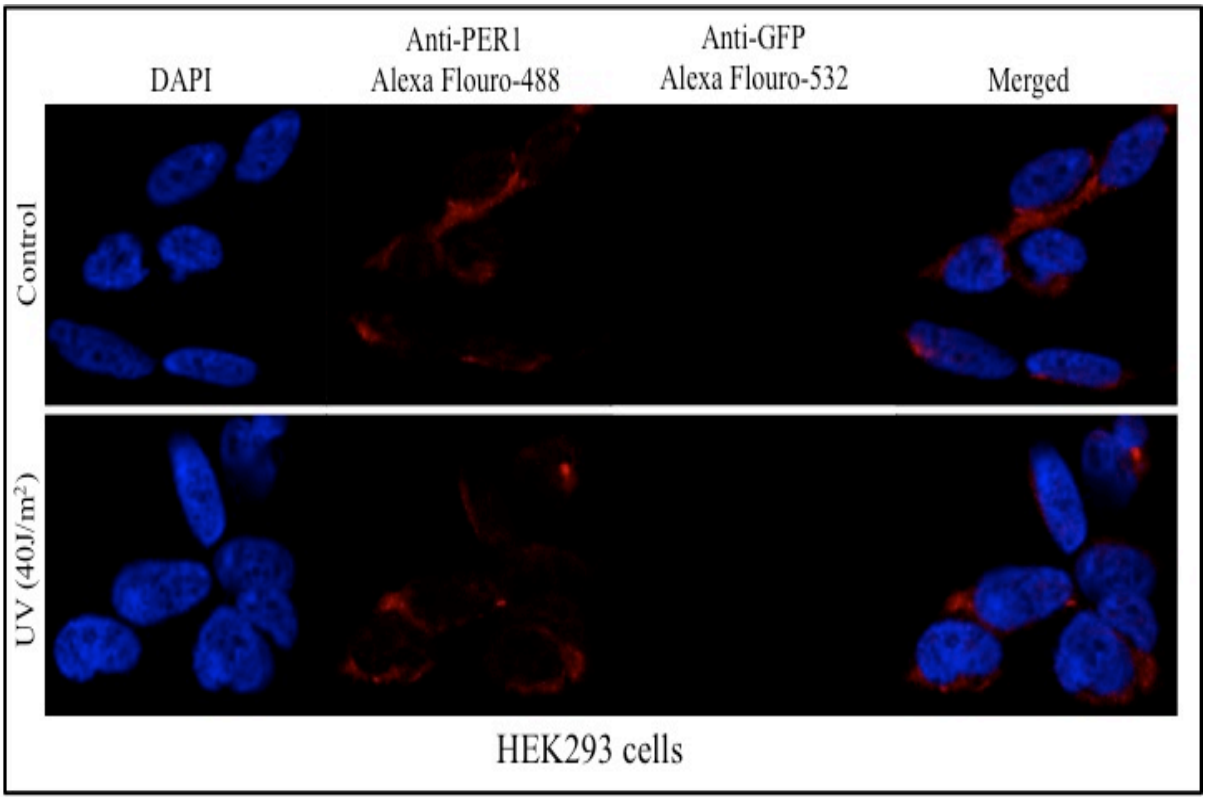

Figure 7–6: Cell localisation of the endogenous hPER1 in un-treated HEK293 cells and cells treated with UV light. Cells were grown on cover slips overnight, exposed to UV light at 40J/m^2 and left to recover for 2 hours at 37°C. They were then fixed with 4% paraformaldehyde (PFA) and stained with anti-PER1 antibody (Pierce/ThermoScientific PA1-524). hPER1 was clearly visible in the cytoplasm under both conditions. The anti-GFP antibody did not result in a positive signal. DNA was stained with DAPI (blue) (Images were obtained by Zeiss LSM710 Confocal Microscope with a 63x objective, and analysed by Zen2010 software and processed by Photoshop [Adobe]).

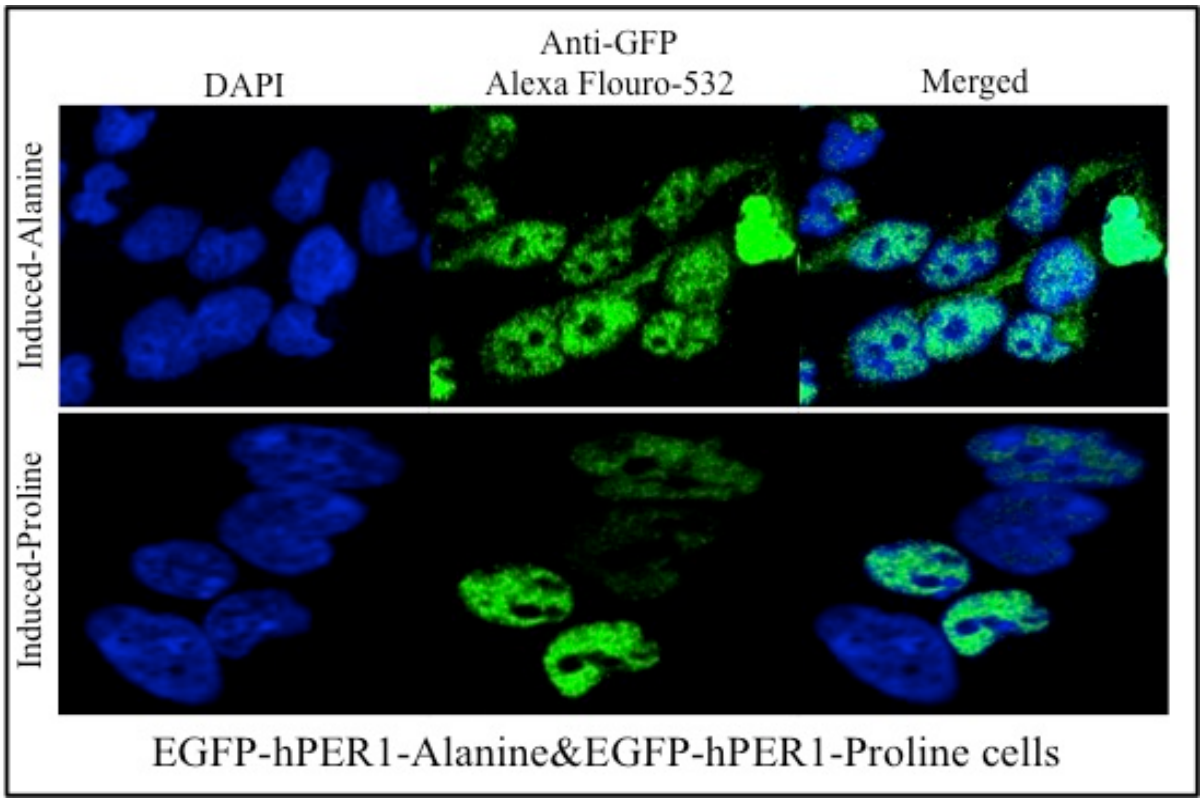

Figure 7–7: Cell localisation of the induced EGFP-PER1-Alanine-962 and EGFP-PER1-Proline-962 cells. Cells were grown on cover slips and induced with 2µg/ml doxycycline overnight, fixed with 4% paraformaldehyde (PFA), then stained with anti-GFP antibody. While EGFP-hPER1-Alanine cells were present in the cytoplasm and the nucleus, EGFP-hPER1-Proline was present only in the nucleus. (Images were obtained by Zeiss LSM710 Confocal Microscope with a 63x objective, analysed by Zen2010 software and processed by Photoshop [Adobe]).

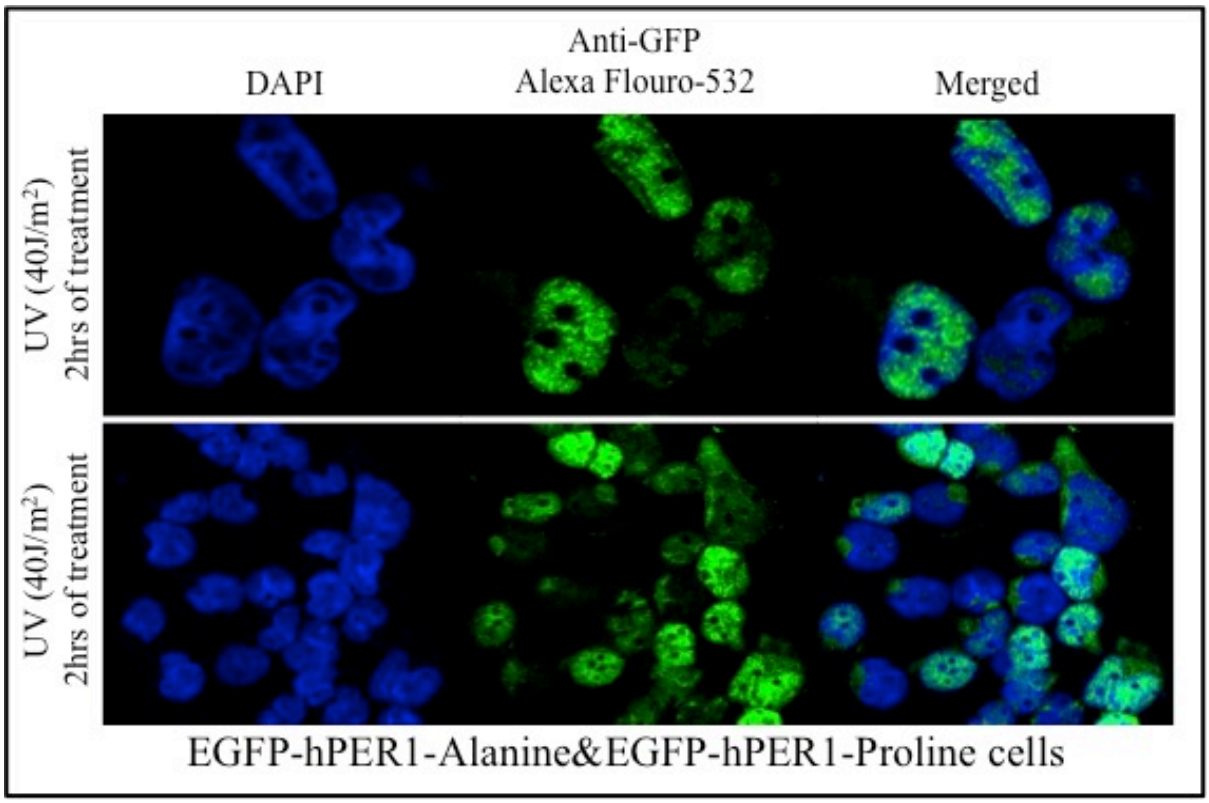

Figure 7–8: Cell localisation of UV-treated EGFP-PER1-Alanine-962 and EGFP-PER1-Proline-962 cells upon UV treatment. Cells were grown on cover slips and induced with 2µg/ml Doxycycline overnight, exposed to UV light at 40J/m2, and left to recover for 2 hours at 37°C. Cells were then fixed with 4% PFA for immune staining. After that, cells were stained with the monoclonal anti-GFP antibody. Top panel: treated EGFP-PER1-Alanine-962 cells were all located in the nucleus after UV-treatment. But, in the second panel: EGFP tagged-hPER1-Proline cells were visible in cytoplasm and nucleus, yet remained largely in the nucleus after UV-treatment. (Images were obtained by Zeiss LSM710 Confocal Microscope with a 63x objective, analysed by Zen2010 software, and processed by Photoshop [Adobe]).

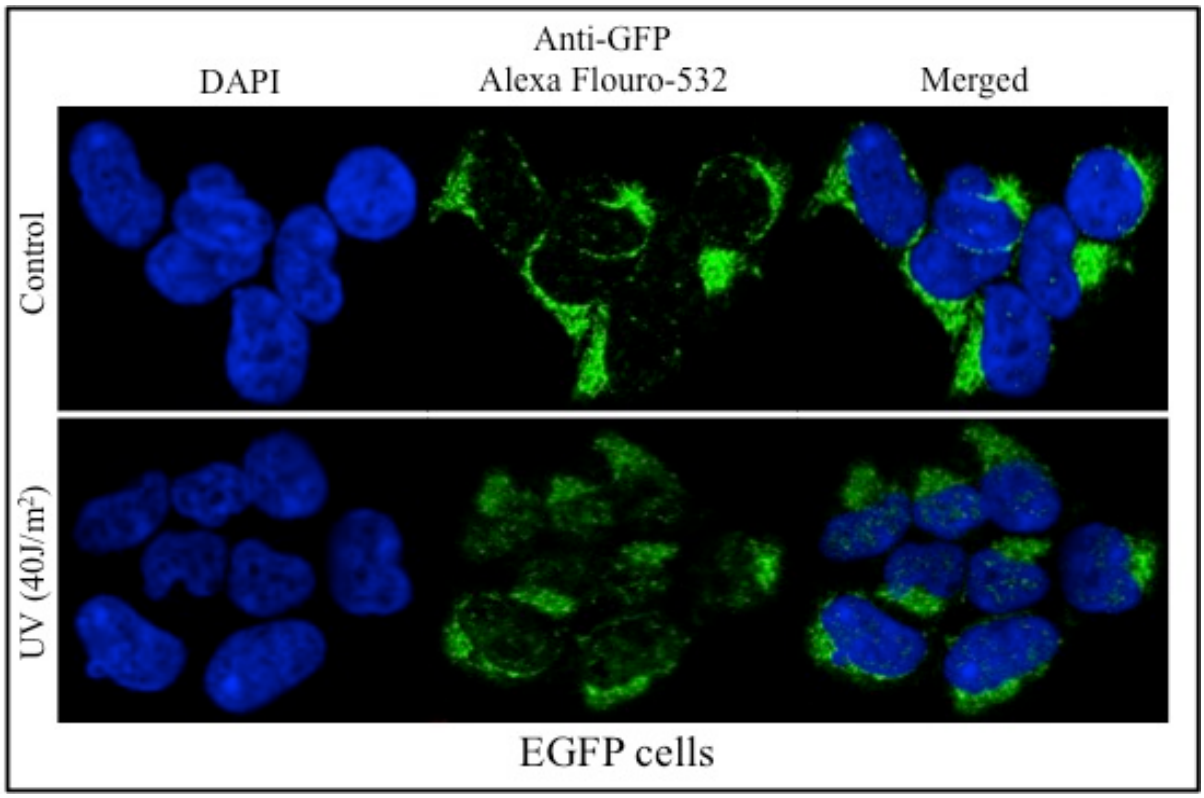

Figure 7–9: Cell localisation of untreated and UV-treated EGFP control cells. Cells were grown on cover slips and induced with 2µg/ml doxycycline overnight, exposed to UV light at 40J/m2, and left to recover for 2 hours at 37°C. Cells were then fixed with 4% PFA for immune staining. After that, cells were stained with the monoclonal anti-GFP antibody. In untreated cells, EGFP were intensely present in the cytoplasm and a very low signal was in the nucleus. After UV treatment, a stronger EGFP signal was in the nucleus, while most of the protein was still in the cytoplasm. (Images were obtained by Zeiss LSM710 Confocal Microscope with a 63x objective, analysed by Zen2010 software, and processed by Photoshop [Adobe]).

Chapter 8: General Discussion

Period 1 (Per1) is well known for its roles as a regulator of the circadian clock. Work by Gery and colleagues (2006) indicated that Per1 also acts as a regulator of the ATM-Chk2 DNA damage checkpoint pathway.

Main findings

The main findings of this thesis are (i) a correlation between elevated Per1 protein levels and increased phosphorylation levels of Chk2 at threonine-68 in colon carcinoma cells (HCT116) (Figure 4-3), (ii) the existence of four Per1 splice variants of which the larger two are a mixture of at least two forms with distinct post-translational modifications (Figure 4-6), (iii) the potential ubiquitinylation of one form of the largest splice variant when DNA replication forks break in the presence of the topoisomerase 1 inhibitor camptothecin (CPT) (Figure 4-6D), (iv) the mainly cytoplasmic localization of Per1 in untreated and UV-irradiated embryonic kidney cells (HEK293) (Figure 6-12), and (v) the ability of the engineered EGFP-PER1-P962 protein to interfere with the phosphorylation of Chk1 at serine-345 in the response to UV light and CPT (Figure 7-2, Figure 7-5).

Subcellular localization of Per1

While the results of this work support a role of Per1 in the regulation of Chk2 and Chk1 phosphorylation in the response to DNA damage, the cytoplasmic localization of the protein was unexpected. There are two possible explanations: Either Per1 shuttles between the cytoplasm and the nucleus and only remains for a brief period of time inside the nucleus, or its acts on the ATM-Chk2 pathway in the cytoplasm.

Mouse Per1 shuttles between the nucleus and the cytoplasm in a manner regulated by the deubiquitinating enzyme USP2 (ubiquitin-specific peptidase 2). Loss of USP2 reduces the amount of the nuclear mPer1 pool (Yang et al., 2014). In the cells of the mouse retina, mPer1 is also localized in the cytoplasm, whereas it is found in the nucleus of the mouse neurons in the suprachiasmatic nucleus (SCN) (García-Fernández, Álvarez-López & Cernuda-Cernuda, 2007). Over-expression of mPer1 in human Hela

cells leads to its nuclear localization, which is dependent on its two C-terminal nuclear import sequences (Loop & Pieler, 2005). In this context, it is noteworthy that both engineered EGFP-PER1 proteins are nuclear after their induction by doxycycline (Figure 7-7). This implies that the N-terminal EGFP fusion domain promotes the nuclear accumulation of human Per1. As shown in Figure 7-9, EGFP on its own did not accumulate in the nucleus of HEK293 cells which suggests that the Per1 part is required for the nuclear localization of the fusion protein. This is consistent with a previous report showing that expression mouse Per1 in HEK293 cells also results in the nuclear accumulation of the recombinant protein (Vielhaber et al., 2000). There are three possible explanations for this finding. First, the EGFP-PER1 protein is not efficiently phosphorylated by Casein kinase 1-ε as it was reported that co-expression of mammalian CK1ε and mPer1 in HEK293 cells, which results in the CK1-dependent phosphorylation of the nuclear localization sequence in mPer1, results in the cytoplasmic accumulation of Per1 (Vielhaber et al., 2000). Also inhibition of CK1ε in mouse cells allows mPer1 to reside for longer inside the nucleus (Miyazaki et al., 2004).

Second, the balance between the nuclear export and nuclear import motifs in hPer1 is not working. It could be that one of the three export signals, especially the first one (138aa-147aa) in the N-terminal part of hPer1, is masked in the fusion protein which would allow for nuclear accumulation via the nuclear import sequence which is closer to the C-terminal section of the protein (Figure 8-1) (Vielhaber et al., 2001).

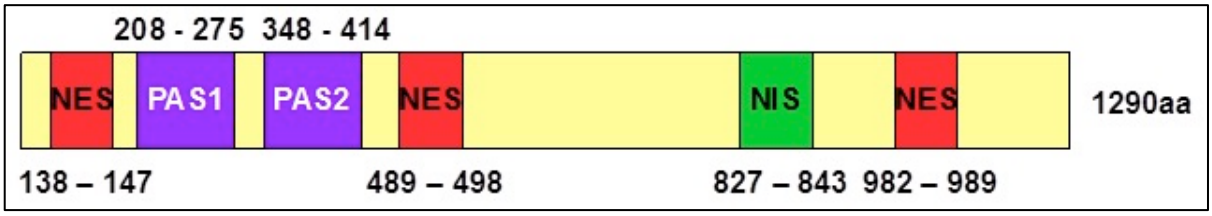

Figure 8–1: Localization of the Nuclear Export Sequences (NES) and the Nuclear Import Sequence (NIS) in human Per1 (variant 001). The two PAS domains are also shown.

It is also interesting in this context that the engineered EGFP-PER1 proteins are both degraded in the response to UV treatment (Figure 7-2, 7-3), since this implies that cells do not want Per1 in the nucleus when the DNA is damaged by UV light. Thirdly, the EGFP-PER1 fusion proteins may not form hetero-dimeric complexes with Cryptochrome that may be required for the nuclear entry (Griffin, Staknis & Weitz, 1999).

Interference of Per1-Proline-962 with Chk1 Serine-345 phosphorylation

The results shown in Figure7-2 show a reduced phosphorylation of Chk1 at serine 345 in HEK293 cells expressing the proline-962 variant of EGFP-PER1. While Chk1 phosphorylation is reduced in the response to UV light it is almost abolished when DNA replication forks break in the presence of camptothecin. This points towards a role of Per1 at collapsed DNA replication forks, a role which is negatively affected by a proline residue at position 962. The key circadian protein physically linked with DNA replication forks is Timeless (Swi1 in *S.pombe*, Tof1 in *S.cerevisiae*). Human Timeless associates with DNA replication proteins and stimulates the activity of DNA helicase DDX11(Cali et al., 2016). Importantly, down-regulation of human Timeless by siRNA reduces Chk1 phosphorylation at Ser-345 when DNA replication stops in the presence of hydroxyurea (HU) or upon irradiation with UV light (Unsal-Kacmaz et al., 2007). Although it is still unclear whether Per1 and Timeless associate in human cells, human Timeless can bind both *Drosophila* and mouse Per1 (Sangoram et al., 1998). This supports the idea that EGFP-PER1 interferes with Chk1 S345 phosphorylation at the level of Timeless at damaged DNA replication forks. Timeless associates with ATR kinase via its interaction with ATRIP which may be a requirement for the modification of Chk1 at S345 (Unsal-Kacmaz et al., 2007). Proline at position 962 in the engineered, nuclear EGFP-PER1-P962 protein may block this signalling cascade thereby reducing Chk1 modification. How a proline residue at position 962 may block the Timeless-ATR-Chk1 pathway is not yet clear. Since human timeless binds to a large section of the N and C-termini of the *Drosophila* Per protein (Figure 8-2), the proline at position 962 may interfere with the association between Per1 and Timeless.

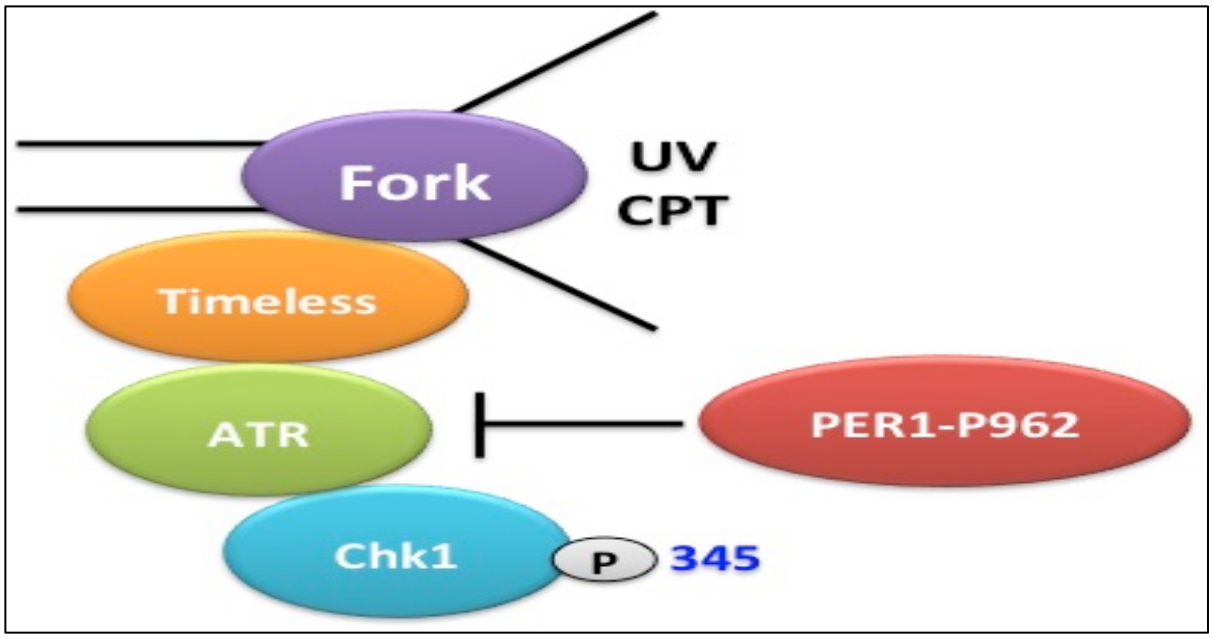

Figure 8–2: Model of how EGFP-Per1-P962 interferes with Chk1 phosphorylation at serine-345. Timeless associates with ATR and Chk1 kinase at a damaged DNA replication fork. While this leads normally to phosphorylation of Chk1 at S345 by ATR kinase, the nuclear EGFP-Per1-P962 may interfere with this modification. CPT = camptothecin, UV = UV light.

While this would explain why there is a strong selection against P962 in Europe and most other parts of the world (Figure 6-1), it would not explain why the proline in found in almost 50% of East Asian genomes. People expression PER1-P962 may have a defect in ATR-Chk1 signalling at damages DNA replication forks that could result in disorders like cancer. One possibility is that the proline-962 SNP is linked with another single nucleotide polymorphism that neutralizes the potential negative impact of the proline residue, either within the *Per1* gene or in other gene associated with ATR-Chk1 signalling. Although the A962P SNP (rs2585405) has been analyzed in a few studies (Hida et al., 2014), no significant correlation with a disorder has been found. Another possibility is that the negative impact on Chk1 phosphorylation is caused by the combination of the N-terminal EGFP fusion partner and the proline at position 962 as the fusion protein accumulated inside the nucleus unlike the endogenous Per1 protein (Figure 7-6, 7-8).

How could cytoplasmic Per1 influence ATM-Chk1 or ATR-Chk1 signalling?

The endogenous Per1 protein localized to the cytoplasm in untreated and UV irradiated HEK293 cells (Figure 6-12). This raises the question how Per1 can affect ATM-Chk2 or ATR-Chk1 signalling in the cytoplasm although DNA damage signalling is initiated in the nucleus. Human ATM kinase has several cytoplasmic activities, especially in neuronal cells (Yang et al., 2011).

In this context it is important to remember that HEK293 cells may be of neuronal origin rather than embryonic kidney cells (Stepanenko & Dmitrenko, 2015). Cytoplasmic ATM regulates insuline-induced AKT phosphorylation at S473 (Viniegra et al., 2005), and activates glucose uptake by GLUT4 into muscle cells (Halaby et al., 2008). ATM also promotes autophagy in the cytoplasm in the response to oxidative stress (Alexander, Kim & Walker, 2010). Like ATM, ATR acts also in the cytoplasm where it binds via its BH3-like domain to the outer membrane of mitochondria to suppress cell death in a manner independent of its subunit ATRIP which recruits ATR to single stranded DNA in the nucleus (Hilton et al., 2015). Interestingly, this cytoplasmic response is triggered by UV light. Chk2 kinase was also found in post-mitotic neurons where it phosphorylates tau (Iijima-Ando et al., 2010). In summary, these findings could provide an explanation for the dominant cytoplasmic localization of Per1 in HEK293 cells. Maybe Per1 influences ATM or ATR activities linked with circadian or cellular stress induced changes to metabolic processes or cell survival. In this context, it is interesting that key enzymes in the mitochondria undergo Per1-dependent oscillation in mouse (Neufeld-Cohen et al., 2016).

What are the functions of the four Per1 splice variants?

As shown in Figure 4-1, human Per1 may exist in five splice variants. While the two larger variants (Per1-001-1290aa, Per1-005-1267 aa) contain the C-terminal domain, which is required for the association with Cryptochrome, this domain is absent in the three smaller variants (Per1-002-859aa, Per1-006-824aa, Per1-003-572aa). Interestingly, Per1-005 (1267aa) carries a small deletion in the first PAS domain, which may affect its ability to form complexes with other PAS domain proteins. HEK293 cells express four Per1 variants, which react with the anti-Per1 antibody (ABCAM-AB174860). The two large proteins (100kDa and 136kDa) could be Per1-001 (1290aa)

and Per1-005 (1267aa), were as the two smaller variants (86KDa) could be Per1-002 (859aa)/Per1-006 (824aa) and the smallest one could be Per1-003 (572aa). Similar splice variants or 140kDa, 110kDa and 75kDa were observed in the SCN of mouse brain cells (García-Fernández, Álvarez-López & Cernuda-Cernuda, 2007). The biological roles of these Per1 variants are currently unknown. It is however very interesting that the two larger variants (100kDa and 136kDa) fractionate in at least two main forms during isoelectric focusing (Figure 4-6). The abundance of the two more positively charged forms strongly increases in the response to DNA damage, which indicates a specific role under stress conditions. Whether these forms arise by de-phosphorylation of the more negatively charged forms or by other means is not yet known. The negative form of the largest Per1 variant may be ubiquitinylated, as two larger forms appear when cells are treated with camptothecin (Figure 4-6D). Since the attachment of ubiquitin to mouse Per1 is linked with nuclear entry (Yang et al., 2014), the two modified forms of Per1 may localize to the nucleus when DNA replication forks are damaged by the topisomerase 1 inhibitor CPT (Figure 8-3). If this were to be correct, only a small amount of modified Per1 would contribute to the DNA damage response. This would be in line with the important role of ubiquitinylation at stalled or damaged DNA replication forks (Garcia-Rodriguez, Wong & Ulrich, 2016).

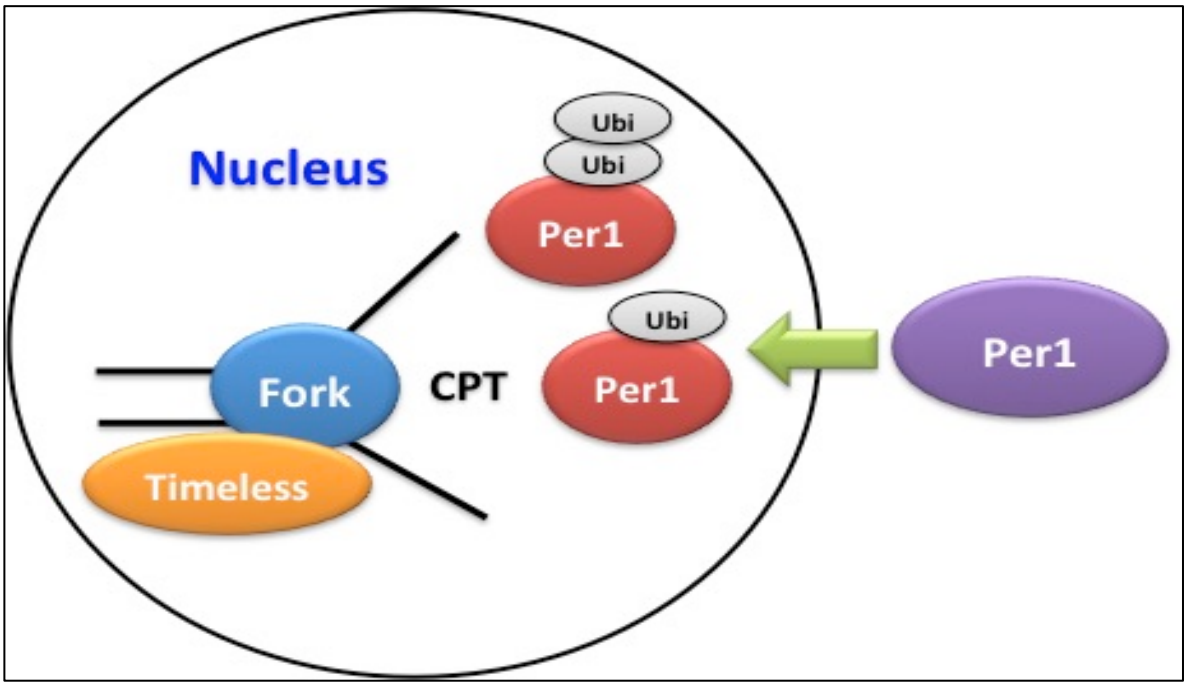

Figure 8–3: Ubiquitinylation may regulate nuclear entry of Per1 when DNA damage occurs.

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Appendix

1. Alignment of human Per1, Per2 and Per3

```
hPer3      ----------------------------------------------------MPRG-----
hPer1      MSGPLEGADGGGDPRPGESFCPGGVPSPGPPQHRP---CPGP-SLADDTDANSNGSSGNE
hPer2      MNGY-------A------EF----PPSPSNPTKEPVEPQPSQVPLQEDV-DMSSGSSGHE
                                                              *

hPer3      ----EAPGPGRRGAKDE--ALGEESGE-----------------R-WSPEFHLQ------
hPer1      SNGHESRGASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQ
hPer2      TNENCSTGRDSQGSDCD--DSGKELGMLVE----------PPDARQSPDTFSLMM-AKSE
                  *            *                            *

                             138      147

hPer3      -RKLADSSHSEQQDRNRVSEELIMVVQEMKKYFPSERRN--KPSTLDALNYALRCVHSVQ
hPer1      DNPSTSGCSSEQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQ
hPer2      HNPSTSGCSSDQSSKVDTHKELIKTLKELKVHLPADKKAKGKASTLATLKYALRSVKQVK
                   * *       **     * *   *          **  *  *** *  *

                                      208

hPer3      ANSEFFQILSQNG--APQADVSMYSLEELATIASEHTSKNTDTFVAVFSFLSGRLVHISE
hPer1      ANQEYYQQWSLEEGEPCSMDMSTYTLEELEHITSEYTLQNQDTFSVAVSFLTGRIVYISE
hPer2      ANEEYYQLLMSSEGHPCGADVPSYTVEEMESVTSEHIVKNADMFAVAVSLVSGKILYISD
           ** *  *           *   *  **     **    * * *    *   *    **

                                          275

hPer3      QAALILNRKKDVLASSHFVDLLAPQDMRVFYAHTARAQLPFWNNWTQRAAARYECAPVKP
hPer1      QAAVLLRCKRDVFRGTRFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKS
hPer2      QVASIFHCKRDAFSDAKFVEFLAPHDVGVFHSFTSPYKLPLWSMCSGADSFTQECMEEKS
           * *     * *      *   *** *  **   *    ** *               *

                                                              348

hPer3      FFCRIRGGEDRKQEKCHSPFRIIPYLIHVHHPAQPELESEPCCLTVVEKIHSGYEAPRIP
hPer1      VFCRIRGGPDRDPGPRYQPFRLTPYVTKIRVS--DGAPAQPCCLLIAERIHSGYEAPRIP
hPer2      FFCRVSVRKSHENEIRYHPFRMTPYLVKVRDQ--QGAESQLCCLLLAERVHSGYEAPRIP
            ***             ***  **               ***   *  **********
```

```
                                                          414
hPer3      VNKRIFTTTHTPGCVFLEVDEKAVPLLGYLPQDLIGTSILSYLHPEDRSLMVAIHQKVLK
hPer1      PDKRIFTTRHTPSCLFQDVDERAAPLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQ
hPer2      PEKRIFTTTHTPNCLFQDVDERAVPLLGYLPQDLIETPVLVQLHPSDRPLMLAIHKKILQ
             ****** *** * *  *** * **********     *  *** ** ** *** * *

hPer3      YAGHPPFEHSPIRFCTQNGDYIILDSSWSSFVNPWSRKISFIIGRHKVRTSPLNEDVFAT
hPer1      LAGQ-PFDHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTP
hPer2      SGGQ-PFDYSPIRFRARNGEYITLDTSWSSFINPWSRKISFIIGRHKVRVGPLNEDVFAA
             *  **  *****   ** *   * **  *  *****  *  ******  *******

hPer3      KIK-KMNDNDKDITELQEQIYKLLLQPVHVSVSSGYGSLGSSGSQEQLVSIASSSEASGH
hPer1      PAPSPAPSLDTDIQELSEQIHRLLLQPVHSPSPTGLCGVGAVTSPGPLHSPGSSSDSNGG
hPer2      HPCTEEKALHPSIQELTEQIHRLLLQPVPHSGSSGYGSLGSNGSHEHLMSQTSSSDSNGH
                        * ** ***  ******     *    *   *   * *  ***   *

hPer3      RVEETK-AEQMTLQQVYASVNKIKNLGQQLYIESMTKSSFKPVTGTRTEPNGGGESANGG
hPer1      DAEGPGPPAPVTFQQICKDVHLVKHQGQQLFIESRARPQSRPRLPA----TGTFK--AKA
hPer2      EDSRR------RRAEICKNGNKTKNRSHYSHESGEQKKKSVTEMQT----NPPAE--KKA
                                  *

                    596
hPer3      GECKTFTSFHQTLKN------NSVYTEPCEDLRNDEHSPSYQQINCIDSVIRYLKSYNIP
hPer1      LPCQSP---DPELEAGSAPVQAPLALVPEEAERKEASSCSYQQINCLDSILRYLESCNLP
hPer2      V---------PAMEKDS-----LGVSFPEELACKNQPTCSYQQISCLDSVIRYLESCNEA
                                    * *         ***** * **  *** * *

hPer3      A-LKRKCISCTNTTSSSSEEDKQNHKADDVQALQAGLQIPAIPKSEMPTNGRSIDTGGGA
hPer1      STTKRKCASSSSYTTSSASDDDRQRTGPVSVGTKKDPPSAALSGEGATPRKEPVVGGTLS
hPer2      ATLKRKCEFPANVPALRSSDKRKATVSPGPHAGEAEPPSRV--------NSRTGVGTHLT
              ****
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```
hPer3      PQILSTAMLSLGSGISQCGYSSTIVHVPPPETARDATLFCEP----------WTLNMQ
hPer1      PLALANKAESVVSVTSQCSFSSTIVHVGDKKPPESDIIMMEDLPGLAPGPAPSPAPSPTV
hPer2      SLALPGKAESVASLTSQCSYSSTIVHVGDKKPQPEL-EMVEDAASGPESLDCLAGPALAC
              *     *  *  ***  *******             *

                                                          815
hPer3      PAPLTSEEFKHVGLTAAVLSAHTQKEEQNYVDKFREKILSSPY----SSYLQQESRSKAK
hPer1      APDPAPDAYRPVGLTKAVLSLHTQKEEQAFLSRFRDLGRLRGLDSSSTAPSALGERGCHH
hPer2      GLSQEKEPFKKLGLTKEVLAAHTQKEEQSFLQKFKEIRKLSIFQSHCHYYLQERSKGQPS
                        ***  **  *******     *

hPer3      YSYFQGDSTSKQTRSAGCRKG--KHKRKKLPEPPDSSSSNTGSGPRRGAHQNAQPCCPSA
hPer1      -----------GPAPPSRRH----HCRSKAKRSRHHQ-NPRAEAPCYVSH-----PSPVP
hPer2      ERTAPGLRNTSGIDSPWKKTGKNRKLKSKRVKPRDSSESTGSGGPVSARP-----PLVGL
                                       *             *

hPer3      ASSPHTSS-------PTFPPAAMVPSQAPYLVPAFPLPAATSPGREYAAPGTAPEGLHG-
hPer1      PSTPWPTPP------ATTPF--PA-VVQPYPLPVFSPR-----GGPQPLPPAP-------
hPer2      NATAWSPSDTSQSSCPAVPF--PAPVPAAYSLPVFPAP-----GTVAA-PPAPPHASFTV
                           *          *  * *        *     *

hPer3      --LPLS---EGLQPYPAFPFPYLDTFMTVFLPDPPVCPLLSPSFLPCPFLGATASSAISP
hPer1      ----------TSVPPAAFPAPLVTPMVALVLPNYLFP-TP-SSYPYGALQ-T----PA--
hPer2      PAVPVDLQHQFAVQPPPFPAPL-APVMAFMLPSYSFP-SG-TPNLPQAFFPSQPQFPSHP
                         ** *         **

                                       SNP-962              982
hPer3      SMSSAMSPTLDPPP----------SVTSQRREEEKWEAQSEGHPFITSRSSSPLQLNLLQ
hPer1      ---------EGPPT------PASHSPSPSLPALAPSPPHRPDSPLFNSRCSSPLQLNLLQ
hPer2      TLTSEMASASQPEFPSRTSIPRQPCACPATRATPPSAMGRASPPLFQSRSSSPLQLNLLQ
                     *                               *   ** **********
```

```
          989
hPer3     EEMPRPSESPDQMRRNTCPQTEYQCVTGNNGSESSPATTGALSTGSPPREN-PSHPTASA
hPer1     LEELPRAEG--------------AAVAGGPGSSA----------GPPPPSAEAAEPEA--
hPer2     LEEAPEGGT--------------GAMGT-TGATE----TAAVGADCKPGTSRDQQPKAP-
           *                          *               *       * *
hPer3     LSTGSPPMKNPSHP-TASALSTGSPPMKNPSHPTASTLSMGLPPSRTPSHPTATVLSTGS
hPer1     -RLA-----EVTESSNQDALSGSSDLL-ELLLQEDSRSGTGSAASGSLGSGLGSGSGSGS
hPer2     LTRD-----EPSDTQNSDALSTSSGLL-NLLLNEDLCSASGSAASESLGSGSLGCDA---
                           ***  *               *   *
hPer3     PPSESPSRTGSAASGSSDSSIYLTSSVYSSKISQNG-QQSQDVQKKETFPNVAEEPIWRM
hPer1     HEGGSTSASITRSSQSSHTSKYFGSIDS-SEAEAGA-ARGGAEPGDQVIKYVLQDPIWLL
hPer2     ------SPSGAGSSDTSHTSKYFGSIDS-SENNHKAKMNTGMEESEHFIKCVLQDPIWLL
                *      *  *  * *  *    *                    *   ***
                         1149
hPer3     IRQTPERILMTYQVPERVKEVVLKEDLEKLESMRQQQPQFSHGQKEELAKVYNWIQSQTV
hPer1     MANADQRVMMTYQVPSRDMTSVLKQDRERLRAMQKQQPRFSEDQRRELGAVHSWVRKGQL
hPer2     MADADSSVMMTYQLPSRNLEAVLKEDREKLKLLQKLQPRFTESQKQELREVHQWMQTGGL
                   **** * *    *** * * *      ** *   *  **  *  *


hPer3     TQEIDIQACVTCENEDSADGAATSC--------GQVLVEDSC------------------
hPer1     PRALDVMACVDCGSSTQDPGHPDDPLFSELDGLGLEPMEEGGGEQGSSGGGSGEGEGCEE
hPer2     PAAIDVAECVYCENKEK--GNICIPYEEDIPSLGLSEVSDTKEDENG----SPLNHRIEE
              *   ** *       *             *


hPer3     -------------------------------------
hPer1     AQGGAKASSSQDLAMEEEEEEGRSSSSPALPTAGNCTS 1290
hPer2     QT-----------------------------------
```

Uniprot numbers: Per1: O15534; Per2: O15055; Per3: P56645

PAS-A domain is shown in blue, PAS-B domain is shown in red, domain required for the association with Cryptochrome is black the domain required for phosphorylation by Casein Kinase 1 is yellow. The two nuclear export signals are shown in green. The SNP A962P is shown in pink. The binding domain of the F box protein beta-TRCP is shown in grey.

2. Alignment of the main hPer1 splice variants

```
hPer1-003_572aa       MSGPLEGADGGGDPRPGESFCPGGVPSPGPPQHRPCPGPSLADDTDANSNGSSGNESNGH
hPer1-002_859aa       ML----------------EVLEEIWSVRARRAHRPCPGPSLADDTDANSNGSSGNESNGH
hPer1-005_1267aa      MSGPLEGADGGGDPRPGESFCPGGVPSPGPPQHRPCPGPSLADDTDANSNGSSGNESNGH
hPer1-001_1290aa      MSGPLEGADGGGDPRPGESFCPGGVPSPGPPQHRPCPGPSLADDTDANSNGSSGNESNGH
hPer1-006_824aa       MSGPLEGADGGGDPRPGESFCPGGVPSPGPPQHRPCPGPSLADDTDANSNGSSGNESNGH
                      *                                ****************************

hPer1-003_572aa       ESRGASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPS
hPer1-002_859aa       ESRGASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPS
hPer1-005_1267aa      ESRGASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPS
hPer1-001_1290aa      ESRGASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPS
hPer1-006_824aa       ESRGASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPS
                      ************************************************************

hPer1-003_572aa       TSGCSSEQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQE
hPer1-002_859aa       TSGCSSEQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQE
hPer1-005_1267aa      TSGCSSEQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQE
hPer1-001_1290aa      TSGCSSEQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQE
hPer1-006_824aa       TSGCSSEQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQE
                      ************************************************************

hPer1-003_572aa       YYQQWSLEEGEPCSMDMSTYTLEELEHITSEYTLQNQDTFSVAVSFLTGRIVYISEQAAV
hPer1-002_859aa       YYQQWSLEEGEPCSMDMSTYTLEELEHITSEYTLQNQDTFSVAVSFLTGRIVYISEQAAV
hPer1-005_1267aa      YYQQWSLEEGEPCSMDMSTYTLEELEHITSEYTLQNQ--------------------AAV
hPer1-001_1290aa      YYQQWSLEEGEPCSMDMSTYTLEELEHITSEYTLQNQDTFSVAVSFLTGRIVYISEQAAV
hPer1-006_824aa       YYQQWSLEEGEPCSMDMSTYTLEELEHITSEYTLQNQDTFSVAVSFLTGRIVYISEQAAV
                      ************************************                   ***

hPer1-003_572aa       LLRCKRDVFRGTRFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKSVFCR
hPer1-002_859aa       LLRCKRDVFRGTRFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKSVFCR
hPer1-005_1267aa      LLRCKRDVFRGTRFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKSVFCR
hPer1-001_1290aa      LLRCKRDVFRGTRFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKSVFCR
hPer1-006_824aa       LLRCKRDVFRGTRFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKSVFCR
                      ************************************************************

hPer1-003_572aa       IRGGPDRDPGPRYQPFRLTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIF
hPer1-002_859aa       IRGGPDRDPGPRYQPFRLTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIF
hPer1-005_1267aa      IRGGPDRDPGPRYQPFRLTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIF
hPer1-001_1290aa      IRGGPDRDPGPRYQPFRLTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIF
hPer1-006_824aa       IRGGPDRDPGPRYQPFRLTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIF
                      ************************************************************

hPer1-003_572aa       TTRHTPSCLFQDVDERAAPLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPF
hPer1-002_859aa       TTRHTPSCLFQDVDERAAPLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPF
hPer1-005_1267aa      TTRHTPSCLFQDVDERAAPLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPF
hPer1-001_1290aa      TTRHTPSCLFQDVDERAAPLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPF
hPer1-006_824aa       TTRHTPSCLFQDVDERAAPLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPF
                      ************************************************************

hPer1-003_572aa       DHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAP
hPer1-002_859aa       DHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAP
hPer1-005_1267aa      DHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAP
hPer1-001_1290aa      DHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAP
hPer1-006_824aa       DHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAP
                      ************************************************************

hPer1-003_572aa       SLDTDIQELSEQIHRLLLQPVHSPSPTGLCGVGAVTSPGPLHSPGSSSDSNGGDAEGPGP
hPer1-002_859aa       SLDTDIQELSEQIHRLLLQPVHSPSPTGLCGVGAVTSPGPLHSPGSSSDSNGGDAEGPGP
hPer1-005_1267aa      SLDTDIQELSEQIHRLLLQPVHSPSPTGLCGVGAVTSPGPLHSPGSSSDSNGGDAEGPGP
hPer1-001_1290aa      SLDTDIQELSEQIHRLLLQPVHSPSPTGLCGVGAVTSPGPLHSPGSSSDSNGGDAEGPGP
hPer1-006_824aa       SLDTDIQELSEQIHRLLLQPVHSPSPTGLCGVGAVTSPGPLHSPGSSSDSNGGDAEGPGP
                      ************************************************************

hPer1-003_572aa       PAPLQARSRPR----------------------PFPANPQTQSWRRVLLPSRPH-------
hPer1-002_859aa       PAPVTFQQICKDVHLVKHQGQQLFIESRARPQSRPRLPATGTFKAKALPCQSPDPELEAG
hPer1-005_1267aa      PAPVTFQQICKDVHLVKHQGQQLFIESRARPQSRPRLPATGTFKAKALPCQSPDPELEAG
hPer1-001_1290aa      PAPVTFQQICKDVHLVKHQGQQLFIESRARPQSRPRLPATGTFKAKALPCQSPDPELEAG
hPer1-006_824aa       PAPVTFQQICKDVHLVKHQGQQLFIESRARPQSRPRLPATGTFKAKALPCQSPDPELEAG
                      ***                                *  * *       **
```

```
hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       SAPVQAPLALVPEEAERKEASSCSYQQINCLDSILRYLESCNLPSTTKRKCASSSSYTTS
hPer1-005_1267aa      SAPVQAPLALVPEEAERKEASSCSYQQINCLDSILRYLESCNLPSTTKRKCASSSSYTTS
hPer1-001_1290aa      SAPVQAPLALVPEEAERKEASSCSYQQINCLDSILRYLESCNLPSTTKRKCASSSSYTTS
hPer1-006_824aa       SAPVQAPLALVPEEAERKEASSCSYQQINCLDSILRYLESCNLPSTTKRKCASSSSYTTS


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       SASDDDRQRTGPVSVGTKKDPPSAALSGEGATPRKEPVVGGTLSPLALANKAESVVSVTS
hPer1-005_1267aa      SASDDDRQRTGPVSVGTKKDPPSAALSGEGATPRKEPVVGGTLSPLALANKAESVVSVTS
hPer1-001_1290aa      SASDDDRQRTGPVSVGTKKDPPSAALSGEGATPRKEPVVGGTLSPLALANKAESVVSVTS
hPer1-006_824aa       SASDDDRQRTGPVSVGTKKDPPSAALSGEGATPRKEPVVGGTLSPLALANKAESVVSVTS


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       QCSFSSTIVHVGDKKPPESDIIMMEDLPGLAPGPAPSPAPSPTVAPDPAPDAYRPVGLTK
hPer1-005_1267aa      QCSFSSTIVHVGDKKPPESDIIMMEDLPGLAPGPAPSPAPSPTVAPDPAPDAYRPVGLTK
hPer1-001_1290aa      QCSFSSTIVHVGDKKPPESDIIMMEDLPGLAPGPAPSPAPSPTVAPDPAPDAYRPVGLTK
hPer1-006_824aa       QCSFSSTIVHVGDKKPPESDIIMMEDLPGLAPGPAPSPAPSPTVAPDPAPDAYRPVGLTK


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       AVLSLHTQKEEQAFLSRFRDLGRLRGLDSSSTAPSALGERGSHLGPPGACPLPSLGLDCW
hPer1-005_1267aa      AVLSLHTQKEEQAFLSRFRDLGRLRGLDSSSTAPSAL---GCHHGPAPPSR----RHHCR
hPer1-001_1290aa      AVLSLHTQKEEQAFLSRFRDLGRLRGLDSSSTAPSALGERGCHHGPAPPSR----RHHCR
hPer1-006_824aa       AVLSLHTQKEEQAFLSRFRDLGRLRGLDSSSTAPSALGERGGGH----------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       GVGLKGGVSAPGTQAGVASTTRPCLGTGPSLASPH--------------------------
hPer1-005_1267aa      SKAKRSR------HHQNPRAEAPCYVSHPSPVPPSTPWPTPPATTPFPAVVQPYPLPVFS
hPer1-001_1290aa      SKAKRSR------HHQNPRAEAPCYVSHPSPVPPSTPWPTPPATTPFPAVVQPYPLPVFS
hPer1-006_824aa       ------------------------------------------------------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       ------------------------------------------------------------
hPer1-005_1267aa      PRGGPQPLPPAPTSVPPAAFPAPLVTPMVALVLPNYLFPTPSSYPYGALQTPAEGPPTPA
hPer1-001_1290aa      PRGGPQPLPPAPTSVPPAAFPAPLVTPMVALVLPNYLFPTPSSYPYGALQTPAEGPPTPA
hPer1-006_824aa       ------------------------------------------------------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       ------------------------------------------------------------
hPer1-005_1267aa      SHSPSPSLPALAPSPPHRPDSPLFNSRCSSPLQLNLLQLEELPRAEGAAVAGGPGSSAGP
hPer1-001_1290aa      SHSPSPSLPALAPSPPHRPDSPLFNSRCSSPLQLNLLQLEELPRAEGAAVAGGPGSSAGP
hPer1-006_824aa       ------------------------------------------------------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       ------------------------------------------------------------
hPer1-005_1267aa      PPPSAEAAEPEARLAEVTESSNQDALSGSSDLLELLLQEDSRSGTGSAASGSLGSGLGSG
hPer1-001_1290aa      PPPSAEAAEPEARLAEVTESSNQDALSGSSDLLELLLQEDSRSGTGSAASGSLGSGLGSG
hPer1-006_824aa       ------------------------------------------------------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       ------------------------------------------------------------
hPer1-005_1267aa      SGSGSHEGGSTSASITRSSQSSHTSKYFGSIDSSEAEAGAARGGAEPGDQVIKYVLQDPI
hPer1-001_1290aa      SGSGSHEGGSTSASITRSSQSSHTSKYFGSIDSSEAEAGAARGGAEPGDQVIKYVLQDPI
hPer1-006_824aa       ------------------------------------------------------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       ------------------------------------------------------------
hPer1-005_1267aa      WLLMANADQRVMMTYQVPSRDMTSVLKQDRERLRAMQKQQPRFSEDQRRELGAVHSWVRK
hPer1-001_1290aa      WLLMANADQRVMMTYQVPSRDMTSVLKQDRERLRAMQKQQPRFSEDQRRELGAVHSWVRK
hPer1-006_824aa       ------------------------------------------------------------


hPer1-003_572aa       ------------------------------------------------------------
hPer1-002_859aa       ------------------------------------------------------------
hPer1-005_1267aa      GQLPRALDVMACVDCGSSTQDPGHPDDDPLFSELDGLGLEPMEEGGGEQGSSGGGSGEGEG
hPer1-001_1290aa      GQLPRALDVMACVDCGSSTQDPGHPDDDPLFSELDGLGLEPMEEGGGEQGSSGGGSGEGEG
hPer1-006_824aa       ------------------------------------------------------------
```

```
hPer1-003_572aa       ----------------------------------------
hPer1-002_859aa       ----------------------------------------
hPer1-005_1267aa      CEEAQGGAKASSSQDLAMEEEEEGRSSSSPALPTAGNCTS
hPer1-001_1290aa      CEEAQGGAKASSSQDLAMEEEEEGRSSSSPALPTAGNCTS
hPer1-006_824aa       ----------------------------------------
```

3. All three-tissue samples align with the cDNA of Per1-005, not with Per1-001

3.1 Alignment with Per1-005 (the ATG start codon is highlighted)

```
Adrenal         ---------GCTAGGA-GTCCTAGAAGAAATTTGGAGTGTGCGTGCGCGCCGAGCGGTAC
Lung            ----------CTAGGA-GTCCTAGAAGAAATTTGGAGTGTGCGTGCGCGCCGAGCGGTAC
Spleen          GCTCTTAATGCTAGGAAGTCCTAGAAGAAATTTGGAGTGTGCGTGCGCGCCGAGCGGTAC
Per1-005        ------------------------------------------------------------


Adrenal         TGGCTGTGATCGAACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGC
Lung            TGGCTGTGATCGAACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGC
Spleen          TGGCTGTGATCGAACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGC
Per1-005        ---------TCGAACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGC
                         **************************************************

Adrenal         CAAGCCTCCAGGCCCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCT
Lung            CAAGCCTCCAGGCCCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCT
Spleen          CAAGCCTCCAGGCCCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCT
Per1-005        CAAGCCTCCAGGCCCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCT
                ************************************************************
                          Start Codon
Adrenal         CCCCATGGCCCAGACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGG
Lung            CCCCATGGCCCAGACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGG
Spleen          CCCCATGGCCCAGACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGG
Per1-005        CCCCATGGCCCAGACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGG
                ************************************************************

Adrenal         CCTGGGGAATCATTTTGTCCTGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCT
Lung            CCTGGGGAATCATTTTGTCCTGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCT
Spleen          CCTGGGGAATCATTTTGTCCTGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCT
Per1-005        CCTGGGGAATCATTTTGTCCTGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCT
                ************************************************************

Adrenal         TGCCCAGGCCCCAGCCTGGCCGA-GA---------------------------------
Lung            TGCCCAGGCCCCAGCCTGGCCGA-G----------------------------------
Spleen          TGCCCAGGCCCCAGCCTGGCCGA-GA---------------------------------
Per1-005        TGCCCAGGCCCCAGCCTGGCCGATGACACCGATGCCAACAGCAATGGTTCAAGTGGCAAT
                ********************** *
```

3.2 Alignment with Per1-001 (the ATG start codon is highlighted)

```
Adrenal         ---------GCTA----------------------GGA-GTCCTA----GAAGAAATTTG
Lung            ----------CTA----------------------GGA-GTCCTA----GAAGAAATTTG
Spleen          GCTCTTAATGCTA----------------------GGAAGTCCTA----GAAGAAATTTG
Per1-001        ----ATTATGCAACCCGCCTCCCCGCCCGCCCGGTGGAGCTTCCACTCGGCTGCGGGCTG
                         * *                     ***  * * *    *  *     **

Adrenal         GAGTGT----------GCGTGCG-----CGC-----CGAGC-GGTACTGGCTGTGATCGA
Lung            GAGTGT----------GCGTGCG-----CGC-----CGAGC-GGTACTGGCTGTGATCGA
Spleen          GAGTGT----------GCGTGCG-----CGC-----CGAGC-GGTACTGGCTGTGATCGA
Per1-001        GAGCGGCGGCGGGCAGGCGTGCGGAGGACACTCCTGCGACCAGGTACTGGCTGTGATCGA
                *** *           *******     * *     *** * ******************

Adrenal         ACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGCCAAGCCTCCAGGC
Lung            ACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGCCAAGCCTCCAGGC
Spleen          ACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGCCAAGCCTCCAGGC
Per1-001        ACTTCTCAACCCTCAGAGACTTAGATCTTCCACCTCACTCCCTCAGCCAAGCCTCCAGGC
                ************************************************************
```

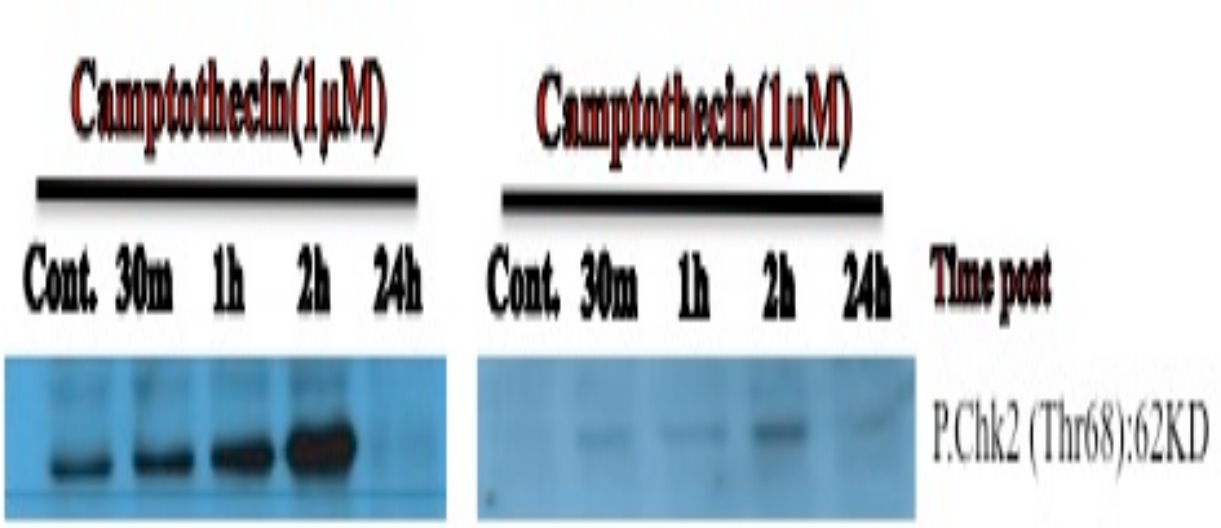
```
Adrenal         CCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCTCCCCATGGCCCAG
Lung            CCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCTCCCCATGGCCCAG
Spleen          CCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCTCCCCATGGCCCAG
Per1-001        CCCCTCGTGCATCCGTGGTGGCCTCTCTGCCTTCTCTGTTCTGTTCTCCCCATGGCCCAG
                ************************************************************

Adrenal         ACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGGCCTGGGGAATCAT
Lung            ACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGGCCTGGGGAATCAT
Spleen          ACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGGCCTGGGGAATCAT
Per1-001        ACATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGGCCTGGGGAATCAT
                ************************************************************

Adrenal         TTTGTCCTGGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCTTGCCCAGGCCCCA
Lung            TTTGTCCTGGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCTTGCCCAGGCCCCA
Spleen          TTTGTCCTGGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCTTGCCCAGGCCCCA
Per1-001        TTTGTCCTGGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCTTGCCCAGGCCCCA
                ************************************************************

Adrenal         GCCTGGCCGA-GA-----------------------------------------------
Lung            GCCTGGCCGA-G------------------------------------------------
Spleen          GCCTGGCCGA-GA-----------------------------------------------
Per1-001        GCCTGGCCGATGACACCGATGCCAACAGCAATGGTTCAAGTGGCAATGAGTCCAACGGGC
                ********** *
```

4. The repeat experiment to check for the phosphorylation of Chk2 in HCT116 and HEK239 cells after CPT treatment. The data relate to Figure 4-2



5. Nucleotide sequence for OriGene's pRFP-C-RS shRNA-29 expression vector

EcoRI

GAATTCCCCAGTGGAAAGACGCGCAGGCAAAACGCACCACGTGACGGAGCGTGACCGCGCGCCGAGCGCGCGCCAA
GGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATA
ATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGT
AGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTT
GGGTTTATATATCTTGTGGAAAGGACGCGGGATCCACTGGACCAGGCAGCAGCGTCAGAAGACTTTTTT**TTGGAA**A
AGCTTGTCGACCCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAG

BamHI-HindIII

GCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCC
CATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAG
GCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAA
AGCTAGCTTACCATGACCGAGTACAAGCCCACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAGGGCCGTACGCA
CCCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCGTCGATCCGGACCGCCACATCGAGCGGGTCAC
CGAGCTGCAAGAACTCTTCCTCACGCGCGTCGGGCTCGACATCGGCAAGGTGTGGGTCGCGGACGACGGCGCCGCG
GTGGCGGTCTGGACCACGCCGGAGAGCGTCGAAGCGGGGGCGGTGTTCGCCGAGATCGGCCCGCGCATGGCCGAGT
TGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCCTCCTGGCGCCGCACCGGCCCAAGGAGCCCGCGTG
GTTCCTGGCCACCGTCGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGCAGCGCCGTCGTGCTCCCCGGAGTG
GAGGCGGCCGAGCGCGCCGGGGTGCCCGCCTTCCTGGAGACCTCCGCGCCCCGCAACCTCCCCTTCTACGAGCGGC
TCGGCTTCACCGTCACCGCCGACGTCGAGGTGCCCGAAGGACCGCGCACCTGGTGCATGACCCGCAAGCCCGGTGC
CTGAGTTTGTTTGAATGAGGCTTCAGTACTTTACAGAATCGATAAAATAAAAGATTTTATTTAGTCTCCAGAAAAA
GGGGGGAATGAAAGACCCCACCTGTAGGTTTGGCAAGCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGAAAAAT
ACATAACTGAGAATAGAGAAGTTCAGATCAAGGTCAGGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATAT
CTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATC
TGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGCCCTCAGCAGTTT
CTAGAGAACCATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATC
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TGGTCTCGCTGTTCCTTGGGAGGGTCTCCTCTGAGTGATTGACTACCCGTCAGCGGGGGTCTTTCATTTCCGACTT
GTGGTCTCGCTGCCTTGGGAGGGTCTCCTCTGAGTGATTGACTACCCGTCAGCGGGGGTCTTCACATGCAGCATGT
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CCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGC
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CCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGC
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CAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGAGAGGACAGTGCCAAGCAAGCAACTCAAATGTCCC
ACCGGTTGGGCATGGCCAGGTAGCCTATGCTGTGTCTGGACGTCCTCCTGCTGGTATAGTTATTTTAAAATCAGAA
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CCGCTTAACGCGTCGGTCCGCTAGCTGCGGCCGCTTCGAACGTTGCGATCGCTACATCGCTCTTCATCTGTGCCCC
AGTTTGCTAGGGAGGTCGCAGTACTTGGCCACAGCCATCTCGTGCTGCTCGACGTAGGTCTCTTTGTCGGCCTCCT
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TCACTAAACGAGCTCTGCTTATATAGACCTCCCACCGTACACGCCTACCGCCCATTTGCGTCAACGGGGCGGGGTT
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CACCAGCTCACCGTCTTTCATTGCCATACGGAACTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAG

GCCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTAT
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TCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCATTAAAGCTAGCGGCCGCT
AGCTTATCGATGATAAGCTGTCAAACATGAGTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAA
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GCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAG
CAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGAGAAC
CATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTCGCTT
CTCGCTTCTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGGGCGCCAGTCC
TCCGATTGACTGAGTCGCCCGGGTACCCGTGTATCCAATAAACCCTCTTGCAGTTGCATCCGACTTGTGGTCTCGC
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TCGGGAGACCCCTGCCCAGGGACCACCGACCCACCACCGGGAGGTAAGCTGGCCAGCAACTTATCTGTGTCTGTCC
GATTGTCTAGTGTCTATGACTGATTTTATGCGCCTGCGTCGGTACTAGTTAGCTAACTAGCTCTGTATCTGGCGGA
CCCGTGGTGGAACTGACGAGTTCTGAACACCCGGCCGCAACCCTGGGAGACGTCCCAGGGACTTTGGGGGCCGTTT
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AACCTAAAACAGTTCCCGCCTCCGTCTGAATTTTTGCTTTCGGTTTGGAACCGAAGCCGCGCGTCTTGTCTGCTGC
AGCATCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTCTGTATTTGTCTGAAAATTAGGGCCAGACTGTTACCACTC
CCTTAAGTTTGACCTTAGATCACTGGAAAGATGTCGAGCGGCTCGCTCACAACCAGTCGGTAGATGTCAAGAAGAG
ACGTTGGGTTACCTTCTGCTCTGCAGAATGGCCAACCTTTAACGTCGGATGGCCGCGAGACGGCACCTTTAACCGA
GACCTCATCACCCAGGTTAAGATCAAGGTCTTTTCACCTGGCCCGCATGGACACCCAGACCAGGTCCCCTACATCG
TGACCTGGGAAGCCTTGGCTTTTGACCCCCCTCCCTGGGTCAAGCCCTTTGTACACCCTAAGCCTCCGCCTCCTCT
TCTTCCATCCGCGCCGTCTCTCCCCCTTGAACCTCCTCTTTCGACCCCGCCTCAATCCTCCCTTTATCCAGCCCTC
ACTCCTTCTCTAGGCGCCGGCCGGATCGGT

Features for pRFP-C-RS vector

Start	End	Description
1	6	EcoRI
75	331	U6 promoter
335	340	BamHI
379	385	HindIII
386	391	Sall
413	604	SV40 promoter

671	1270	Puromycin-N-acetyl transferase sequence
1349	1942	3' LTR
2299	2918	pBR322 ORI
2977	3563	Poly A signal
3604	3667	Multiple Cloning Site [PmeI (3604), NotI (3619, 3649), MluI (3630) AsisI (3667)]
3648	4380	tRFP
4467	5192	CMV promoter
5263	5922	CAM^r for Chlormphenicol resistance
6104	6574	5' LTR

6. Protein Sequence of PER1-HEK293

MSGPLEGADGGGDPRPGESFCPGGVPSPGPPQHRPCPGPSLADDTDANSNGSSGNESNGHESR
GASQRSSHSSSSGNGKDSALLETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPSTSGCSS
EQSARARTQKELMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQEYYQQWSLEE
GEPCSMDMSTYTLEELEHITSEYTLQNQDTFSVAVSFLTGRIVYISEQAAVLLRCKRDVFRGT
RFSELLAPQDVGVFYGSTAPSRLPTWGTGASAGSGLRDFTQEKSVFCRIRGGPDRDPGPRYQP
FRLTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIFTTRHTPSCLFQDVDERAA
PLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPFDHSPIRFCARNGEYVTMDTSW
AGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAPSLDTDIQELSEQIHRLLLQPVHSP
SPTGLCGVGAVTSPGPLHSPGSSSDSNGGDAEGPGPPAPVTFQQICKDVHLVKHQGQQLFIES
RARPQSRPRLPATGTFKAKALPCQSPDPELEAGSAPVQAPLALVPEEAERKEASSCSYQQINC
LDSILRYLESCNLPSTTKRKCASSSSYTTSSASDDDRQRTGPVSVGTKKDPPSAALSGEGATP
RKEPVVGGTLSPLALANKAESVVSVTSQCSFSSTIVHVGDKKPPESDIIMMEDLPGLAPGPAP
SPAPSPTVAPDPAPDAYRPVGLTKAVLSLHTQKEEQAFLSRFRDLGRLRGLDSSSTAPSALGE
RGCHHGPAPPSRRHHCRSKAKRSRHHQNPRAEAPCYVSHPSPVPPSTPWPTPPATTPFPAVVQ
PYPLPVFSPRGGPQPLPPAPTSVPPAAFPAPLVTPMVALVLPNYLFPTPSSYPYGALQTPAEG

PPTPASHSPSPSLPALAPSPPHRPDSPLFNSRCSSPLQLNLLQLEELPRAEGAAVAGGPGSSA
GPPPPSAEAAEPEARLAEVTESSNQDALSGSSDLLELLLQEDSRSGTGSAASGSLGSGLGSGS
GSGSHEGGSTSASITRSSQSSHTSKYFGSIDSSEAEAGAARGGAEPGDQVIKYVLQDPIWLLM
ANADQRVMMTYQVPSRDMTSVLKQDRERLRAMQKQQPRFSEDQRRELGAVHSWVRKGQLPRAL
DVMACVDCGSSTQDPGHPDDPLFSELDGLGLEPMEEGGGEQGSSGGGSGEGEGCEEAQGGAKA
SSSQDLAMEEEEEGRSSSSPALPTAGNCTS

SNP position in HEK293 cells

Per1_HEK AAFPAPLVTPMETVALVLPNYLFPTPSSYPYGALQTPAEGPPTPASHSPSPSLPALPPSP 965

Per1_001_database AAFPAPLVTPMETVALVLPNYLFPTPSSYPYGALQTPAEGPPTPASHSPSPSLPALAPSP 965

**********.***

Per1_HEK PHRPDSPLFNSRCSSPLQLNLLQLEELPRAEGAAVAGGPGSSAGPPPPSAEAAEPEARLA 1020

Per1_001_database PHRPDSPLFNSRCSSPLQLNLLQLEELPRAEGAAVAGGPGSSAGPPPPSAEAAEPEARLA 1020

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7. Alignment of Per1 clone with hPer1-001 in Ensembl database

hPer1_clone ATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGGCCTGGGGAATCATTT 60
hPer1-001_Ensembl ATGAGTGGCCCCCTAGAAGGGGCTGATGGGGGAGGGGACCCCAGGCCTGGGGAATCATTT 60
****

hPer1_clone TGTCCTGGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCTTGCCCAGGCCCCAGC 120
hPer1-001_Ensembl TGTCCTGGGGGGCGTCCCATCCCCTGGGCCCCCACAGCACCGGCCTTGCCCAGGCCCCAGC 120
****

hPer1_clone CTGGCCGATGACACCGATGCCAACAGCAATGGTTCAAGTGGCAATGAGTCCAACGGGCAT 180
hPer1-001_Ensembl CTGGCCGATGACACCGATGCCAACAGCAATGGTTCAAGTGGCAATGAGTCCAACGGGCAT 180
****

hPer1_clone GAGTCTAGAGGCGCATCTCAGCGGAGCTCACACAGCTCCTCCTCAGGCAACGGCAAGGAC 240
hPer1-001_Ensembl GAGTCTAGAGGCGCATCTCAGCGGAGCTCACACAGCTCCTCCTCAGGCAACGGCAAGGAC 240
****

hPer1_clone TCAGCCCTGCTGGAGACCACTGAGAGCAGCAAGAGCACAAACTCTCAGAGCCCATCCCCA 300
hPer1-001_Ensembl TCAGCCCTGCTGGAGACCACTGAGAGCAGCAAGAGCACAAACTCTCAGAGCCCATCCCCA 300
****

hPer1_clone CCCAGCAGTTCCATTGCCTACAGCCTCCTGAGTGCCAGCTCAGAGCAGGACAACCCGTCC 360
hPer1-001_Ensembl CCCAGCAGTTCCATTGCCTACAGCCTCCTGAGTGCCAGCTCAGAGCAGGACAACCCGTCC 360
****

hPer1_clone ACCAGTGGCTGCAGCAGTGAACAGTCAGCCCGGGCAAGGACTCAGAAGGAACTCATGACA 420
hPer1-001_Ensembl ACCAGTGGCTGCAGCAGTGAACAGTCAGCCCGGGCAAGGACTCAGAAGGAACTCATGACA 420
****

hPer1_clone GCACTTCGAGAGCTCAAGCTTCGACTGCCGCCAGAGCGCCGGGGCAAGGGCCGCTCTGGG 480
hPer1-001_Ensembl GCACTTCGAGAGCTCAAGCTTCGACTGCCGCCAGAGCGCCGGGGCAAGGGCCGCTCTGGG 480
****

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hPer1_clone          ACCCTGGCCACGCTGCAGTACGCACTGGCCTGTGTCAAGCAGGTGCAGGCCAACCAGGAA 540
hPer1-001_Ensembl    ACCCTGGCCACGCTGCAGTACGCACTGGCCTGTGTCAAGCAGGTGCAGGCCAACCAGGAA 540
                     ************************************************************************

hPer1_clone          TACTACCAGCAGTGGAGCCTGGAGGAGGGCGAGCCTTGCTCCATGGACATGTCCACCTAT 600
hPer1-001_Ensembl    TACTACCAGCAGTGGAGCCTGGAGGAGGGCGAGCCTTGCTCCATGGACATGTCCACCTAT 600
                     ************************************************************************

hPer1_clone          ACCCTGGAGGAGCTGGAGCACATCACGTCTGAGTACACACTTCAGAACCAGGATACCTTC 660
hPer1-001_Ensembl    ACCCTGGAGGAGCTGGAGCACATCACGTCTGAGTACACACTTCAGAACCAGGATACCTTC 660
                     ************************************************************************

hPer1_clone          TCAGTGGCTGTCTCCTTCCTGACGGGCCGAATCGTCTACATTTCGGAGCAGGCAGCCGTC 720
hPer1-001_Ensembl    TCAGTGGCTGTCTCCTTCCTGACGGGCCGAATCGTCTACATTTCGGAGCAGGCAGCCGTC 720
                     ************************************************************************

hPer1_clone          CTGCTGCGTTGCAAGCGGGACGTGTTCCGGGGTACCCGCTTCTCTGAGCTCCTGGCTCCC 780
hPer1-001_Ensembl    CTGCTGCGTTGCAAGCGGGACGTGTTCCGGGGTACCCGCTTCTCTGAGCTCCTGGCTCCC 780
                     ************************************************************************

hPer1_clone          CAGGATGTGGGAGTCTTCTATGGTTCCACTGCTCCATCTCGCCTGCCCACCTGGGGCACA 840
hPer1-001_Ensembl    CAGGATGTGGGAGTCTTCTATGGTTCCACTGCTCCATCTCGCCTGCCCACCTGGGGCACA 840
                     ************************************************************************

hPer1_clone          GGGGCCTCAGCAGGTTCAGGCCTCAGGGACTTTACCCAGGAGAAGTCCGTCTTCTGCCGT 900
hPer1-001_Ensembl    GGGGCCTCAGCAGGTTCAGGCCTCAGGGACTTTACCCAGGAGAAGTCCGTCTTCTGCCGT 900
                     ************************************************************************

hPer1_clone          ATCAGAGGAGGTCCTGACCGGGATCCAGGGCCTCGGTACCAGCCATTCCGCCTAACCCCG 960
hPer1-001_Ensembl    ATCAGAGGAGGTCCTGACCGGGATCCAGGGCCTCGGTACCAGCCATTCCGCCTAACCCCG 960
                     ************************************************************************

hPer1_clone          TATGTGACCAAGATCCGGGTCTCAGATGGGGCCCCTGCACAGCCGTGCTGCCTGCTGATT 1020
hPer1-001_Ensembl    TATGTGACCAAGATCCGGGTCTCAGATGGGGCCCCTGCACAGCCGTGCTGCCTGCTGATT 1020
                     ************************************************************************

hPer1_clone          GCAGAGCGCATCCATTCGGGTTACGAAGCTCCCCGGATACCCCCTGACAAGAGGATTTTC 1080
hPer1-001_Ensembl    GCAGAGCGCATCCATTCGGGTTACGAAGCTCCCCGGATACCCCCTGACAAGAGGATTTTC 1080
                     ************************************************************************

hPer1_clone          ACTACGCGGCACACACCCAGCTGCCTCTTCCAGGATGTGGATGAAAGGGCTGCCCCCCTG 1140
hPer1-001_Ensembl    ACTACGCGGCACACACCCAGCTGCCTCTTCCAGGATGTGGATGAAAGGGCTGCCCCCCTG 1140
                     ************************************************************************

hPer1_clone          CTGGGCTACCTGCCCCAGGACCTCCTGGGGGCCCCAGTGCTCCTGTTCCTGCATCCTGAG 1200
hPer1-001_Ensembl    CTGGGCTACCTGCCCCAGGACCTCCTGGGGGCCCCAGTGCTCCTGTTCCTGCATCCTGAG 1200
                     ************************************************************************

hPer1_clone          GACCGACCCCTCATGCTGGCTATCCACAAGAAGATTCTGCAGTTGGCGGGCCAGCCCTTT 1260
hPer1-001_Ensembl    GACCGACCCCTCATGCTGGCTATCCACAAGAAGATTCTGCAGTTGGCGGGCCAGCCCTTT 1260
                     ************************************************************************

hPer1_clone          GACCACTCCCCTATCCGCTTCTGTGCCCGCAACGGGGAGTATGTCACCATGGACACCAGC 1320
hPer1-001_Ensembl    GACCACTCCCCTATCCGCTTCTGTGCCCGCAACGGGGAGTATGTCACCATGGACACCAGC 1320
                     ************************************************************************

hPer1_clone          TGGGCTGGCTTTGTGCACCCCTGGAGCCGCAAGGTAGCCTTCGTGTTGGGCCGCCACAAA 1380
hPer1-001_Ensembl    TGGGCTGGCTTTGTGCACCCCTGGAGCCGCAAGGTAGCCTTCGTGTTGGGCCGCCACAAA 1380
                     ************************************************************************

hPer1_clone          GTACGCACGGCCCCCCTGAATGAGGACGTGTTCACTCCCCCGGCCCCCAGCCCAGCTCCC 1440
hPer1-001_Ensembl    GTACGCACGGCCCCCCTGAATGAGGACGTGTTCACTCCCCCGGCCCCCAGCCCAGCTCCC 1440
                     ************************************************************************

hPer1_clone          TCCCTGGACACTGATATCCAGGAGCTGTCAGAGCAGATCCACCGGCTGCTGCTGCAGCCC 1500
hPer1-001_Ensembl    TCCCTGGACACTGATATCCAGGAGCTGTCAGAGCAGATCCACCGGCTGCTGCTGCAGCCC 1500
                     ************************************************************************

hPer1_clone          GTCCACAGCCCCAGCCCCACGGGACTCTGTGGAGTCGGCGCCGTGACATCCCCAGGCCCT 1560
hPer1-001_Ensembl    GTCCACAGCCCCAGCCCCACGGGACTCTGTGGAGTCGGCGCCGTGACATCCCCAGGCCCT 1560
                     ************************************************************************

hPer1_clone          CTCCACAGCCCTGGGTCCTCCAGTGATAGCAACGGGGGTGATGCAGAGGGGCCTGGGCCT 1620
hPer1-001_Ensembl    CTCCACAGCCCTGGGTCCTCCAGTGATAGCAACGGGGGTGATGCAGAGGGGCCTGGGCCT 1620
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hPer1_clone       CCTGCGCCAGTGACTTTCCAGCAGATCTGTAAGGATGTGCATCTGGTGAAGCACCAGGGC 1680
hPer1-001_Ensembl CCTGCGCCAGTGACTTTCCAGCAGATCTGTAAGGATGTGCATCTGGTGAAGCACCAGGGC 1680
                  ********************************************************************************|*

hPer1_clone       CAGCAGCTTTTTATTGAGTCTCGGGCCCGGCCTCAGTCCCGGCCCCGCCTCCCTGCTACA 1740
hPer1-001_Ensembl CAGCAGCTTTTTATTGAGTCTCGGGCCCGGCCTCAGTCCCGGCCCCGCCTCCCTGCTACA 1740
                  ************************************************************

hPer1_clone       GGCACGTTCAAGGCCAAGGCCCTTCCCTGCCAATCCCCAGACCCAGAGCTGGAGGCGGGT 1800
hPer1-001_Ensembl GGCACGTTCAAGGCCAAGGCCCTTCCCTGCCAATCCCCAGACCCAGAGCTGGAGGCGGGT 1800
                  ************************************************************

hPer1_clone       TCTGCTCCCGTCCAGGCCCCACTAGCCTTGGTCCCTGAGGAGGCCGAGAGGAAAGAAGCC 1860
hPer1-001_Ensembl TCTGCTCCCGTCCAGGCCCCACTAGCCTTGGTCCCTGAGGAGGCCGAGAGGAAAGAAGCC 1860
                  ************************************************************

hPer1_clone       TCCAGCTGCTCCTACCAGCAGATCAACTGCCTGGACAGCATCCTCAGGTACCTGGAGAGC 1920
hPer1-001_Ensembl TCCAGCTGCTCCTACCAGCAGATCAACTGCCTGGACAGCATCCTCAGGTACCTGGAGAGC 1920
                  ************************************************************

hPer1_clone       TGCAACCTCCCCAGCACCACTAAGCGTAAATGTGCCTCCTCCTCCTCCTATACCACCTCC 1980
hPer1-001_Ensembl TGCAACCTCCCCAGCACCACTAAGCGTAAATGTGCCTCCTCCTCCTCCTATACCACCTCC 1980
                  ************************************************************

hPer1_clone       TCAGCCTCTGACGACGACAGGCAGAGGACAGGTCCAGTCTCTGTGGGGACCAAGAAAGAT 2040
hPer1-001_Ensembl TCAGCCTCTGACGACGACAGGCAGAGGACAGGTCCAGTCTCTGTGGGGACCAAGAAAGAT 2040
                  ************************************************************

hPer1_clone       CCGCCGTCAGCAGCGCTGTCTGGGGAGGGGGCCACCCCACGGAAGGAGCCAGTGGTGGGA 2100
hPer1-001_Ensembl CCGCCGTCAGCAGCGCTGTCTGGGGAGGGGGCCACCCCACGGAAGGAGCCAGTGGTGGGA 2100
                  ************************************************************

hPer1_clone       GGCACCCTGAGCCCGCTCGCCCTGGCCAATAAGGCGGAGAGTGTGGTGTCCGTCACCAGT 2160
hPer1-001_Ensembl GGCACCCTGAGCCCGCTCGCCCTGGCCAATAAGGCGGAGAGTGTGGTGTCCGTCACCAGT 2160
                  ************************************************************

hPer1_clone       CAGTGTAGCTTCAGCTCCACCATCGTCCATGTGGGAGACAAGAAGCCCCCGGAGTCGGAC 2220
hPer1-001_Ensembl CAGTGTAGCTTCAGCTCCACCATCGTCCATGTGGGAGACAAGAAGCCCCCGGAGTCGGAC 2220
                  ************************************************************

hPer1_clone       ATCATCATGATGGAGGACCTGCCTGGTCTAGCCCCAGGCCCAGCCCCCAGCCCAGCCCCC 2280
hPer1-001_Ensembl ATCATCATGATGGAGGACCTGCCTGGCCTAGCCCCAGGCCCAGCCCCCAGCCCAGCCCCC 2280
                  ************************************************************

hPer1_clone       AGCCCCACAGTAGCCCCTGACCCAGCCCCAGACGCCTACCGTCCAGTGGGGCTGACCAAG 2340
hPer1-001_Ensembl AGCCCCACAGTAGCCCCTGACCCAGCCCCAGACGCCTACCGTCCAGTGGGGCTGACCAAG 2340
                  ************************************************************

hPer1_clone       GCCGTGCTGTCCCTGCACACGCAGAAGGAAGAGCAAGCCTTCCTCAGCCGCTTCCGAGAC 2400
hPer1-001_Ensembl GCCGTGCTGTCCCTGCACACACAGAAGGAAGAGCAAGCCTTCCTCAGCCGCTTCCGAGAC 2400
                  ************************** ************************************************

hPer1_clone       CTGGGCAGGCTGCGTGGACTCGACAGCTCTTCCACAGCTCCCTCAGCCCTTGGCGAGCGA 2460
hPer1-001_Ensembl CTGGGCAGGCTGCGTGGACTCGACAGCTCTTCCACAGCTCCCTCAGCCCTTGGCGAGCGA 2460
                  ************************************************************

hPer1_clone       GGCTGCCACCACGGCCCCGCACCCCCAAGCCGCCGACACCACTGCCGATCCAAAGCCAAG 2520
hPer1-001_Ensembl GGCTGCCACCACGGCCCCGCACCCCCAAGCCGCCGACACCACTGCCGATCCAAAGCCAAG 2520
                  ************************************************************

hPer1_clone       CGCTCACGCCACCACCAGAACCCTCGGGCTGAAGCGCCCTGCTATGTCTCACACCCCTCA 2580
hPer1-001_Ensembl CGCTCACGCCACCACCAGAACCCTCGGGCTGAAGCGCCCTGCTATGTCTCACACCCCTCA 2580
                  ************************************************************

hPer1_clone       CCCGTGCCACCCTCCACCCCCTGGCCCACCCCACCAGCCACTACCCCCTTCCCAGCGGTT 2640
hPer1-001_Ensembl CCCGTGCCACCCTCCACCCCCTGGCCCACCCCACCAGCCACTACCCCCTTCCCAGCGGTT 2640
                  ************************************************************

hPer1_clone       GTCCAGCCCTACCCTCTCCCAGTGTTCTCTCCTCGAGGAGGCCCCCAGCCTCTTCCCCCT 2700
hPer1-001_Ensembl GTCCAGCCCTACCCTCTCCCAGTGTTCTCTCCTCGAGGAGGCCCCCAGCCTCTTCCCCCT 2700
                  ************************************************************

hPer1_clone       GCTCCCACATCTGTGCCCCCAGCTGCTTTCCCCGCCCCTTTGGTGACCCCAATGGTGGCC 2760
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hPer1-001_Ensembl   GCTCCCACATCTGTGCCCCCAGCTGCTTTCCCCGCCCCTTTGGTGACCCCAATGGTGGCC 2760
                    ********************************************************************************

hPer1_clone         TTGGTGCTCCCTAACTATCTGTTCCCAACCCCATCCAGCTATCCTTATGGGGCACTCCAG 2820
hPer1-001_Ensembl   TTGGTGCTCCCTAACTATCTGTTCCCAACCCCATCCAGCTATCCTTATGGGGCACTCCAG 2820
                    ********************************************************************************

hPer1_clone         ACCCCTGCTGAAGGGCCTCCCACTCCTGCCTCGCACTCCCCTTCTCCATCCTTGCCCGCC 2880
hPer1-001_Ensembl   ACCCCTGCTGAAGGGCCTCCCACTCCTGCCTCGCACTCCCCTTCTCCATCCTTGCCCGCC 2880
                    ********************************************************************************

hPer1_clone         CTCCCCCCGAGTCCTCCTCACCGCCCGGACTCTCCACTGTTCAACTCGAGATGCAGCTCT 2940
hPer1-001_Ensembl   CTCGCCCCGAGTCCTCCTCACCGCCCGGACTCTCCACTGTTCAACTCGAGATGCAGCTCT 2940
                     ***  ********************************************************************************

hPer1_clone         CCACTCCAGCTCAATCTGCTGCAGCTGGAGGAGCTCCCCCGTGCTGAGGGGGCTGCTGTT 3000
hPer1-001_Ensembl   CCACTCCAGCTCAATCTGCTGCAGCTGGAGGAGCTCCCCCGTGCTGAGGGGGCTGCTGTT 3000
                    ********************************************************************************

hPer1_clone         GCAGGAGGCCCTGGGAGCAGTGCCGGGCCCCCACCTCCCAGTGCGGAGGCTGCTGAGCCA 3060
hPer1-001_Ensembl   GCAGGAGGCCCTGGGAGCAGTGCCGGGCCCCCACCTCCCAGTGCGGAGGCTGCTGAGCCA 3060
                    ********************************************************************************

hPer1_clone         GAGGCCAGACTGGCGGAGGTCACTGAGTCCTCCAATCAGGACGCACTTTCCGGCTCCAGT 3120
hPer1-001_Ensembl   GAGGCCAGACTGGCGGAGGTCACTGAGTCCTCCAATCAGGACGCACTTTCCGGCTCCAGT 3120
                    ********************************************************************************

hPer1_clone         GACCTGCTCGAACTTCTGCTGCAAGAGGACTCGCGCTCCGGCACAGGCTCCGCAGCCTCG 3180
hPer1-001_Ensembl   GACCTGCTCGAACTTCTGCTGCAAGAGGACTCGCGCTCCGGCACAGGCTCCGCAGCCTCG 3180
                    ********************************************************************************

hPer1_clone         GGCTCCTTGGGCTCTGGCTTGGGCTCTGGGTCTGGTTCAGGCTCCCATGAAGGGGGCAGC 3240
hPer1-001_Ensembl   GGCTCCTTGGGCTCTGGCTTGGGCTCTGGGTCTGGTTCAGGCTCCCATGAAGGGGGCAGC 3240
                    ********************************************************************************

hPer1_clone         ACCTCAGCCAGCATCACTCGCAGCAGCCAGAGCAGCCACACAAGCAAATACTTTGGCAGC 3300
hPer1-001_Ensembl   ACCTCAGCCAGCATCACTCGCAGCAGCCAGAGCAGCCACACAAGCAAATACTTTGGCAGC 3300
                    ********************************************************************************



hPer1_clone         ATCGACTCTTCCGAGGCTGAGGCTGGGGCTGCTCGGGGCGGGGCTGAGCCTGGGGACCAG 3360
hPer1-001_Ensembl   ATCGACTCTTCCGAGGCTGAGGCTGGGGCTGCTCGGGGCGGGGCTGAGCCTGGGGACCAG 3360
                    ********************************************************************************

hPer1_clone         GTGATTAAGTACGTGCTCCAGGATCCCATTTGGCTGCTCATGGCCAATGCTGACCAGCGC 3420
hPer1-001_Ensembl   GTGATTAAGTACGTGCTCCAGGATCCCATTTGGCTGCTCATGGCCAATGCTGACCAGCGC 3420
                    ********************************************************************************

hPer1_clone         GTCATGATGACCTACCAGGTGCCCTCCAGGGACATGACCTCTGTGCTGAAGCAGGATCGG 3480
hPer1-001_Ensembl   GTCATGATGACCTACCAGGTGCCCTCCAGGGACATGACCTCTGTGCTGAAGCAGGATCGG 3480
                    ********************************************************************************

hPer1_clone         GAGCGGCTCCGAGCCATGCAGAAGCAGCAGCCTCGGTTTTCTGAGGACCAGCGGCGGGAA 3540
hPer1-001_Ensembl   GAGCGGCTCCGAGCCATGCAGAAGCAGCAGCCTCGGTTTTCTGAGGACCAGCGGCGGGAA 3540
                    ********************************************************************************

hPer1_clone         CTGGGTGCTGTGCACTCCTGGGTCCGGAAGGGCCAACTGCCTCGGGCTCTTGATGTGATG 3600
hPer1-001_Ensembl   CTGGGTGCTGTGCACTCCTGGGTCCGGAAGGGCCAACTGCCTCGGGCTCTTGATGTGATG 3600
                     ********************************************************************************

hPer1_clone         GCCTGTGTGGACTGTGGGAGCAGCACCCAAGATCCTGGTCACCCTGATGACCCACTCTTC 3660
hPer1-001_Ensembl   GCCTGTGTGGACTGTGGGAGCAGCACCCAAGATCCTGGTCACCCTGATGACCCACTCTTC 3660
                     ********************************************************************************

hPer1_clone         TCAGAGCTGGATGGACTGGGGCTGGAGCCCATGGAAGAGGGTGGAGGCGAGCAGGGCAGC 3720
hPer1-001_Ensembl   TCAGAGCTGGATGGACTGGGGCTGGAGCCCATGGAAGAGGGTGGAGGCGAGCAGGGCAGC 3720
                     ********************************************************************************

hPer1_clone         AGCGGTGGCGGCAGTGGTGAGGGAGAGGGCTGCGAGGAGGCCCAAGGCGGGGCCAAGGCT 3780
hPer1-001_Ensembl   AGCGGTGGCGGCAGTGGTGAGGGAGAGGGCTGCGAGGAGGCCCAAGGCGGGGCCAAGGCT 3780
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hPer1_clone          TCAAGCTCTCAGGACTTGGCTATGGAGGAGGAGGAAGAAGGCAGGAGCTCATCCAGTCCA 3840
hPer1-001_Ensembl    TCAAGCTCTCAGGACTTGGCTATGGAGGAGGAGGAAGAAGGCAGGAGCTCATCCAGTCCA 3840
                     ********************************************************************************

hPer1_clone          GCCTTACCTACAGCAGGAAACTGCACCAGCTAG 3873
hPer1-001_Ensembl    GCCTTACCTACAGCAGGAAACTGCACCAGCTAG 3873
                     *********************************
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1-Position of the Silent SNP (rs2253820) **2-** Position of the non-silent SNP (rs2585405)

8. Sequence of pcDNA5-Flag-EGFP

FRT-F primer

KpnI

EGFP

NotI

XhoI

BGH-R Primer

CMV Promotor

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATGC
CGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGC
AAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTT
AGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTA
GTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTAC
ATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATA
ATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATT
TACGGTAAACTGCCCACTTGGCAGTACATCAAGTGT

ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTA
TGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCT
ATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGG
GGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACC

AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGAC**GCAAATGGGCG**

GTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCCCTATCAGTGATAGAGATC

TCCCTATCAGTGATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAG

ACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGGACTCTA

GCGTTTAAACTTAAGCTTGGTACCgtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagct

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CAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCA

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CTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTT

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GACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGT

TGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGC

GATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGT

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